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Nuclear Complementation Restores mtDNA Levels in Cultured Cells from a Patient with mtDNA Depletion

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Summary

We have studied cultured skin fibroblasts from a patient with a fatal mitochondrial disease manifesting soon after birth. These fibroblasts were found to grow only in the presence of pyruvate and uridine, a characteristic of cells lacking mtDNA (rho^0 cells). Southern blot and PCR analyses confirmed that the patient's fibroblasts contained less than 2% of control levels of mtDNA. Biochemical analyses indicated that the activities of all the respiratory-chain enzymes were severely decreased in mitochondria isolated from these fibroblasts. In order to elucidate the underlying molecular defect, cell fusions were performed between enucleated fibroblasts from this patient and a human-derived rho^0 cell line (rho^{0}A549.B2). The resulting cybrids were plated in medium lacking pyruvate and uridine, to select for the restoration of respiratory-chain function. Complementation was observed between the nuclear genome of the rho^{0}A549.B2 cells and the mtDNA of the patient's cells, restoring mtDNA levels and respiratory-chain function in the cybrid cells. These results indicate that mtDNA depletion in our patient is under the control of the nuclear genome.

Introduction

It is now well established that mutations of mtDNA are associated with several mitochondrial myopathies and encephalomyopathies (Harding 1991; Wallace 1992). Recently, a new class of mitochondrial disease has been reported which does not involve qualitative errors in mtDNA but quantitative depletion of mtDNA in affected tissues (Moraes et al. 1991; Mazziotta et al. 1992; Tritschler et al. 1992). In this disorder, individuals exhibit variable levels of mtDNA depletion (up to 98%) in affected tissues, while unaffected tissues have relatively normal levels of mtDNA. In addition, different tissues may be involved in related patients (Moraes et al. 1991; Tritschler et al. 1992). The molecular defect in this disorder has not been identified. It is possible that the primary defect may reside in either the mtDNA or a nuclear gene whose product is involved in mtDNA replication. Moraes et al. (1991) did not find evidence of a mtDNA mutation in the areas surrounding the origin of replication of the heavy strand (H-strand) or light strand (L-strand) of mtDNA. There is also no evidence of maternal inheritance, often found in mtDNA diseases (Moraes et al. 1991; Tritschler et al. 1992). It has been suggested that mtDNA depletion is inherited as an autosomal recessive (Moraes et al. 1991) or an autosomal dominant trait with incomplete penetrance (Mazziotta et al. 1992), both of which support the involvement of a nuclear gene. Alternatively, it was also suggested that this phenotype could be the result of a dominant nuclear mutation which is expressed only when combined with a certain mitochondrial genotype (Moraes et al. 1991).

We have studied fibroblasts from a patient (patient 1) who developed a fatal mitochondrial disease soon after birth (Leonard et al. 1991). The clinical presentation and family history were similar to those reported for cases of mtDNA depletion. Studies on cultured skin fibroblasts from patient 1 revealed a decrease in activities of the respiratory-chain enzymes and a quantitative decrease in mtDNA. It was also observed that, for growth, the fibroblasts were dependent on uridine and

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pyruvate. This requirement is well characterized for rho^0 cells which have been depleted of mtDNA by long-term exposure to low concentrations of ethidium bromide (Desjardins et al. 1986; King and Attardi 1989). This property of the patient's fibroblasts provided a selectable marker for our experiments, as complementation of these metabolic requirements indicated the reconstitution of mitochondrial function. We have used complementation analysis after somatic cell fusion to investigate the underlying defect in this disorder. This paper provides evidence that mtDNA depletion is controlled by the nuclear genome.

Patients and Methods

Clinical History

The clinical details of this patient (patient 1) have been described elsewhere (Leonard et al. 1991). In brief, he was the fourth child of healthy unrelated parents and was born after an uncomplicated full-term pregnancy. Soon after birth he developed progressive liver failure, widespread edema, hyponatremia, hypoalbuminemia, marked prolongation of clotting times, and lactic acidosis. He became progressively more hypotonic and unresponsive and died at 4 mo of age. The patient's eldest sibling was unaffected. An older sister (referred to as "patient 2" in this study) had a clinically similar illness and died at 4 mo of age. One older brother was noted to be hypertonic and jittery on the second day of life and died suddenly and unexpectedly at 4 wk of age (Clayton et al. 1986; Leonard et al. 1991). Initial studies conducted on cultured skin fibroblasts from patient 1 and patient 2 revealed a generalized mitochondrial defect (Clayton et al. 1986; Leonard et al. 1991). Respiratory-chain function as assessed in these previous studies suggested a less severe defect than was detected in our analyses (see Results). The explanation for this discrepancy is not known; however, it may relate to the suboptimal growth and assay conditions used previously.

Cell Culture

Fibroblast cultures were established by standard methods (Martin 1973). Two control fibroblast cell lines (161-Br and 171-Br) were acquired from the European Collection of Animal Cell Cultures. The rho^{0}A549.B2 cell line was derived from the human lung carcinoma cell line (A549) by a modified method of Desjardins et al. (1986).

Cells were normally grown in supplemented growth medium consisting of Dulbecco's modified Eagle medium (DMEM) containing 4.5 g of glucose/liter, supplemented with 10% FCS, 50 units of penicillin/ml, and 50 μg of streptomycin/ml, 50 μg of uridine/ml, and 110 μg of sodium pyruvate/ml. Selective growth medium was used when indicated and consisted of DMEM containing 4.5 g of glucose/liter, 5% dialyzed FCS, 50 units of penicillin/ml, and 50 μg streptomycin/ml.

Enucleation and Cell Fusion

For enucleation, fibroblasts were grown on collagen-coated plastic disks (2-cm diameter) prepared from the bottoms of tissue culture dishes (Veomett 1982). Enucleation was performed by inverting the disks in prewarmed DMEM containing 10 μg of cytochalsin B/ml, followed by centrifugation at 25,000 g_{max} for 19 min at 35°C (Veomett 1982). Under these conditions, greater than 90% enucleation was achieved, as determined by fixing the cells/cytoplasts (ethanol:acetic acid, 3:1) and staining with Mayer's hematoxylin. The disks containing cytoplasts were placed in 35-mm tissue culture dishes and were plated with 3×10^5 to 6×10^5 cells for 3 h prior to fusion. The cytoplasts and cells were fused as described elsewhere (Norwood and Zeigler 1982). Twenty-four hours after fusion the cells were plated in selective medium, and growing colonies were selected 10–14 d later.

Mitochondria Preparation and Enzyme Assays

Mitochondria were prepared from 10–20 tissue culture dishes (10-cm diameter) of confluent cells, essentially as described elsewhere (Rickwood et al. 1987), except that the homogenization buffer consisted of 10 mM Tris-Cl (pH 7.4), 0.25 M sucrose, and 1 mM EDTA. Rotenone-sensitive NADH-CoQ_1 reductase (complex I) was measured according to the method of Krige et al. (1992). The succinate cytochrome *c* reductase (complex II/III) assay was performed as described by King (1967). Cytochrome oxidase (complex IV) activity was determined by the procedure of Wharton and Tzagoloff (1967). Citrate synthase was assayed according to the method described by Coore et al. (1971), and malate dehydrogenase was measured as described elsewhere (Robinson et al. 1987). Protein concentrations were determined by the method of Lowry et al. (1951), with BSA used as standard.

Analysis of DNA

Total DNA was isolated from cultured cells by the procedure of Hauswirth et al. (1987). For Southern blot analysis of mtDNA, 2–4 μg of total DNA was digested overnight with the appropriate restriction enzymes, and

the resultant fragments were separated on either 0.8% or 1.0% agarose gels. The DNA was transferred to nylon membranes as described elsewhere (Ford et al. 1989). mtDNA for the probe was isolated from human placenta by the protocol of Welter et al. (1989). mtDNA prepared by the procedure of Palva and Palva (1985) was contaminated with some nuclear DNA, and a band of 24 kb was isolated after *Bam*HI digestion for the nuclear probe. The identity of this 24-kb segment of DNA is not known, but it consistently copurified with mtDNA isolated by this protocol. The mtDNA and nuclear DNA probes were isolated from agarose gels by the Gene Clean kit (Bio 101) and were labeled with fluorescein by the Random Primer Labeling kit (Amersham, U.K.). The nylon filters were prehybridized, hybridized, and developed according to the enhanced chemiluminescence (ECL) procedure (Amersham, U.K.).

The PCR amplification reaction contained 10 mM Tris-Cl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 200–500 ng of total DNA, and 1 μM each primer. The primers used for the amplification of mtDNA were M4 (nucleotides [nt] 2928–2947) and M5 (nt 3558–3539), which generated a 630-bp fragment. The primers for amplification of nuclear DNA (N1 and N2) hybridized to the P450 CYP2D6 gene and have been described by Smith et al. (1992). Primer N1 is located at nt 1828–1846, and N2 is located at nt 2163–2182 of this gene, generating a 354-bp product. The reaction was initially incubated at 94°C for 4 min, prior to the addition of 2.5 units of *Taq* DNA polymerase (Promega, U.K.). PCR was performed for 30 cycles of 1 min at 92°C, 1 min at 55°C, and 1 min at 72°C, followed by a final extension, of 10 min at 72°C. For quantitation, the PCR was performed on a dilution series of DNA. The mtDNA and nuclear DNA primers were used independently for these reactions, and the reactions were spiked with 2.5 μCi [α-^{32}P]dCTP (3,000 Ci/mmol). After amplification, the products were separated by agarose gel electrophoresis. Gel slices containing bands of interest were excised, and the radioactivity was eluted by Solvable (Du Pont, U.K.) prior to liquid scintillation counting.

Results

Growth curves of fibroblasts from patient 1 and of control fibroblasts are shown in figure 1*A* and *B*. It was observed that the cells from patient 1 did not grow in the absence of uridine and pyruvate, whereas control

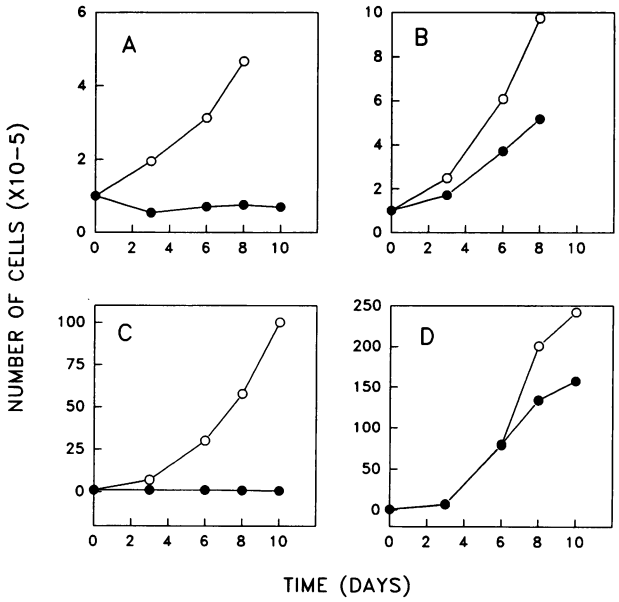

Figure 1 Growth of various cell lines in supplemented and selective growth medium. Cells were plated in a series of 10-cm tissue culture dishes (1×10^5 cells/dish) and were grown in either supplemented growth medium (○) or selective growth medium (lacking pyruvate and uridine) (●). At various times, the cells from individual plates were trypsinized and counted. *A*, Fibroblasts from patient 1. *B*, Control fibroblasts. *C*, rho^{0}A549.B2 cells. *D*, A549 cells (the parent cells used for construction of the rho^0 cell line).

fibroblasts could divide under these conditions. This dependence on pyruvate and uridine was also seen for the rho^0 cell line (rho^{0}A549.B2) but not for the parent cell line (A549), from which the rho^0 cells were created (fig. 1*C* and *D*).

Mitochondria were isolated from fibroblasts of patient 1 and were assayed for enzymes of the respiratory chain, as well as for citrate synthase and malate dehydrogenase (table 1). The activities of the respiratory-chain enzymes (complex I, complex II/III, and complex IV) were severely decreased compared with those in the controls. In contrast, both citrate synthase and malate dehydrogenase activities did not differ significantly from those in the controls. These data indicated that the defect specifically affected enzymes of the respiratory chain, which contain mtDNA encoded subunits, and a similar result was observed for the rho^{0}A549.B2 cells (table 1).

Southern blot analysis of mtDNA from two control fibroblast cell lines, fibroblasts of patient 1, and the rho^{0}A549.B2 cell line is shown in figure 2*A*. Total DNA was digested with *Pvu*II, and the blot was simulta-

Table 1

Enzyme Analyses of Mitochondria Isolated from Human Cell Lines

Enzyme	Control Fibroblasts (n = 5)	Patient 1 Fibroblasts (n = 2)	F1 Cells[a] (n = 3)	rho^{0}A549 Cells (n = 3)	A549 Cells (n = 3)
Complex I	25.6 ± 6.5	.93	...[b]	...[b]	...[b]
Complex II/III	59.1 ± 16.5	Not detected	74.3 ± 31.6	Not detected	96.0 ± 28.7
Complex IV	3.24 ± .90	.06	2.98 ± .85	.02 ± .01	4.18 ± .51
Citrate synthase	188 ± 55	181	176 ± 47	156 ± 5	231 ± 33
Malate dehydrogenase ...	2,708 ± 299 (n = 3)	2,271	1,947 ± 370	2,366 ± 235	3,008 ± 483

NOTE.—The activities of complex I, complex II/III, citrate synthase, and malate dehydrogenase are expressed as nmol/min/mg of protein, while that of complex IV is expressed as the first-order rate constant k/min/mg.

[a] Cybrid cells created by fusing enucleated fibroblasts from patient 1 and rho^{0}A549 cells.

[b] Rotenone-sensitive complex I activity was difficult to interpret in the A549-derived cell lines. This characteristic of these cells is under further investigation.

neously hybridized with a mtDNA probe (16.6 kb), as well as with a fragment of nuclear DNA (24 kb). The nuclear probe serves as an internal control which ensures that DNA is present and intact in each lane of the gel. The control fibroblasts showed a band at 16.6 kb, representing mtDNA; however, this band was not detected in the DNA from either patient 1 or the rho^{0}A549.B2 cells (fig. 2*A*). This same result was obtained with the restriction enzymes *Bam*HI and *Apa*I (data not shown), eliminating the possibility of a *Pvu*II polymorphism.

To investigate this result further, PCR analysis was performed on the same DNA samples (fig. 2*B*). PCR was performed with two sets of primers; one set, specific to mtDNA, resulted in a fragment of 630 bp, and the other set, specific to a single-copy nuclear gene (P450-CYP2D6), yielded a DNA fragment of 354 bp (Smith et al. 1992). When both sets of primers were included in the PCR, there was a preferential amplification of mtDNA; therefore, quantitation of mtDNA levels was performed by using samples where the mtDNA and nuclear DNA primers were used independently. Quantitation was performed by PCR on a dilution series of DNA (data not shown). These results showed that there was less than 2% of mtDNA (0.91 ± 0.27% of controls; n = 3) in the DNA from fibroblasts of patient 1 and confirmed that there was no detectable mtDNA in the DNA extracted from the rho^{0}A549.B2 cells.

In order to determine whether the underlying defect was in the mtDNA, nuclear DNA, or both genomes, a number of cell fusion experiments were conducted. The first experiment was designed to determine whether the mitochondria from control fibroblasts could replicate in the patient's cells. To this end, control fibroblasts were enucleated, and the resulting cytoplasts were fused to fibroblasts from patient 1. The fusion products were plated in selective medium (lacking pyruvate and uridine). Since the cytoplasts cannot survive in culture, and since the patient's cells cannot grow without added pyruvate and uridine, any growing colonies would indicate restored mitochondrial function. No growing colonies were observed over the next 2 mo. Although not unequivocal, this result may indicate that the mtDNA from control cells could not replicate in the cells of patient 1, suggesting that the abnormality in these cells was caused by a nuclear-encoded product. To investigate this further, a second experiment was designed to determine whether the residual mtDNA of patient 1 could replicate and restore mitochondrial function in a normal nuclear environment. Fibroblasts from patient 1 were enucleated, and the cytoplasts were fused to rho^{0}A549.B2 cells which are devoid of mtDNA. As both these cell lines require pyruvate and uridine for growth, complementation was measured by plating the fusion products in selective medium (lacking uridine and pyruvate). Growing colonies were observed 10–14 d after fusion, and one of these colonies (F1) was ring cloned and grown up for further investigations. The F1 cybrid cells had the morphology and growth characteristics of the A549 cells (parent cells used to create the rho^0 cell line) and quickly generated sufficient cells for the isolation of mitochondria. Mitochondria were isolated from this cybrid cell line and were assayed for respiratory-chain enzymes, as well as for citrate synthase and malate dehydrogenase. Table 1 illustrates that the activities of the respiratory-chain enzymes (complex II/III and complex

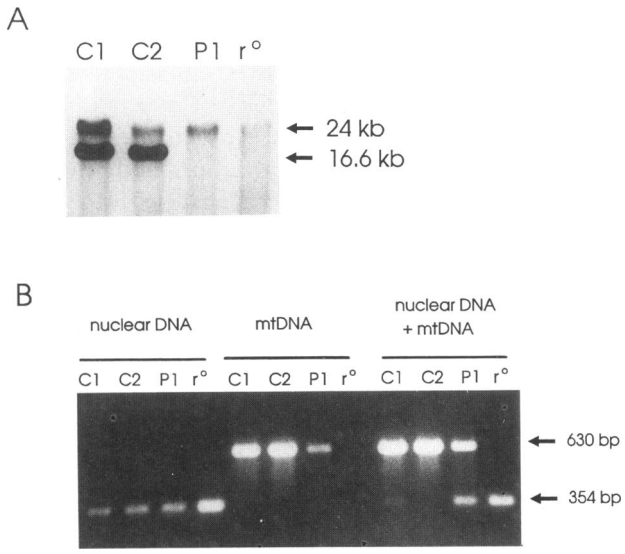

Figure 2 Southern blot and PCR analysis of DNA extracted from the cell lines indicated. *A*, Southern blot analysis using total DNA isolated from two control fibroblast cell lines (C1 and C2), fibroblasts of patient 1 (P1), and rho^{0}A549.B2 cells (r^0). Three micrograms of each DNA sample was digested with *Pvu*II, electrophoresed through a 0.8% agarose gel, and transferred to a nylon membrane. The blot was hybridized simultaneously with fluorescein-labeled mtDNA (16.6 kb) and a fluorescein-labeled 24-kb nuclear fragment and was developed by the ECL procedure. *B*, PCR amplification of mtDNA and nuclear DNA. Amplification was as follows: for nuclear DNA, 500 ng of DNA was used, and the primers were designed to generate a 354-bp fragment; for mtDNA, 200 ng of DNA was used, and the primers were designed to generate a 630-bp fragment; and for nuclear and mtDNA, 200 ng of DNA was used, and both sets of primers were included in the reaction.

IV) had been restored in the F1 cell line. Southern blot analysis indicated that the mtDNA levels had also been restored (fig. 3). The level of mtDNA in the F1 fusion product was equal to that of the A549 cell line (fig. 3). To confirm that the mtDNA in the cybrid was indeed that of patient 1 and not the result of the unlikely conversion of the rho^{0}A549.B2 cell line to its original mtDNA genotype (A549), we developed a method of distinguishing the mtDNA of patient 1's fibroblasts from that of the A549 cell line. Since there was not enough mtDNA present in fibroblasts of patient 1 to detect a signal on Southern blots, for these experiments we used DNA isolated from fibroblasts of the patient's sister (patient 2). The rationale for this was that mtDNA is exclusively maternally inherited in humans (Giles et al. 1980), and therefore patient 1's mtDNA should be identical to that of his sister. Fibroblasts from the sister (also clinically affected) showed a decrease in mtDNA, but not to the extent of that in her brother, and it therefore gave a visible, although weak, signal on Southern blot. An RFLP was detected between the sister's mtDNA and the mtDNA of the A549 cells when the restriction enzyme *Alu*I was used, generating an additional band at 0.9 kb in the sister's mtDNA. When DNA from the F1 cybrid cell line was digested with *Alu*I and blotted, the mtDNA generated the same restriction pattern as that in the patient's sister (fig. 3*B*). This result confirms that the mtDNA of the patient's cells had been replicated up to normal levels under the control of the rho^{0}A549.B2 nucleus in the fusion cell line.

Discussion

We have characterized cultured skin fibroblasts from an infant (patient 1) who was previously reported to have a fatal mitochondrial disease (Leonard et al. 1991). These fibroblasts exhibited a severe reduction (more than 98%) in mtDNA levels and a concomitant reduction in respiratory-chain enzyme activities. In an effort

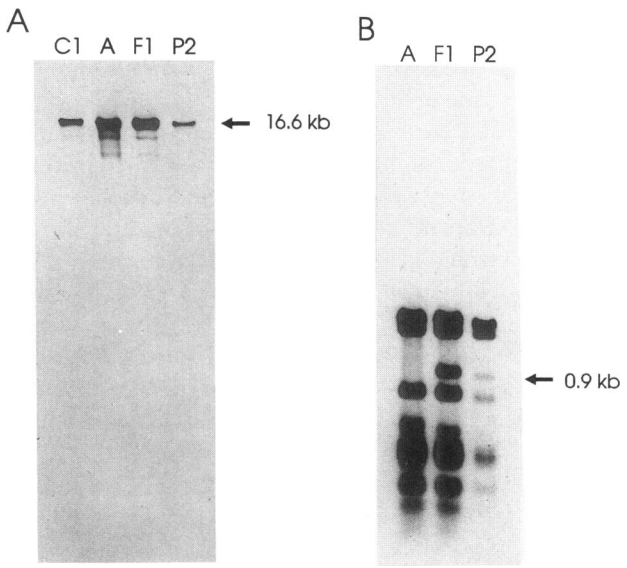

Figure 3 Southern blot analysis of the F1 cybrid cell line. Total DNA was isolated from a control fibroblast cell line (C1), the A549 cells (A), the F1 cybrid cell line (F1), and patient 2 (P2, the sister of patient 1). DNA (2 μg for C1, A549, and F1; and 4 μg for P2) was digested with *Pvu*II (panel *A*) or *Alu*I (panel *B*) and was separated on a 1% agarose gel. After transfer of the DNA to nylon membranes, the blots were hybridized with fluorescein-labeled mtDNA and were developed by the ECL procedure. Linearized mtDNA is indicated in panel *A* (16.6 kb), and the RFLP in P2's cells and also in the F1 cybrid cells generated an additional 0.9-kb fragment (panel *B*).

to elucidate the underlying genetic defect, cell fusion experiments were performed. These experiments were facilitated by the fact that, for growth, the patient's fibroblasts were dependent on uridine and pyruvate. This property of patient 1's cells provided a selectable marker, as complementation of these metabolic requirements indicated the restoration of mitochondrial function.

In the first cell fusion experiment, the addition of normal mitochondria into cells of patient 1 failed to restore mitochondrial function. This indicated that the defect may be located in the nuclear genome of patient 1 and that this defect prevents normal mtDNA from replicating. In the second fusion experiment, mitochondria from the cells of patient 1 could restore mitochondrial function in rho^{0}A549.B2 cells. The cybrid cell line (i.e., F1) was found to have restored mtDNA levels and respiratory-chain function. These results eliminated the possibility of a defect in the patient's mtDNA in this disease, as his mtDNA can replicate and function in a normal nuclear environment. Therefore, mtDNA depletion in these cells is due to a defect in a nuclear gene, probably one involved with mtDNA replication.

The basic mechanism of mtDNA replication is known; however, many of the factors involved remain undefined (Clayton 1982, 1991). Replication of mtDNA is initiated at the origin of H-strand replication, located within the displacement loop (D-loop), and proceeds unidirectionally. When the L-strand origin of replication is exposed as a single-stranded template, replication is subsequently initiated from this strand. The proteins involved in this process are encoded by nuclear genes (Clayton 1991). Replication is primed with RNA and involves DNA polymerase gamma, topoisomerase, single-stranded DNA-binding proteins, and possibly an mtRNA-processing ribonucleoprotein (Clayton 1982, 1991; Kiss and Filipowicz 1992; Topper et al. 1992). Errors in replication could arise from mutations in the genes encoding any of these factors; however, different levels of mtDNA in different tissues are difficult to explain on this basis. Differential tissue involvement within patients and between patients could be explained if the nuclear factor is involved in processes such as those which control the mtDNA copy number in somatic tissues. Little is known about the mechanisms which set mtDNA copy number in various somatic tissues or about those which coordinate mtDNA levels with changes in organelle or cell populations (Clayton 1982).

It has been suggested that differential tissue involvement may result from an error in the timing of the resumption of mtDNA replication in early embryogenesis (Moraes et al. 1991). Somatic cells contain 2–10 copies of mtDNA per organelle; however, during oogenesis the number decreases to approximately 1–2 copies and does not increase after fertilization, until the blastocyst stage (Moraes et al. 1991). If the gene(s) controlling the resumption of mtDNA replication is not expressed at the appropriate time in development, it is conceivable that individual stem-cell populations could possess abnormally reduced numbers of mtDNA (Moraes et al. 1991). This explanation could be used to understand both the variable expression of mtDNA depletion within tissues of an individual and the variable expression among different members of the same family (Moraes et al. 1991). However, for this to hold true, expression of the gene(s) involved in the resumption of mtDNA replication must be expressed to different extents in different subpopulations of stem cells, or there must exist a mechanism for preventing the residual mtDNA from repopulating the various tissues up to normal levels.

Further characterization of the cell lines from patients 1 and 2 will provide a better understanding, not only of the molecular mechanisms underlying this disorder but also of the interactions between the nuclear and mitochondrial genomes in the establishment and maintenance of mtDNA levels. We are currently using a mammalian expression library to select a cDNA which complements the molecular defect in patient 1's cells and thereby restores mtDNA levels and respiratory-chain function.

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