# REVIEW ARTICLE
# Mitochondrial threshold effects

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The study of mitochondrial diseases has revealed dramatic variability in the phenotypic presentation of mitochondrial genetic defects. To attempt to understand this variability, different authors have studied energy metabolism in trans-mitochondrial cell lines carrying different proportions of various pathogenic mutations in their mitochondrial DNA. The same kinds of experiments have been performed on isolated mito-chondria and on tissue biopsies taken from patients with mitochondrial diseases. The results have shown that, in most cases, phenotypic manifestation of the genetic defect occurs only when a threshold level is exceeded, and this phenomenon has been named the 'phenotypic threshold effect'. Subsequently, several authors showed that it was possible to inhibit considerably the activity of a respiratory chain complex, up to a critical value, without affecting the rate of mitochondrial respiration or ATP synthesis. This phenomenon was called the 'biochemical threshold effect'. More recently, quantitative analysis of the effects of various mutations in mitochondrial DNA on the rate of mitochondrial protein synthesis has revealed the existence of a 'translational threshold effect'. In this review these different mitochondrial threshold effects are discussed, along with their molecular bases and the roles that they play in the presentation of mitochondrial diseases.

Key words: complementation, Metabolic Control Analysis, mitochondrial diseases, mitochondrial DNA, oxidative phos-phorylation, threshold effect.

## INTRODUCTION

Mitochondrial function in mammalian cells is generally presented as the central pathway for energy metabolism, but several other aspects of mitochondrial function have been described, such as key roles in apoptosis, free radical production, thermogenesis and calcium signalling. Since 1962 [1], impairment of mito-chondrial function has been associated with a wide range of severe human disorders, which have been regrouped under the name of mitochondrial diseases [2]. Most of these diseases are due to a defect within the respiratory chain, which consists of five enzyme complexes each composed of different subunits (Figure 1) encoded by either nuclear DNA or mitochondrial DNA (mtDNA). As a consequence, genetic defects in both genomes can be responsible for mitochondrial cytopathies, even though most of the known mutations have been identified in mtDNA [3–5]. However, despite improved characterization of the genetic defects that lead to mitochondrial disorders, the pathogenetic mechanisms of these diseases are for the most part not under-stood. In particular, the relationship between the presence of a pathogenic mutation in mtDNA (or the presence of a defect in a given oxidative phosphorylation complex) and the occurrence of specific clinical signs (phenotype) remains problematic [6,7]. Indeed, the study of mitochondrial diseases has shown that the same clinical features can be caused by different mutations in mtDNA and, conversely, that the same genetic defect in mtDNA can lead to different clinical manifestations (Table 1). Functional studies have also shown that biochemical defects affecting the same respiratory chain complex can lead to different clinical manifestations (Table 2). This variability in the expression of mitochondrial genetic or biochemical defects can be observed between different patients (who present with different clinical manifestations but possess the same defect), and also between different tissues in a given individual (tissue specificity).



**Figure 1  Oxidative phosphorylation network and composition of mito-chondrial respiratory chain enzyme complexes**

Mitochondria of normal tissues usually oxidize fatty acids, pyruvate or amino acids (or combinations of these substrates) into NADH and/or FADH$_2$. These reduced equivalents are oxidized further by the mitochondrial respiratory chain to establish an electrochemical gradient of protons, which is finally used by the F$_1$F$_0$-ATP synthase to produce ATP, the only form of energy used by the cell. IM, mitochondrial inner membrane; OM, mitochondrial outer membrane; CI (etc.), complex I (etc.); Cyt c, cytochrome c; nDNA, nuclear DNA.

| Respiratory chain complex | nDNA subunits | mtDNA subunits | Redox cofactors |
|---|---|---|---|
| I (EC 1.6.6.3) | 38 | 7 | FMN, [Fe–S] centres, ubiquinones |
| II (EC 1.3.5.1) | 0 | 4 | FAD, [Fe–S] centres, cytochrome $b_{560}$ |
| III (EC 1.10.2.2) | 10 | 1 | Cytochromes $b$ and $c1$, Rieske protein |
| IV (EC 1.9.3.1) | 10 | 3 | [Cu$_a$] centre, [Cu$_b$-haem $a3$] centre |
| V (EC 3.6.1.34) | 14 | 2 | None |

**Table 1   The same mitochondrial pathology (same clinical features) can be caused by various mutations in mtDNA or nuclear DNA affecting different tRNAs or different respiratory chain complex subunits**

PEO, progressive external opthalmoplegia; cyt. *b*, cytochrome *b*; LHON, Leber's hereditary optic, neuropathy (Leber's disease); NDUFS, NADH dehydrogenase ubiquinol iron–sulphur protein; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase; SURF-1, *Homo sapiens* surfeit 1 gene.

| Mitochondrial pathology or clinical features | Mutations in mtDNA or in nuclear DNA |
|---|---|
| MELAS | tRNA$^{Leu\ (UUR)}$ (A3243G, A3252G, A3260G, T3271C, T3291C), tRNA$^{Phe}$ (G583A), tRNA$^{Val}$ (G1642A), tRNA$^{Cys}$ (A5814G), ND1 (T3308C), ND5 (G13513A), COX III (T9957C) |
| MERRF | tRNA$^{Lys}$ (A8344G, T8356C, G8363A) |
| PEO | Single deletions, tRNA$^{Leu\ (UUR)}$ (A3243G, A3251G, C3256T, T12311C), tRNA$^{Leu\ (UCN)}$ (T12311C, G12315A), tRNA$^{Lys}$ (A8344G, G8342A), tRNA$^{Ile}$ (T4274C, T4285C, G4298A, G4309A), tRNA$^{Asn}$ (A5692G, G5703A), ANT1 |
| Myopathy | tRNA$^{Leu\ (UUR)}$ (A3243G, T3250C, C3254G, A3260G, A3288G, T3291C, A3302G, A12320G), tRNA$^{Met}$ (T4409C), tRNA$^{Phe}$ (T618C), tRNA$^{Lys}$ (A8344G), tRNA$^{Glu}$ (T14709C), tRNA$^{Pro}$ (C15990T), tRNA$^{Trp}$ (G5521A), tRNA$^{Leu\ (CUN)}$ (A12320G), cyt. *b* (G15762A), ND1 (7 bp inversion) |
| Cardiomyopathy | 12 S rRNA (A1555G), tRNA$^{Leu}$ (C3303T, C3254G, A3260G), tRNA$^{Lys}$ (A8344G, G8363A, A8296G), tRNA$^{Ile}$ (A4295G, A4300G, A4269G, C4320T), tRNA$^{Gly}$ (T9997C), cyt. *b* (G15243A), NDUFS2 (S314P, A228Q, P229Q) |
| Leigh disease | tRNA$^{Lys}$ (A8344G), tRNA$^{Val}$ (A1644T), tRNA$^{Trp}$ (5537T), ND6 (G14459A), ATPase 6 (T8993C, T8993G, T9176C), single deletion, PDH (A555G, 963–965ins15, 1004–1005ins36, 1159–1160ins16, 1162–1163ins4), fp SDH, SURF-1, NDUFS7 (V122M), NDUFS8 (P79L/R102H) |
| Deafness | 12 S rRNA (A1555G), tRNA$^{Ser\ (UCN)}$ (7445, 7472), tRNA$^{Leu\ (UUR)}$ (A3243G, C3271T), single large deletion |
| Diabetes | tRNA$^{Leu}$ (A3243G, A3252G, C3256T), tRNA$^{Ser}$ (C12258A), tRNA$^{Glu}$ (T14709C), mutations at nucleotides 1310, 1438 and 12026 |
| LHON | ND1 (G3460A, T3394C, T4160C, T4216C), ND2 (A4917G), ND4 (A11696G, G11778A), ND5 (G13708A, G13513A, G14459A), ND6 (T14484C, T14596A, G14459A), cyt. *b* (G15257A, G15812A) |

**Table 2   Activity defects in the same respiratory chain complex can lead to different clinical manifestations**

LHON, Leber's hereditary optic neuropathy (Leber's disease); NARP, neurogenic weakness ataxia and retinitis pigmentosa; MILS, maternally inherited Leigh syndrome.

| Respiratory chain complex affected | Clinical phenoype |
|---|---|
| Complex I | MELAS, bilateral striatal necrosis/MELAS, LHON, myopathy and exercise intolerance, Parkinsonism, LHON/dystonia, Leigh's disease, LHON/MELAS, exercise-intolerant myoglobinuria, leukodystrophy/myoclonic epilepsy |
| Complex III | Parkinsonism/MELAS, exercise intolerance, cardiomyopathy, myopathy, exercise intolerance myoglobinuria |
| Complex IV | Sideroblastic anaemia, myoclonic ataxia, deafness, myopathy, MELAS, exercise intolerance, mitochondrial encephalomyopathy, motor neuron disease-like, exercise-intolerant myoglobinuria |
| Complex V | Leigh's disease, NARP, NARP/MILS, bilateral striatal necrosis |

To understand this variability, one must first consider the characteristics of mitochondrial genetics. In mammalian cells, the mitochondrion consists of an interacting mobile network [8,9] that contains thousands of copies of mtDNA. In tissues of patients, mutated and wild-type molecules of mtDNA can co-exist. The proportion of mutant to wild-type mtDNA is the percentage heteroplasmy, and this can vary between different individuals, or between cells or tissues within a patient [10,11]. This genetic variability is one explanation of why a particular mutation in mtDNA can lead to different clinical manifestations in different patients. This variable segregation of mutant and wild-type mtDNA during embryogenesis can also explain the frequently observed tissue specificity of mitochondrial pathologies, i.e. the higher the mutant load in certain tissues, the more severe the clinical phenotype. However, heteroplasmy is not sufficient to explain (i) the numerous cases where there is no correlation between the percentage of mutant mtDNA and phenotype [12–14]; (ii) the tissue specificity observed when the mtDNA mutation is homoplasmic in all tissues [15–18]; or (iii) the phenotypic variability associated with nuclear mutations affecting an oxidative phosphorylation complex [19]. Therefore, while the study of the segregation and replication of mtDNA during oogenesis and embryogenesis has contributed to a better understanding of the mechanisms involved in the variation in heteroplasmy levels between tissues or individuals [20], it does not explain how and to what extent a particular proportion of mutated mtDNA molecules can affect mitochondrial function and cell activity. As we argue here, to explain this requires a study of the effects of quantitative expression of mutations in mtDNA on mitochondrial metabolism and cell phenotype.

## PHENOTYPIC THRESHOLD EFFECT

The study of genotype–phenotype relationships in mitochondrial diseases has been made possible by the development of techniques whereby mitochondria from cells obtained from patients are transferred to a cell line lacking mtDNA (rho$^0$ cells) [21,22]. In this manner, it is possible to create transmitochondrial cell lines (cybrids) containing different proportions of mutated mtDNA from 0 to 100 %, and to study the effects of a given 'mutant load' on the activity of respiratory chain complexes, mitochondrial respiration and cell growth. In 1986, Wallace [23] used such techniques to study the expression of a mutation in the mtDNA 16 S rRNA gene. This mutation conferred chloramphenicol resistance when the mutant load reached 15 %. More recently, the relevance of such thresholds in the expression of pathogenic mutations in human mtDNA has been studied using cybrids constructed from cell lines derived from patients with the diseases MERRF (myoclonic epilepsy and ragged-red fibres) or MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) (Table 3). These studies have shown clearly the existence of a 'phenotypic threshold effect', which can be characterized by the following: (i) a low proportion of wild-type mtDNA co-existing with mutated mtDNA allows a normal phenotype to be

**Table 3  Summary of quantitative relationships at different levels of expression of genetic or biochemical mitochondrial defects**

Tested relationships were as follows: 1, mutant RNA and mutant mtDNA; 2, subunit expression and mutant mtDNA; 3, complex activity and subunit level; 4, complex activity and mutant mtDNA; 5, respiration or ATP synthesis and defective subunit; 6, respiration or ATP synthesis and complex activity; 7, respiration or ATP synthesis and mutant mtDNA; 8, observed phenotype and mutant mtDNA. T, threshold effect; P, proportional; sf, results observed at the single-fibre level; n.c., no correlation; OXPHOS, oxidative phosphorylation; NDUFS and SURF are defined in Table 1; ↓, decreased level of.

| Mutation in mtDNA or inhibited complex | Relationship | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
| Deletion | P | T (60%) or P (sf) | P | T (60–85%) | T (> 50%) | | T (50–60%) | T (72–82%) | [26,27,31,35,116–123] |
| A8344G tRNA^Lys | ↓ mutant tRNA | T (50%) or P | P | T (85–95%) | T (50%) | T (85%) | T (86–90%) | T (73–98%) | [12,24,32,38,39,41,61,124–126] |
| A3243G tRNA^Leu | ↓ mutant tRNA | | | T (87–95%) or P | | | T (90–94%) | T (60–90%) | [14,25,30,33,37,88,127–134] |
| T8993G or T9176C ATP6 | | | | | | | | T (> 80%) Leigh | [135,136] |
| T7445C tRNA^Ser | ↓ mutant tRNA | T (40%) | | | | | | n.c. | [17,137,138] |
| G3460A (complex I) | | | | | | T (> 90%) | | | [63] |
| G11778A | | | | | | | | | [139] |
| 11778 (ND4) | | n.c. | | n.c. | | | | n.c. | [90] |
| Various OXPHOS complexes | | | | | | T (20–95%) | | | [48,55–60,62,64] |
| Complex I (ND5) | P | T (40%) | | T (60%) | | | T (40%) | | [28] |
| Complex I (NDUFS 1, 2, 4, 7, 8) | | | P or T | | | | | | [46] |
| Complex I and complex IV | | | | | | T | | | [141] |
| Complex IV (SURF I, COX III) | | | P | | | | | | [45] |
| Complex IV (Stop codon) | | | P | T | | | T (30%) | | [36] |
| Complex IV (G6930A) | | | T | | | | | | [142] |

maintained, but (ii) a small decrease in this proportion below a threshold value alters the phenotype (Figure 2). Similar observations have also been reported in experiments on muscle fibres taken from MERRF [24] or MELAS patients [25] (the respective threshold values were 90% and 65%). Accordingly, the clinical manifestations of MERRF occur only when a threshold of mutant mtDNA in muscle is reached [24]. Studies performed on cell lines derived from patients with chronic progressive external opthalmoplegia ('CPEO') or on *Drosophila* carrying a deletion in mtDNA have also shown a phenotypic threshold effect for the expression of large-scale mtDNA deletions [26,27]. Finally, Bai et al. [28] showed that cybrids containing a mutation in the complex I (ND5) subunit gene exhibited an impairment of glutamate/malate-dependent mitochondrial respiration only after the percentage heteroplasmy had reached 60%.

According to Shoffner and co-workers [24,29], the phenotypic threshold value is around 60% for mtDNA deletions, and around 90% for other mtDNA mutations. The results grouped in Table 3 show that all phenotypic threshold values reported are greater than 60%, and vary in level above this, depending on the type of mutation or the tissue being evaluated. Studies performed directly on individual muscle fibres have confirmed the existence of the phenotypic threshold effect at the single-cell level [30–33]. Thus the proportion of mutated mtDNA varies between the different muscle fibres, with only the ones containing concentrations of mutated mtDNA over a threshold value showing a deficiency in complex IV activity, as assessed by histochemical staining [cytochrome *c* oxidase (COX)-negative fibres]. Petruzzella et al. [30] suggested that the differential distribution of these COX-negative fibres in the skeletal muscle of patients with chronic progressive external ophthalmoplegia or MELAS could explain why the same mean heteroplasmy level of the A3243G tRNA^Leu mutation in muscle lead to different phenotypes in different individuals.

## MOLECULAR BASES OF THE PHENOTYPIC THRESHOLD EFFECT

The phenotypic threshold effect observed at the single-cell level could arise when the products of the wild-type mtDNA can no longer 'complement' the effects of the mutated ones. For instance, a heteroplasmic mutation in mtDNA will result in the co-existence of mutated mRNAs, mutated tRNAs and defective respiratory chain subunits along with their wild-type homologues. These wild-type molecules may be sufficient to support normal function of the organelle until their levels fall below a critical value (threshold), at which point they can no longer compensate for the effect of the mutation, leading to impairment of mitochondrial function (Figure 2). This hypothesis implies that, in the absence of mutations, there is an excess of mRNAs, tRNAs and active respiratory chain complexes compared with what is required to permit 'normal' respiration. The phenotypic threshold effect is based on this reserve of different macromolecules (mRNAs, tRNAs, subunits), and can then be considered as a protective mechanism providing a safety margin against the effects of deleterious mutations.

Depending on the gene affected by the mutation in mtDNA, complementation can occur at different levels of mitochondrial gene expression. For instance, a mutation affecting a tRNA gene can be complemented at the translational level by wild-type tRNA genes to ensure a normal rate of mitochondrial protein synthesis. Alternatively, the effect of a mutation affecting a subunit of a respiratory chain complex could be complemented by the presence of an excess of wild-type subunits compared with the levels of the enzyme complex. Thus genetic and functional complementation of mitochondrial defects can occur, either exclusively or simultaneously, at the following levels: transcription, translation, enzyme assembly and activity, mitochondrial respiration and ATP synthesis, cell activity and, finally, organ function. In the end, all of these different compensatory mechanisms can combine to result in the observed phenotypic
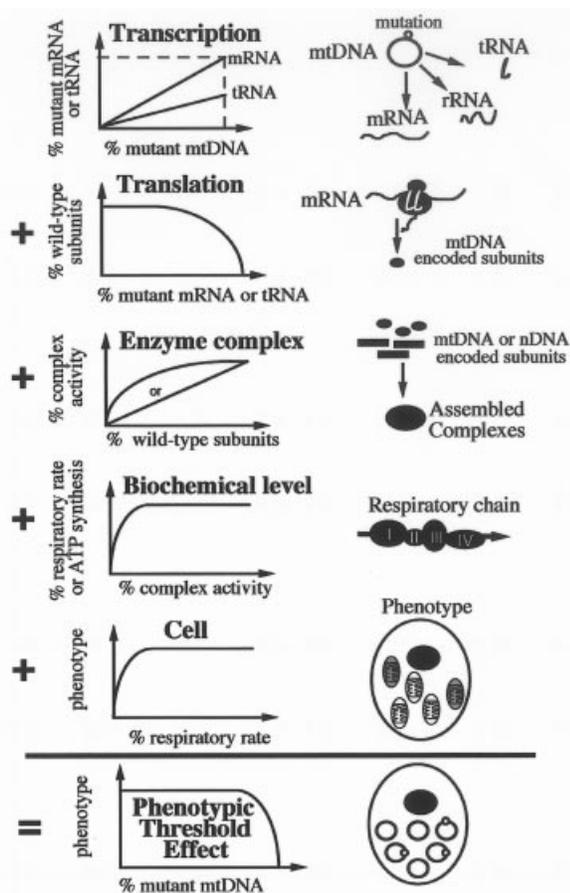
**Figure 2    Components of the phenotypic threshold effect**

This figure illustrates the quantitative relationships occurring at six different levels of the expression of a given mtDNA mutation: transcription, translation, enzyme activity, respiratory flux, cell activity and clinical manifestations (phenotype). The deleterious effects of the mutation can be assessed at each level by determining respectively the proportion of mutant mRNA or tRNA, the proportion of defective subunits, enzyme complex activity, mitochondrial respiration or ATP synthesis, cell growth and the emergence of particular clinical features. nDNA, nuclear DNA.

threshold effect (Figure 2). Thus, in order to understand the molecular bases of the phenotypic threshold effect, it is necessary to determine the importance of the compensation occurring at each level of the expression of a mutation in mtDNA. This can be realized by following and quantifying the effect of a given heteroplasmic mutation in mtDNA at the different levels of its genetic and functional expression. Experimentally, the deleterious effect of such a mutation can be assessed by determining the proportions of mutated mRNA and possibly mutated tRNA, the proportion of defective subunits, enzyme complex levels and activity, mitochondrial respiration or ATP synthesis rate, cell growth, and the emergence of particular clinical features. Different authors have partially studied these parameters for various mutations in mtDNA, and in the following we have regrouped and compared the numerous data that support the existence of different mitochondrial threshold effects.

## TRANSCRIPTION LEVEL

The mitochondrial genome (mtDNA) consists of a 16.6 kb circular double-stranded DNA that encodes various subunits of

the respiratory chain complexes I, III, IV and V, tRNAs and rRNAs [34]. Briefly, the transcription of mtDNA occurs independently and in the opposite direction for each strand of the molecule (the heavy strand and the light strand), resulting in three polycistronic transcripts that are cleaved further to release 13 mRNAs, 22 tRNAs and two rRNAs. By this mechanism, a given percentage of mutated mtDNA will lead to a proportionate number of mutated mRNAs or tRNAs. Accordingly, it has been shown experimentally that some heteroplasmic point mutations in mtDNA structural genes give rise to proportionate amounts of mutated mRNAs. This has been observed for mtDNA deletions [26,35] as well as for a point mutation in complex I subunit ND5 [28] or in complex IV subunit COX I [36].

However, the situation seems to be different for mutations affecting mitochondrial genes encoding tRNAs. In this case, the proportion of defective tRNAs is not related to the percentage of heteroplasmy. For instance, the results reported by Chomyn et al. [37] showed that two different cybrid clones harbouring the A3243G tRNA[Leu] MELAS mutation in a quasi-homoplasmic manner (almost 100% mutant mtDNA) contained only 50% inactive (uncharged) tRNA[Leu], implying a lower transcription efficiency or an increased rate of degradation of the mutated tRNA[Leu] gene. For example, the mutation in the tRNA could destabilize its tertiary structure and make it more susceptible to hydrolysis, a possibility supported by the fact that the total amount of tRNA[Leu] measured in these cybrids was decreased by half [37]. A decrease in the total amount of mutant tRNA (compared with the wild type) was also reported for the MERRF A8344G tRNA[Lys] mutation [38] and the tRNA[Ser] 7445 mutation [39]. In these studies, a given percentage heteroplasmy for a mitochondrial tRNA mutation always resulted in a lower proportion of mutant tRNAs. This instability of the mutant tRNAs could explain why it is necessary to have a mutant load greater than a threshold value before a decrease in mitochondrial protein synthesis or in mitochondrial respiratory rate is observed (Figure 2).

## TRANSLATION LEVEL

The translation of mtDNA-encoded proteins occurs at the level of the 'mitoribosomes', and uses a specific genetic code to synthetize respiratory chain subunits [34]. However, differences in the sequences involved in the initiation of translation, or in associations with translation factors, are responsible for different levels of translation that contribute to maintain a stable stoichiometry between the different respiratory chain complexes [40]. A mutation within mitochondrial structural genes (in mtDNA or in nuclear DNA) could partially alter their expression by increasing the rate of degradation of the mRNA and/or the protein. Bai et al. [28] demonstrated that a point mutation in mtDNA affected the level of expression of a complex I subunit (ND5). In that work, the authors observed a diminished rate of synthesis for this subunit only when the proportion of mRNA carrying the mutation was greater than 40%. According to the authors, when this threshold was reached, the rate of degradation of the ND5 subunit exceeded its rate of synthesis. In the same manner, Hayashi et al. [26] observed that, when more than 40% of mtDNA was deleted, the translation of respiratory chain proteins was stopped. This could be due to the fact that this deletion encompassed elements necessary for translation (tRNA and rRNA), and that above the threshold value (40% of deleted mtDNA) the quantity of these elements was no longer sufficient to perform translation. Finally, Boulet et al. [41] reported that impairment of the rate of mitochondrial protein translation occurred only when the percentage heteroplasmy for the MERRF

A8344G tRNA$^{Lys}$ mutation was above 50 %. In this case, at least 50 % of wild-type tRNA$^{Lys}$ would be necessary to achieve normal translation.

What is clear from these different studies [26,28,41] is that impairment of mitochondrial protein translation is apparent only when the percentage of mutant mRNA or tRNA exceeds a threshold value (Figure 2). This 'translational threshold effect' is based either (i) on the instability of mutated mRNAs (mutation in a structural gene) and/or (ii) on the requirement for a critical amount of wild-type tRNAs for translation (mutations in mitochondrial tRNAs [42]). As suggested by Enriquez and Attardi [43], the balance between wild-type (aminoacylated) and mutated (uncharged) tRNAs plays a crucial role in controlling the translation process. As shown in Table 3, the translational threshold values reported so far are similar, ranging between 40 % and 50 % of mutated mRNA or tRNA, indicating that the amount of mRNA and tRNA must exceed the requirement for mitochondrial protein synthesis by a factor of two. Taken together, these data argue that there is a reserve for supporting a normal rate of oxidative phosphorylation in case of (i) an increase in energy demand or (ii) a respiratory chain deficiency. Chomyn [39] suggested that the translational threshold effect could be derived from ancient mechanisms involved in the regulation of aminoacyl-tRNA levels in bacteria. Indeed, when *Escherichia coli* are placed under moderately limiting conditions of growth, the level of uncharged tRNA increases, and when uncharged tRNAs are in sufficient excess over charged tRNAs (over a threshold value), the specific interactions of uncharged tRNAs with the ribosomes lead to the stringent response, with pleiotropic consequences for the cell [44].

## ENZYME LEVEL

Pathogenic mutations in both mtDNA and nuclear DNA can affect respiratory chain activity by (i) decreasing the total amount of assembled active enzyme complexes (default in synthesis or in assembly) and/or (ii) decreasing the intrinsic kinetic parameters of these complexes. Therefore, in order to understand the functional consequences of a point mutation in a given subunit-encoding gene, it is necessary to determine not only the steady-state level of expression of this subunit, but also the proportion of assembled complex as well as enzyme activity. Such an in-depth analysis has been achieved by various groups working on patient-derived cell lines harbouring various mutations in mtDNA or nuclear DNA. Their results show that the relationship between the level of expression of a respiratory chain subunit and the activity of the corresponding complex depends on the subunit studied and/or on the type of mutation in mtDNA. For instance, Hanson et al. [45] observed that in fibroblasts from patients with an assembly defect (Surf-1 or COX III mutation), COX activity was correlated with the steady-state level of assembled subunits. Similarly, D'Aurelio et al. [36] described a direct correlation between the proportion of synthesized COX I subunit and COX activity in cybrids harbouring a COX I stop-codon mutation. Finally, Triepels et al. [46] also showed a linear relationship between the level of expression of two different complex I subunits (30 kDa and 39 kDa subunits) and complex I activity in fibroblasts obtained from 11 patients with mitochondrial diseases. However, this last study also showed that, for two other subunits (8 kDa and 15 kDa), this relationship was not proportional (Figure 3); thus 30 % of the normal level of the 8 kDa subunit (40 % for the 15 kDa subunit) was sufficient to maintain 70 % of complex I activity. Another example of this phenomenon was reported by Spelbrink et al. [35], who studied the expression of a mtDNA deletion in a cell line obtained from a patient with
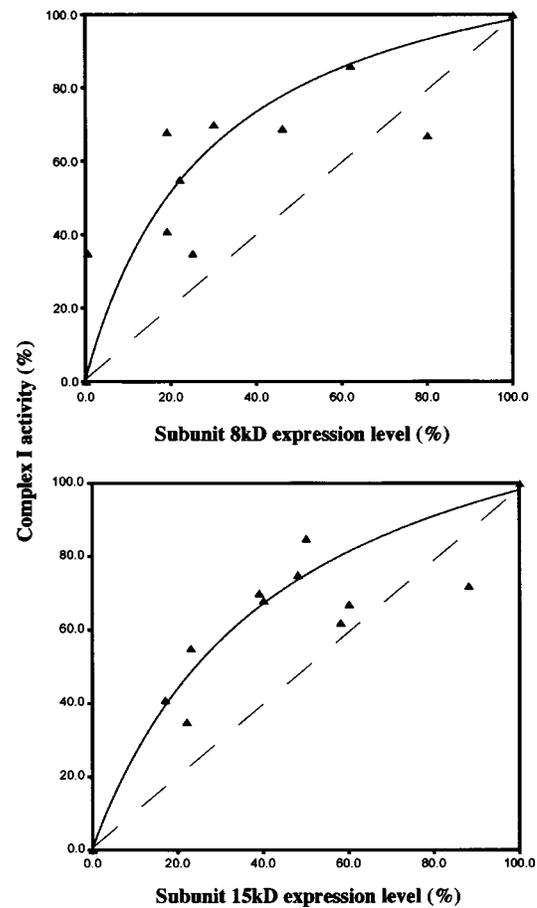


**Figure 3    Relationship between the levels of expression of two different complex I subunits (8 kDa and 15 kDa), and complex I activity**

These curves were constructed from the data reported by Triepels et al. [46].

Pearson's syndrome. In that work, the amount of COX II subunit was decreased by half, but COX activity and the rate of ATP synthesis were maintained at normal levels. Recently, Gatterman et al. [47] reported that cybrids containing 95 % of a stop-codon mutation in the COX I subunit presented 70 % of wild-type enzyme activity. In these last three studies, a marked decrease in the level of expression of a given subunit did not necessarily lead to a proportionate (if any) decrease in the activity of the corresponding respiratory chain complex. One possible explanation for this phenomenon could be that the mutation affects a regulatory subunit that is inactive in the conditions of the activity assay. Another possibility is the existence of excess subunits that could constitute a back-up, or play a regulatory role in cellular metabolism [48].

There is some experimental evidence that different compensatory mechanisms – i.e. at both the translational and the enzymic levels – can exist simultaneously. Thus Boulet et al. [41] observed that mitochondrial protein translation was impaired only when the proportion of the MERRF mutation was greater than 50 % in myotubes. In the same work, complex IV activity remained unaffected until the proportion of the MERRF mutation reached 85 %. This indicates that even with more than 50 % mutated mtDNA, the decrease in the level of expression of the respiratory chain subunit had no consequences for COX activity. Therefore a compensatory mechanism must exist at the enzyme function

level. This study illustrates the efficiency of combining translational and enzymic threshold effects (Figure 2).

## BIOCHEMICAL LEVEL

As we have described above, different mutations in mtDNA can decrease the activity of each individual enzyme complex of the respiratory chain. What will be the effects of these mutations on overall mitochondrial respiration and ATP production? For instance, if a mutation inhibits complex I activity by 50%, will mitochondrial respiration also be diminished to the same extent? The application of Metabolic Control Analysis [49–51] to the oxidative phosphorylation network has helped to provide a quantitative answer to this question [52,53]. The main results of this approach are that: (i) the control of mitochondrial respiration is shared between all of the oxidative phosphorylation complexes (no rate-limiting step *per se*); (ii) the importance of the control exercised by a particular enzyme on the respiratory flux is measured by its control coefficient; and (iii) distribution of control between the different complexes depends on the steady-state rate of respiration [54].

However, although Metabolic Control Analysis has helped us to understand the control of mitochondrial respiration at a given steady state of respiration, it cannot be used to study the effects of pathogenic mutations that lead to substantial enzyme deficiencies and important changes in the steady state. To analyse the effects of different levels of inhibition of a given respiratory chain complex on mitochondrial respiration, it is possible to use increasing concentrations of a specific inhibitor of this complex, and to measure individual complex activity and overall mitochondrial respiration simultaneously. In this manner, we observed that a decrease in complex IV activity had to exceed a critical value (approx. 75% inhibition) before a decrease in mitochondrial respiration could be observed [55]. This phenomenon was called the 'biochemical threshold effect', and was further described for complexes I, III and IV, $F_1F_0$-ATP synthase, adenine nucleotide translocator (ANT), phosphate carrier and pyruvate carrier, with isolated rat mitochondria and with permeabilized human cells [48,55–60].

The biochemical threshold effect has been examined using cell lines harbouring various pathogenic mutations in mtDNA. For instance, James et al. [61] used human cell lines with various levels of complex I inhibition (due to different levels of the MERFF mutation) to show that mitochondrial respiration was impaired only when the inhibition of complex I activity was greater than 85%. A similar threshold effect in the expression of complex I deficiency was reported by Barrientos and Moraes [62]. Cybrids harbouring different levels of mutated mtDNA (COX I G6930A stop-codon mutation) showed normal cell respiration and ATP synthesis, whereas COX activity was inhibited by 60% [36]. Finally, the G3460A mutation in Leber's disease has been found to be associated with a severe decrease in complex I activity, but a normal rate of mitochondrial ATP production was measured in fibroblasts and skeletal muscle *in vivo* [63]. All of these results demonstrate clearly the existence of a biochemical threshold effect for the expression of respiratory chain deficiencies and mitochondrial energy production. The threshold values obtained at the biochemical level indicate that there is considerable variability according to the type of mutation or the tissue (Table 3). Moreover, the importance of this phenomenon can also vary according to the experimental conditions for a given complex. For instance, various authors have determined the biochemical threshold value for complex IV with regard to mitochondrial respiration or ATP synthesis. The first studies performed on rat mitochondria (isolated from skeletal

muscle or brain) showed a high threshold value of approx. 80% [55,56,58]. These results were in agreement with the observation of a large excess of COX in isolated mitochondria [65]. The subsequent work of Villani and Attardi [59] on human cell lines revealed a lower threshold (approx. 40%), in agreement with the measurement of a small excess capacity for COX *in vivo* by the same authors [60]. More recently, a low biochemical threshold value for COX was reported in human permeabilized muscle fibres [66] and in human cybrids [36]. Finally, Wiedemann and Kunz [67] observed an increase in the control coefficient (a decrease in the biochemical threshold value) for COX on mitochondrial respiration under conditions of low oxygen partial pressure. These different results illustrate clearly the variability of the biochemical threshold effect for a given respiratory chain complex, depending on the tissue studied and/or the experimental conditions.

A possible mechanism involved in the biochemical threshold effect could be the existence of an excess of active respiratory chain complexes that could be used as a reserve to compensate for a deficit [48]. The plateau phase of the threshold curves (Figure 2) indicates that this reserve can be decreased, most of the time to a large extent, without any effect on the global flux of respiration. This compensation could be explained by the mobilization of a pool of active respiratory chain enzymes. For instance, complex I has been shown to exist in two kinetically and structurally distinct (slowly interconvertible) active and inactive forms [68]. Grivennikova et al. [69] have also demonstrated the possibility of a transition between these two forms of complex I in intact mitochondria. Interestingly, a reversible transition between different stable spatial conformations of complex I was observed in *E. coli* [70]. Taken together, such mechanisms could play an important role in the regulation of mitochondrial energy production, and could also participate in the compensation of an oxidative phosphorylation defect by turning some inactive complexes into active ones.

Other observations suggest that the compensation of a defect in oxidative phosphorylation activity may also depend on respiratory chain organization. Indeed, Schägger and co-workers [71–73] have demonstrated that, in yeast and bovine heart mitochondria, respiratory chain complexes exist as free holoenzymes or are organized in supercomplexes. Supercomplex organization could be modulated by the level of cardiolipin synthesis [74]. In this situation, it is likely that differences in these superstructures (composition, stoichiometry) could affect the importance of the mechanisms of reserve and attenuation. For instance, the supercomplexes will favour the channelling of intermediary substrates, and this could decrease the possibility of 'network attenuation' (see below). In the same manner, free respiratory chain complexes could represent a reserve of activity, while complexes assembled in a superstructure could only be those participating in mitochondrial respiration. This could be the case for ANT, which is engaged in the permeability transition pore [75], or $F_1F_0$-ATP synthase, which is involved in control of the biogenesis of the mitochondrial inner membrane through auto-association [76].

Thus observed differences in threshold values measured in different tissues could be related to differences in the organization, composition and/or content of enzyme complexes. For example, the content of ATP synthase (determined by titration with oligomycin) is 200 pmol/mg of protein in rat heart mitochondria and 70 pmol/mg in rat brain mitochondria [53]. Accordingly, the biochemical threshold is higher in heart (80%) and much lower in brain (60%) [48]. Such correlations can be found for the other oxidative phosphorylation complexes in different tissues [48,53]. The threshold values also depend upon the respiratory substrate,
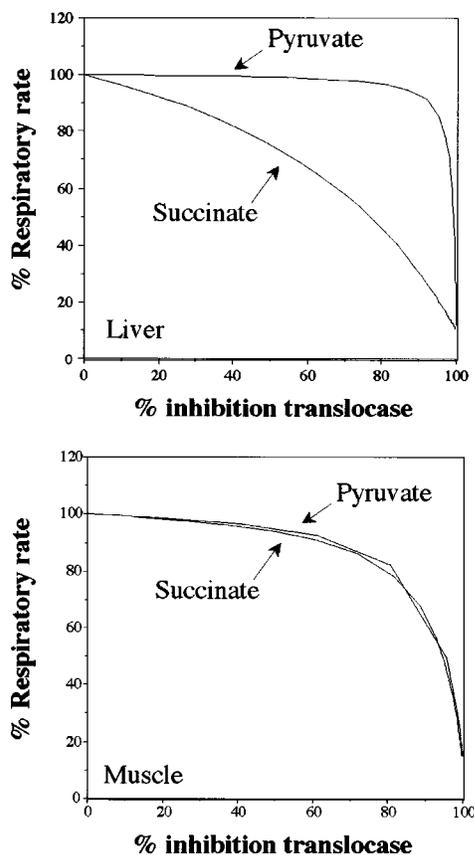
**Figure 4 Dependence of biochemical threshold value and reserve of activity for the ANT in rat liver and muscle mitochondria respiring on different energy substrates**

i.e. the steady state of respiration. For example, a different reserve was observed for ANT by comparing rat liver and muscle mitochondria respiring on different substrates [48]. Figure 4 shows the threshold curves obtained for ANT in rat muscle or liver mitochondria respiring on pyruvate/malate or succinate. In liver the threshold is much higher with pyruvate compared with succinate, while in muscle it does not vary. This observation can be explained by the fact that the respiratory rate was greater with succinate ($211 \pm 13$ natoms of O/min per mg of protein) than with pyruvate ($142 \pm 20$ natoms of O/min per mg), and in liver the amount of ANT [290 pmol/mg of mitochondrial protein, estimated by the quantity of carboxyatractylate that completely inhibited phosphorylation] allowed a reserve with pyruvate respiration, but not with succinate [48]. As a result, the threshold value obtained with pyruvate ($92 \pm 1.6\%$) was considerably higher than that when succinate was used ($58.6 \pm 6.62\%$). Conversely, in muscle, there was no modification of the threshold curve with succinate, because the higher concentration of ANT in this tissue (1600 pmol/mg of protein) allows a reserve with both pyruvate and succinate (Figure 4). This 'reserve of activity' used in response to an increase in energy demand could also be used to compensate for the effect of a mutation, in order to maintain a normal steady state of respiration and ATP synthesis.

The second mechanism that could explain the compensation of an activity defect is a direct chemical regulation of oxidative phosphorylation enzyme activity in response to a decrease in energy production. In that case, a modification of the apparent kinetic properties of the active complexes [$K_m$ (app) or $V_{max}$ (app)] could be observed. For instance, stimulation of complex I activity can be realized via cAMP-dependent phosphorylation of the 18 kDa protein, as demonstrated in human fibroblasts [77]. It has also been reported that complex IV activity can be regulated by cAMP-dependent phosphorylation [78] or ATP binding [79]. Finally, the activity of respiratory chain complexes could also be regulated directly by the mitochondrial transmembrane eletro-chemical gradient of protons [80].

The final mechanism that could explain the biochemical threshold effect is what we call 'network attenuation'. This concept derives from metabolic control theory [49–51], and considers the kinetic buffering (compensation) of the effect of a perturbation in the activity of an enzyme on the overall flux by the metabolic network through variations in intermediary metabolite concentrations. According to this theory, at a given steady-state rate of respiration, moderate inhibition of the activity of a respiratory chain complex leads to variations in the concentrations of intermediary metabolites (coenzyme Q, cytochrome $c$, electrochemical gradient of protons), which in turn modulates the activity of the other enzymes of the network (respiratory chain complexes) to keep the respiratory flux unchanged. Such modulation is only possible because, at any steady state of mitochondrial coupled respiration, each individual respiratory chain complex is working at a velocity substantially lower than their maximal velocity ($V_{max}$). The difference between the $V_{max}$ and the steady-state velocity ($V_{state\,3}$) of these complexes is their 'excess capacity'. Therefore the notion of excess capacity can be considered as one component of the network attenuation mechanism. The limit in the capacity for attenuation depends on the steady-state rate of respiration (flux value), the structure of the metabolic network (organization, composition), the concentrations of the pools of intermediary substrates, and the kinetic parameters ($V_{max}$) of the different complexes. Variations in these parameters between different tissues could explain in part the tissue specificity observed in mitochondrial diseases [48].

In summary, the biochemical threshold effect can be explained by three different mechanisms: (i) mobilization of a pool of inactive respiratory chain enzyme complexes (reserve of enzyme activity); (ii) chemical modification of the apparent kinetic properties of the active complexes (kinetic regulation); and (iii) modulation of the activity of respiratory chain complexes by variations in the concentrations of intermediary metabolites (network attenuation). It remains to be shown whether these different mechanisms operate simultaneously or alternatively, and also to what extent they participate in the biochemical threshold effect in different tissues.

## MULTIPLE THRESHOLD EXPRESSION SYSTEM AND MITOCHONDRIAL CYTOPATHIES

The threshold effects that are localized at different levels of the expression of mtDNA mutations constitute an efficient safety mechanism conferring to the cell, and to the organism, greater stability against mutations in mtDNA and also nuclear DNA (Figure 2). Such an intregrated compensatory system can partly overcome the high mutation rate of mtDNA and its limited capacity for repair [81]. Furthermore, some mutations affecting oxidative phosphorylation components have, in addition to their direct structural effect on enzyme activity, a secondary deleterious effect characterized by increased production of reactive oxygen species (ROS), which are responsible for oxidative damage to both mtDNA and respiratory chain components [82]. Cells have

developed specific systems to detect excessive ROS, which lead to the activation of specific transcription factors and the expression of appropriate target genes [83]. In addition, the biochemical threshold effect can directly help to compensate for ROS-induced decreases in the activity of respiratory chain complexes. However, it cannot prevent the telomere shortening or the accumulation of mutations and deletions in mtDNA that occur as a result of hydroxyl damage [82].

The biochemical threshold effect can also explain a part of the phenotypic variability in relation to pathogenic mtDNA mutations. For instance, as we have shown in rat tissues, the threshold value for a given respiratory chain complex is not the same in muscle, heart, liver, kidney and brain [48]. These observations previously led us to propose an explanation for the tissue specificity observed in mitochondrial diseases [48]. Thus the biochemical threshold value will depend on the origin and distribution of the mutation (nuclear or mitochondrial; homogeneous or not), and on the energy demands of the cell [84]. Ventura et al. [85] recently observed a marked decrease in the biochemical threshold value of complex I with aging in rat liver (80 % in 4-month-old rats compared with 30 % in 8-month-old rats). This observation clearly shows that a similar decrease in complex I activity (between 30 and 50 % inhibition) will lead to a dramatic decrease in the rate of mitochondrial respiration in old rats, while having no effect in young animals. The transcriptional, translational and enzymic threshold values may also vary according to the nature of the tissue (nuclear background, i.e. presence of different enzyme isoforms, substrate channelling, and distribution of mutated mtDNA within the mitochondriome, the cell and the tissue) and to the metabolic steady state (flux value, concentrations of metabolites, control and regulation of oxidative phosphorylation, nature and accessibility of the different substrates, energy demands, aging).

The existence of different threshold effects has important implications for the study and diagnosis of human mitochondrial diseases. For instance, the biochemical threshold effect explains why a significant defect in the activity of a component of the respiratory chain does not always lead to a mitochondrial pathology. More importantly, it may not be necessary to completely correct an activity defect in a respiratory chain complex: it may be sufficient to bring this activity over the threshold value, or to modify the threshold value itself [86]. According to Taylor et al. [87], understanding the mechanisms that determine the phenotypic threshold effect could provide the basis for gene therapy of mtDNA disorders. In addition to the different threshold effects operating at the cellular level, the intercellular and inter-organellar distribution of mutated mtDNA and the absolute mtDNA copy number also play a role in determining the phenotypic presentation of a mtDNA mutation. Indeed, Bentlage and Attardi [88] observed a proportionality between mitochondrial respiratory rate and total mtDNA copy number in transmitochondrial cell lines harbouring different heteroplasmy levels for the A3243G tRNA[Leu] mutation. According to these authors, this suggests a contribution of the mutant mtDNA to cell respiratory competence, and points to an important role for mtDNA copy number in determining the phenotype of the cell.

In summary, a phenotypic threshold effect (expressed in terms of the percentage of mutant mtDNA) has been observed in transmitochondrial cell lines harbouring various proportions of mutant compared with wild-type mtDNA, but containing the same absolute amount of total mtDNA (Table 3). It is thus clear that the threshold effect observed in these studies is determined by competition between mutant and wild-type mtDNA. On the other hand, studies of the influence of a decrease in total mtDNA

content on oxidative phosphorylation function show a proportionate decrease in respiratory rate [88]. It can thus be hypothesized that a decrease in mtDNA content (as observed in mtDNA depletion syndrome) would affect the respiratory steady state, which in turn can modify the biochemical threshold value.

## ADDITIONAL COMPENSATORY MECHANISMS OF DEFICIENCIES IN OXIDATIVE PHOSPHORYLATION

In addition to the threshold effects that occur at the mitochondrial level, several other mechanisms can take place at the organellar, cellular and tissue levels to compensate for a defect in mitochondrial energy production. Firstly, a decrease in mitochondrial ATP production can be directly compensated for by an equivalent increase in glycolysis. In most cancer cell lines, where the mitochondrial oxidative phosphorylation system is deficient, the energy for growth is derived almost exclusively through glycolysis [89]. Similarly, fibroblast cell lines derived from patients with a mitochondrial disease can grow as fast as control cells in glucose medium by using glycolysis to produce energy, whereas they hardly grow in galactose media, where they are forced to derive energy through oxidative phosphorylation [90]. More generally, in mammalian cells the balance between glycolysis and oxidative phosphorylation depends on substrate availability (glucose, fatty acids, oxygen, etc.) and energy demand. In different tissues with variable energy requirements and substrate utilization, glycolysis and oxidative phosphorylation are used to different extents. One reason for this is that glycolysis provides ATP at a low yield but a high rate, while oxidative phosphorylation produces ATP at a low rate but a higher yield [91]. In the cytosol, the [ATP][ADP]/[$P_i$] ratio serves as the primary sensor of altered energy production, while in the mitochondrial matrix the [NADH]/[NAD$^+$] ratio reflects substrate levels and respiratory state [92]. According to Korzeniewski [93], the physiological stimulation of oxidative phosphorylation in response to an increase in energy demand (low [ATP]/[ADP] ratio) can be realized through (i) direct regulation of oxidative phosphorylation complexes by external effectors such as [Ca$^{2+}$], or (ii) metabolite-mediated regulation (such as an increase in ADP concentration). In this manner, compensation of defects in oxidative phosphorylation can occur at the cellular level through stimulation of mitochondrial ATP production by the action of various hormones (vasopressin, glucagon, adrenaline) and/or neurotransmitters [93]. A molecular hypothesis on the physiological regulation of oxidative phosphorylation also considers a hormonally controlled dynamic equilibrium between a relaxed state of energy metabolism with low ROS formation, and an excited state with elevated formation of ROS. This hypothesis is based on the allosteric inhibition of COX by ATP at high intramitochondrial ATP/ADP ratios, which is switched on by cAMP-dependent phosphorylation and switched off by calcium-induced dephosphorylation of the enzyme [94].

At the tissue level, defects in oxidative phosphorylation can be further compensated for by an increase in oxygen delivery by the circulatory system via an acceleration of cardiac output and breathing, as measured in some patients with mitochondrial diseases [95]. According to Haller [96], the mitochondrial capacity to extract oxygen is in excess compared with the capacity of the circulatory system to deliver oxygen. Another means of compensating for an oxidative phosphorylation defect in muscle is to increase the rate of phosphocreatine resynthesis or to recruit more glycolytic fibres. The staining of mitochondria in muscle fibres from patients with mitochondrial diseases has revealed the existence of so-called 'ragged red fibres', characterized by a subsarcolemmal and interfibrillar increase in mitochondrial

number and volume [97]. Increased mitochondrial mass has also been observed in mice with mitochondrial myopathy [98] and in rats treated with high levels of glucocorticoid hormone [99]. It is suspected that such an increase in mitochondrial volume could help to compensate for an oxidative phosphorylation defect, by facilitating both oxygen uptake and ATP delivery in the cytosol.

Finally, genetic studies have demonstrated a co-ordinate induction of the expression (transcription) of genes involved in energy metabolism in tissues of some patients with mitochondrial diseases [100]. This could be mediated through inducible pro-motor regions, such as the OXBOX/REDOX complex found in the genes coding for ANT1 and the $\beta$ subunit of ATP synthase [101]. These motifs provide one mechanism whereby mammalian energy metabolism can be adapted to developmental and en-vironmental demands. More generally, expression of metabolic genes can be modulated by several effectors, such as oxygen and its reactive species [83], fatty acids [102], cAMP [103], ATP [104], redox state [105], thyroid hormone [106] and nutrients [107]. Accordingly, analysis of the promoters of several genes involved in oxidative phosphorylation reveals a great diversity and hetero-geneity of transfactor-binding elements, suggesting that no single regulatory feature could account for the co-ordinated expression of all oxidative phosphorylation genes [108].

## CONCLUSION

Phenotypic manifestations of mitochondrial defects occur only when a threshold level is exceeded, and this phenotypic threshold effect can be explained by various mechanisms localized at several levels of expression of a mutation in mtDNA or in nuclear DNA. This situation is certainly not specific to mito-chondria, and over 20 years ago Kacser and Burns [109] described the same phenomena in a variety of biological systems. These authors explained by this means the apparent evolutionary paradox of the heterozygote, which carries roughly half of the normal activity of the wild-type homozygote but nevertheless maintains normal fluxes. They concluded that, in metabolism, it cannot be avoided that most of the enzymes appear as if they are in excess.

Mitochondrial threshold effects may be based on both the existence of reserves of various macromolecules and the possi-bility of mitochondrial transcomplementation. The latter is a matter of controversy, as illustrated by the debate between the groups of Attardi and Hayashi [110–112]. In fact, both authors agree on the potential of mitochondria to fuse and mix, but they observed striking differences in the frequency of transmito-chondrial complementation in their respective experiments. Attardi and co-workers [38,43,113] regard complementation as a rare process, while Hayashi and colleagues [112,114] believe that it is a general phenomenon. These latter authors demonstrated the existence of inter-mitochondrial complementation in hybrids, in cybrids and in mouse tissues carrying a deletion in mtDNA [112,114]; however, as suggested by Attardi et al. [111], the frequency of mitochondrial complementation *in vivo* is likely to be controlled by mitochondrial dynamic organization. Indeed, recent advances in the study of mitochondrial structure have led to reconsideration of the mitochondrion as a single functional unit that can be organized as a complex reticular network or fragmented into smaller ovoid structures [8,115]. Therefore conformational modifications of the mitochondrial network could affect the capacity for genetic and functional complemen-tation, and thus modify the importance of the phenotypic threshold effect. To better understand the molecular bases of mitochondrial threshold effects, it will be necessary to study the relationships between mitochondrial function and the structure of the organelle in living cells.

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