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The Myoclonic Epilepsy and Ragged-Red Fiber Mutation Provides New Insights into Human Mitochondrial Function and Genetics

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Of the ~50 mtDNA point mutations, reported so far, that cause disease in humans, ⩾35 occur in tRNA genes (Schon et al. 1997). Of these, perhaps the best-studied is the A→G transition at position 8344 in the $tRNA^{Lys}$ gene, the mutation that causes myoclonic epilepsy and ragged-red fiber syndrome (MERRF; Shoffner et al. 1990). This disorder is an encephalomyopathy characterized by myoclonic epilepsy, ataxia, and mitochondrial myopathy, with additional dysfunction, in some individuals, in tissues other than brain and skeletal muscle (Schon et al. 1997). Histological analysis of transverse sections of a MERRF patient's skeletal muscle, stained with Gomori's modified trichrome stain, reveals the presence of "ragged red fibers," muscle fibers that exhibit peripheral blotchy, red patches that represent subsarcolemmal accumulations of mitochondria.

The mammalian mitochondrial genome contains 13 reading frames, which encode subunits of respiratory-chain enzyme complexes and the H^+-ATPase (Anderson et al. 1981; Attardi et al. 1986). mtDNA also contains genes for the large and small rRNAs and for 22 tRNAs. The latter genes encode a complete set of tRNAs, sufficient to support mitochondrial protein synthesis. Each mitochondrial tRNA gene is essential for translation; that is, there is no redundancy, and no tRNA appears to be imported into mitochondria.

The 8344 $tRNA^{Lys}$ mutation, like many deleterious tRNA mutations, occurs in a heteroplasmic state: a MERRF patient carries both the mutant and the wild-type alleles of the gene. The proportion of mutant DNA varies among individuals within a MERRF maternal lineage—and even among the tissues of the same individual (reviewed in Shoffner and Wallace 1992; see also Poulton 1998 [in this issue]). The relatives of the proband who carry the mutation are often unaffected, or they may exhibit only milder symptoms such as hearing loss or ataxia. These relatives generally have a lower proportion of mutant mtDNA than does the proband.

Enzymological analysis of muscle-biopsy material from MERRF patients has revealed reduced activities of respiratory-chain enzymes (Wallace et al. 1988). Especially affected are the rotenone-sensitive NADH dehydrogenase, or complex I, which contains seven subunits synthesized in mitochondria, and cytochrome *c* oxidase, or complex IV, which contains three such subunits.

Any deleterious mtDNA mutation would have, as its ultimate effect, the decreased production of ATP. In this regard, one of the most intriguing questions regarding MERRF—and, for that matter, other mtDNA-linked diseases—concerns the mechanism by which a specific mtDNA point mutation brings about loss of cellular function in a tissue-specific pattern (Schon et al. 1997; Zhou et al. 1997), so as to cause a disease different from that caused by another mtDNA mutation. It behooves us then to understand in depth the pathogenetic mechanisms associated with a particular point mutation, as well as the biochemical consequences of different proportions of mutant DNA and of its distribution among the mitochondria of a cell. One important experimental tool for addressing these questions, at a fundamental level, is the use of cellular models of mitochondrial diseases. Biochemical and molecular studies carried out on the 8344 mutation and on other mtDNA mutations in such cellular models have, over the past 7 years, revealed surprising phenomena regarding mitochondrial biogenesis and genetics, and several general principles have emerged.

Use of Transmitochondrial Cell Lines for the Analysis of the Molecular Pathogenetic Mechanism of the MERRF Mutation

About a year before the identification of the MERRF 8344 mutation, the first human mtDNA–less cell lines were isolated by King and Attardi (1989). These mtDNA-less cells, or "ρ^0" cells (to use the designation given to the equivalent yeast mutants), do have mitochondria, which maintain a membrane potential, using the ATP/ADP translocase and glycolytically produced

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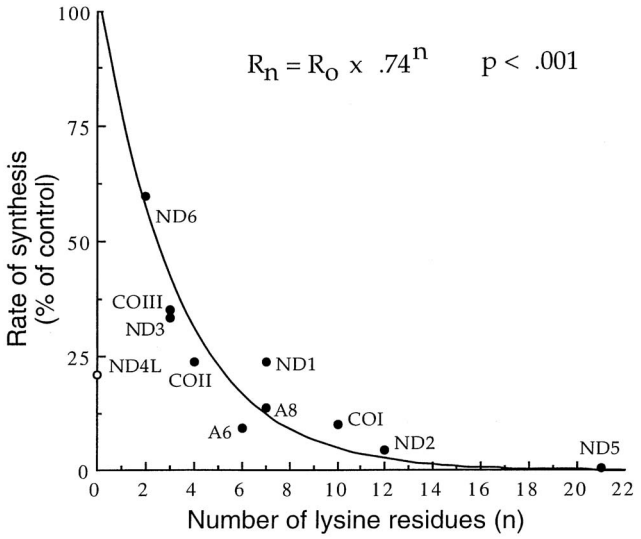

Figure 1 Relationship between rate of synthesis of the individual mitochondrial translation products and number of lysine residues that they contain in pT1, a transmitochondrial cybrid line that carries the 8344 mutation in nearly homoplasmic form. The protein-synthesis data were derived from an experiment in which the mutant and control cells were labeled with ^{35}S-methionine for 30 min in the presence of emetine. The labeling of the individual polypeptides in pT1 cells is expressed as a percentage of the labeling of the same polypeptides in pT3, a cybrid line that lacks the 8344 mutation. R_n = relative rate of synthesis; $R_0 = 1.05$; n = number of lysine residues of the individual polypeptides. COI, COII, and COIII are subunits of cytochrome *c* oxidase; ND1, ND2, ND3, ND4L, ND5, and ND6 are subunits of NADH dehydrogenase; and A6 and A8 are subunits of the H^+-ATPase. Reprinted from the study by Enriquez et al. (1995).

ATP as well as other carriers. However, lacking the 13 mtDNA-encoded polypeptides, the ρ^0 cells do not respire. ρ^0 cells grow at an almost-normal rate in glucose-containing culture medium supplemented with both pyruvate, necessary for oxidizing the cytosolic NADH via lactate dehydrogenase (King and Attardi 1996), and uridine, to compensate for the lack of pyrimidine biosynthesis resulting from the absence of a functional respiratory chain (Grégoire et al. 1984).

ρ^0 cells can be repopulated with exogenous mitochondria, containing wild-type mtDNA, either by polyethylene glycol-mediated fusion with cytoplasts (enucleated cells) or with platelets or by microinjection—thereby restoring pyruvate- and uridine-independent growth (see Parker and Swerdlow 1998 [in this issue]). This provides a powerful selection for transmitochondrial cybrids. Even cybrids constructed with mitochondria carrying a deleterious mutation in their DNA can be selected, but generally only under the less-stringent selection conditions provided by pyruvate-supplemented uridine-free medium.

When enucleated myoblasts from a MERRF patient were fused with ρ^0 cells, the isolated cybrid clones exhibited two phenotypes: wild type, as judged by biochemical and molecular criteria, and mutant, as revealed by the extremely low levels of respiration and cytochrome *c* oxidase activity (Chomyn et al. 1991). The cybrids exhibiting the mutant phenotype carried the 8344 mutation in predominant form, whereas the cybrids displaying no phenotype carried only wild-type mtDNA. These results provided a direct link between the biochemical defect and the mtDNA mutation, in a constant nuclear background. The isolation of two types of clones derived from a single patient indicated that, either in vivo or during culture of the myoblasts, intercellular segregation of the wild-type and mutant genomes had occurred. This segregation made available, in the convenient form of immortal cell lines, mutant cells and their perfectly matched controls: namely, cells with the same nuclear background, carrying the patient's mitochondrial genome or haplotype, but without the MERRF mutation. This was a fortunate situation because mtDNA is highly polymorphic; the mtDNA sequence varies between individuals by 0.3%, on average (Cann et al. 1987).

Effects of the 8344 Mutation on Mitochondrial Protein Synthesis

^{35}S-methionine pulse-labeling experiments, carried out to investigate the effects of the mutation on mitochondrial protein synthesis in transmitochondrial cybrids, revealed that, in addition to the 13 proteins normally synthesized in mitochondria, several abnormal polypeptides were also produced (Chomyn et al. 1991; Masucci et al. 1995). The most highly labeled one was also observed in myoblasts and myotubes from MERRF patients (Chomyn et al. 1991; Boulet et al. 1992). Furthermore, the rate of labeling of the 13 normal translation products in the mutant cybrids was considerably decreased, a finding that was not surprising, considering that the mutation occurred in a tRNA gene.

A great advantage of working with the human mitochondrial system is that the entire genome has been sequenced (Anderson et al. 1981); thus, the lysine content of each polypeptide is known. Furthermore, the 13 polypeptides can be resolved electrophoretically in a one-dimensional SDS-polyacrylamide gel, and the correspondence of the polypeptides with the individual genes is well established (Attardi et al. 1986). Thus, using the ^{35}S-methionine pulse-labeling data from cybrids carrying the 8344 mutation, Enriquez et al. (1995) were able to correlate the relative synthesis rate of each polypeptide with the number of lysine codons in the corresponding reading frame (fig. 1). The data fit an exponential curve that described a model of defective protein synthesis whereby, at each lysine codon, there was an ~26% probability that chain elongation would termi-

nate. Thus, polypeptides with a relatively large number of lysine residues—such as ND2 (a subunit of the NADH dehydrogenase), with 12 lysines—would have only a small probability of being completed. In fact, the full-length ND2 in the mutant cybrids was synthesized at only ~4% of the normal rate, which is close to the predicted 2.8% ($= 1.05 \times 0.74^{12}$). Three subunits of complex I and one subunit of complex IV have $\geqslant 10$ lysine residues and were synthesized at $\leqslant 10\%$ of the normal rate in mutant cybrids, which explains the major respiratory and biochemical defects observed in cybrids and in muscle biopsies of MERRF patients.

Peptide mapping and immunoprecipitation experiments showed that the most highly labeled abnormal polypeptide, pMERRF, corresponded to a truncated form of an mtDNA-encoded subunit of cytochrome *c* oxidase, COI, and several other proteins were identified as N-terminal fragments of ND2 (Enriquez et al. 1995). Thus, the abnormal polypeptides appeared indeed to be products of premature termination of translation.

How Is the $tRNA^{Lys}$ Affected by the Mutation?

The 8344 mutation occurs in the $tRNA^{Lys}$ loop equivalent of the universal TΨC loop. An effect of the mutation on the accuracy of processing of the polycistronic transcript from which the $tRNA^{Lys}$ is derived was ruled out by the results of RNA gel-blot analysis. Both the mature mRNAs corresponding to the adjacent genes and the mature $tRNA^{Lys}$ were of the correct size in the mutant cybrids (Enriquez et al. 1995). However, the same type of analysis revealed that the steady-state level of the mutant $tRNA^{Lys}$ was decreased by 18%–35%. Furthermore, using conditions of isolation and electrophoresis that protected the amino acid–tRNA bond, Enriquez et al. (1995) found that the proportion of $tRNA^{Lys}$ that was charged was decreased in the mutant cells by 30%–40%. The combination of the two factors (amount and aminoacylation efficiency) produced a level of lysyl-$tRNA^{Lys}$ that was 50%–60% lower in mutant cells than in control cells.

Did the mutant charged $tRNA^{Lys}$ have any elongator activity? The mtDNA in the mutant cybrid cell line used most extensively in the work by Enriquez et al. (1995), pT1, had $\geqslant 98\%$ mutant mtDNA; furthermore, only mutant $tRNA^{Lys}$ could be detected in pT1 cells. Yet the cells were able to translate, albeit at a low rate, most mitochondrial mRNAs. In the case of the polypeptide least affected in its rate of synthesis, ND6, which has only two lysine residues, the rate of formation of the finished protein was reduced in pT1 cells by only 40%, relative to the control cell line (fig. 1). It is unlikely that the vanishingly small amount of wild-type $tRNA^{Lys}$ gene possibly present in pT1 cells could account for the protein synthesis observed in these cells. Indeed, pT1 cells were able to incorporate ^{3}H-lysine into all the various mitochondrial translation products except ND4L, which has no lysine codon in its reading frame. When compared to the labeling pattern of a control cybrid, the polypeptides most affected in their labeling rates by the mutation were the same as those showing the most differences after ^{35}S-methionine labeling (Enriquez et al. 1995). Not only did these results indicate the substantial functionality of the mutant tRNA in elongation, but they also ruled out any significant misincorporation of lysine residues or incorporation of other amino acids at lysine codons. The latter finding is in agreement with the lack of evidence of misacylated $tRNA^{Lys}$ in electrophoretic analysis of $tRNA^{Lys}$ charged in vivo.

It is likely that the decrease in the level of charged $tRNA^{Lys}$ in cells with the 8344 mutation can completely account for the observed premature termination of translation. This premature termination may be due to ribosomal frameshifting or to premature release of peptidyl-tRNA. Unfortunately, there is limited information, from other systems, on premature termination of translation resulting from reduced levels of charged tRNA. In bacteria, protein synthesis rates decrease rapidly when the ratio of charged tRNA to uncharged tRNA drops below a certain level, and the possibility has been raised that uncharged tRNA binding to the A site might lead to premature termination of translation (Goldman and Jakubowski 1990).

Like the MERRF mutation at position 8344, a mutation at position 8356 in the $tRNA^{Lys}$ gene causes transmitochondrial cell lines to produce an abnormal translation product of the size of pMERRF (Masucci et al. 1995). Apart from these two $tRNA^{Lys}$ mutations, no other mitochondrial tRNA mutation has been observed to cause premature termination of translation. Lymphoblastoid cell lines carrying a deafness-associated mtDNA mutation at position 7445 (Reid et al. 1994) process the $tRNA^{SerUCN}$ inefficiently, and the steady-state level of this tRNA is consequently diminished by 60%–75% (Guan et al. 1997; Reid et al. 1997). The absence of any truncated polypeptides in these cells and the results of analysis of the translation rates of various UCN-containing mRNAs suggest that the $tRNA^{SerUCN}$ causes a transient pause, rather than premature termination, in translation (M.-X. Guan, J. A. Enriquez, and G. Attardi, personal communication). The mutation at position 3243 in the $tRNA^{LeuUUR}$ gene, which is associated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) or with non–insulin-dependent diabetes mellitus (NIDDM; see Schon et al. 1997), causes a similar decrease (~65%) in the level of leucyl-$tRNA^{LeuUUR}$, but, again, no correlation has been found between the decrease in the synthesis rate of a given polypeptide and the number of UUR codons in its reading frame, nor has any truncated polypeptide been observed (Chomyn et

al. 1997). Thus, in the human mitochondrial system, lysine codons appear to be unique in their ability to cause premature termination of translation when the supply of charged tRNA is low.

Is There Normally an Excess of Mitochondrial tRNA?

Is the normal level of mitochondrial tRNAs in cells in culture rate limiting for protein synthesis, or is it in excess and, if so, how much? These questions are difficult to address in the organism, but it is possible to make an estimate of the amount of functional mitochondrial tRNA required to support protein synthesis in human cells in culture.

Hayashi et al. (1991) constructed a series of heteroplasmic transmitochondrial cell lines carrying both wild-type mtDNA and, in varying proportions, the mutant mtDNA that is associated with chronic progressive external ophthalmoplegia. The latter DNA contains a 5-kb deletion that eliminates three reading frames and five tRNA genes, and it creates a fusion reading frame containing ATPase 6 and ND5 sequences. In a mitochondrion containing only mutant genomes, no protein synthesis would occur, because of the lack of five tRNA genes. On the contrary, Hayashi et al. (1991) found that the fusion reading frame was translated in cells in which $\leqslant 55\%$ of their mitochondrial genomes carried a deletion. This complementation indicated that the products of the wild-type mitochondrial genomes mixed with the products of the deletion genomes. Hayashi et al. (1991) also observed a dramatic effect of varying the proportion of wild-type and mutant mtDNAs on the rate of synthesis of proteins encoded by the nondeleted genes. When $\geqslant 45\%$ of the mitochondrial genomes were wild type, synthesis of cytochrome *c* oxidase subunit II proceeded at the wild-type rate. With lower proportions of wild-type mtDNA, the synthesis rate declined sharply. Thus, assuming that the amount of a given wild-type tRNA is proportional to the number of genes for that tRNA, normal protein-synthesis rates required that one or more of the five tRNAs be expressed at a level $\geqslant 45\%$ of the wild-type level.

In a study using transmitochondrial cybrids that carried different proportions of wild-type and MERRF mtDNA, Yoneda et al. (1994) found that respiration rates were normal if $\geqslant 10\%$ of the mitochondrial genomes were wild type (fig. 2). This result agreed with the findings of an earlier study in which cultured myotubes heteroplasmic for the 8344 mutation showed an ~15% threshold for cytochrome *c* oxidase activity and cytochrome *c* oxidase subunit I synthesis (Boulet et al. 1992). Moreover, these results were fully consistent with data from muscle biopsies from MERRF families, which showed that the mutant DNA had to be present in high proportion, >85%, before clinical symptoms appeared

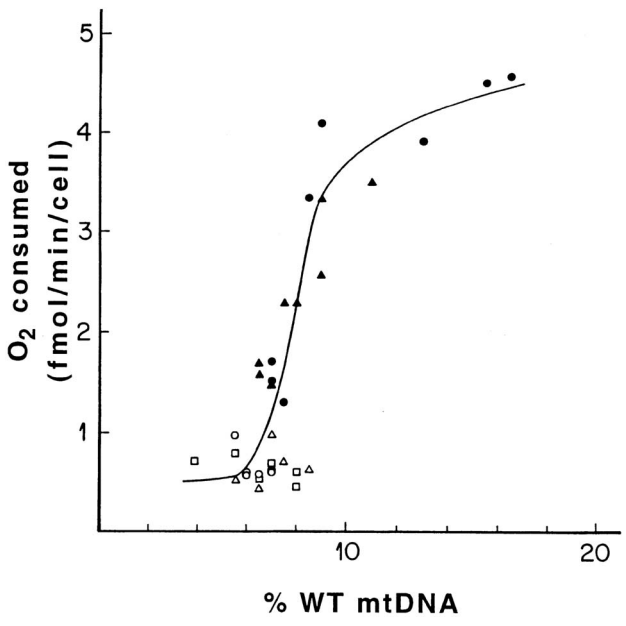

Figure 2 A small minority of wild-type mtDNA can correct the defective respiration caused by the 8344 MERRF mutation in different heteroplasmic transformants. Each type of symbol represents a different transmitochondrial clone. Individual clones were sampled over an extended period of time during which the genotype of some of the clones drifted toward a higher proportion of mutant DNA. Modified from the study by Yoneda et al. (1994).

(Shoffner and Wallace 1992). These thresholds for the 8344 MERRF mutation seemed to be at odds with the finding of Hayashi et al. (1991) that $\geqslant 45\%$ of the genomes in cells heteroplasmic for the large deletion must be wild type for full complementation to occur. However, this apparent discrepancy simply reflects the leakiness of the $tRNA^{Lys}$ mutation in MERRF cells, where $tRNA^{Lys}$ is inefficiently charged but, once charged, participates normally in protein synthesis.

According to this interpretation, the primary basis for the decreased protein-synthesis rate in homoplasmic or nearly homoplasmic MERRF mutant cells is the shortage of charged $tRNA^{Lys}$ molecules (or the lower ratio of charged to uncharged $tRNA^{Lys}$). In heteroplasmic cells, complementation occurs when enough wild-type lysyl-$tRNA^{Lys}$ is present to bring the level of total charged $tRNA^{Lys}$ up to the minimum level (or to the correct ratio of charged to uncharged tRNA) required to support normal protein-synthesis rates. In the investigations of the MERRF mutation described above, the level of charged $tRNA^{Lys}$ in the nearly homoplasmic mutant cybrid cell lines was 40%–50% of the control level. The capacity of 10% wild-type genomes to restore in the cells a normal rate of mitochondrial protein synthesis would imply that, under the assumption of an intrinsically normal elongator activity of the mutant $tRNA^{Lys}$, the threshold

for the amount of lysyl-tRNA^{Lys} required for normal translation is ~50% of the control level—that is, very close to the threshold for a wild-type tRNA gene level determined by the data of Hayashi et al. (1991).

With regard to other mitochondrial tRNAs, the mutation at position 7445, which was mentioned above, revealed a threshold for the tRNA^{SerUCN} of ~40% as being required to support a normal rate of protein synthesis (M.-X. Guan, J. A. Enriquez, and G. Attardi, personal communication). Therefore, it appears from the available data that the mitochondrial tRNA^{Lys}, the mitochondrial tRNA^{SerUCN}, and at least one other mitochondrial tRNA are normally present in cultured human cells in amounts not much in excess over what is needed in the cell. Further work is necessary to establish whether this conclusion can be extended to most, if not all, mitochondrial tRNAs, as seems reasonable.

Can mtDNA with the MERRF Mutation Complement Other Mutant Genomes?

A minority (10%) of wild-type genomes in transmitochondrial cybrids containing the 8344 MERRF mutation or the 3243 MELAS/NIDDM mutation in the tRNA^{LeuUUR} gene is sufficient to complement the mutant genomes and to support a normal respiration rate (Yoneda et al. 1994). In this reflected mitochondrial fusion, one would expect that, when cytoplasts containing virtually only mutant mtDNA in the tRNA^{Lys} gene were fused with cybrids carrying predominantly mutant mtDNA in the tRNA^{LeuUUR} gene, full protein-synthetic capacity and respiration would be restored. However, when Yoneda et al. (1994) carried out such a fusion, of 116 clones isolated without selection for respiration, 5 were found to contain $\geqslant$12% of each type of mtDNA, but none of them exhibited any complementation. All five clones had markedly low respiration rates and low protein-synthesis rates. These results were interpreted as indicating that the products of the MERRF and the MELAS genomes did not mix, so as to allow complementation to occur, and that the genomes were compartmentalized, perhaps within autonomous mitochondria.

A reasonable explanation of why complementation occurred between the mutant and wild-type genomes in cells derived from the same individual in the threshold experiments described earlier, is that the mutant genome originally arose among the wild-type genomes within one mitochondrion. Thereafter, this mutant genome was maintained with wild-type genomes within the same mitochondrial compartments during growth and division, for the obvious selective advantage that intramitochondrial complementation would provide.

The number of clones analyzed in the work of Yoneda et al. (1994) was small, and it was quite possible that, if selection for respiration had been applied on a large population of newly constructed cybrids, some clones with complementing genomes would have been isolated. Takai et al. (1997), applying very stringent selection conditions, were able to isolate cybrids in which complementation of two mtDNA mutations had occurred. More recently, J. Cabezas-Herrera, J. A. Enriquez, and G. Attardi (personal communication) have applied selection to a large-scale ρ^0 × cytoplast fusion that was made between cells carrying the 8344 MERRF mutation in >99% of their genomes and cells carrying a homoplasmic frameshift mutation in the ND4 gene. Very few cells survived the selection. These survivors, clones that carried both mitochondrial genomes and in which complementation between them had presumably occurred, represented only a very small fraction (<1%) of the total number of cybrids formed. These were quantified by screening clones from the same fusion mixture for the simultaneous presence of the two mtDNAs. These results, therefore, clearly confirmed the earlier finding that mixing of mitochondrial genomes originating in distinct organelles is infrequent, at least in established cell lines. Mitochondrial fusion is, however, well documented in certain developmental situations—namely, in gametogenesis (Hales and Fuller 1997) and in muscle development (Bakeeva et al. 1981; Boulet et al. 1992).

Future research will be aimed at understanding the behavior of the mitochondria and their DNA during growth and cell division and in postmitotic cells, and the factors that control mitochondrial fusion and mtDNA complementation and segregation. This understanding may someday provide the basis for therapies aimed at restoring the predominance of wild-type mtDNA in a heteroplasmic cell. Furthermore, we may learn the basis for the occurrence of distinct diseases produced by different mutations and for the variety of clinical phenotypes produced by the same mutation. Of course, the site of the mutation is important. Even mutations in tRNA genes will affect the four oxidative phosphorylation complexes containing mtDNA-encoded subunits differently (see fig. 1, for example). This bias in affected complexes was explicitly demonstrated by Villani and Attardi (1997) for cells bearing the 8344 MERRF mutation. Aside from this very fundamental aspect, the factors that are important for producing a particular phenotype may include the extent of leakiness of a given mutation, whether the mutation is recessive or dominant in a heteroplasmic situation, whether it can be complemented by wild-type mtDNA in the same organelle, and, if the mutation can be complemented, what the threshold of complementation of mutant mtDNA by wild-type genomes is. Also of major importance are the distribution of the mitochondrial genotypes among the mitochondria and the capacity of mitochondrial genomes carried within distinct organelles to interact with each other.

Superimposed on these phenomena would be growth control of the cells and cellular energy needs. In a proliferating tissue, such as lymphocytes, segregation of the mutation may lead to selection against cells having more homoplasmic mutant mitochondria. This segregation could account for the finding that lymphocytes often have lower proportions of mutant mtDNA than does muscle (Shoffner and Wallace 1992). Also, the role of segregation of wild-type and mutant mitochondrial genomes, during development, in generating a specific phenotype is of key importance and has been discussed (Schon et al. 1997). Nuclear factors clearly play a role in many of the factors mentioned above. Finally, because mitochondrial gene products interact directly with nuclear gene products to form enzyme complexes, an individual's nuclear makeup could also influence the severity of the disease phenotype. Not all of the above questions can be addressed by the use of cells in culture, but, certainly, cellular models of mitochondrial diseases isolated by repopulation of mtDNA-less cells, by immortalization of patients' cells, or by myoblast fusion, will continue to play an important role in these investigations.

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