Respiratory-deficient human fibroblasts exhibiting defective mitochondrial DNA replication

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We have characterized cultured skin fibroblasts from two siblings affected with a fatal mitochondrial disease caused by a nuclear genetic defect. Mitochondrial respiratory-chain function was severely decreased in these cells. Southern-blot analysis showed that the fibroblasts had reduced levels of mitochondrial DNA (mtDNA). The mtDNA was unstable and was eliminated from the cultured cells over many generations, generating the *rho*^{0} genotype. As the mtDNA level decreased, the cells became more dependent upon pyruvate and uridine for growth. Nuclear-encoded subunits of respiratory-chain complexes were synthesized and imported into the mitochondria of the mtDNA-depleted cells, albeit at reduced levels compared with the controls. Mitochondrial protein synthesis directed by the residual mtDNA indicated that the mtDNA was expressed and that the defect specifically involves the replication or maintenance of mtDNA. This is a unique example of a respiratory-deficient human cell line exhibiting defective mtDNA replication.

INTRODUCTION

The biogenesis and function of mitochondria depend on the complementation of both the nuclear and mitochondrial genomes. The human mitochondrial genome is a double-stranded circular molecule of 16569 bp containing 37 genes encoding two rRNAs, 22 tRNAs and 13 polypeptides. All 13 polypeptides are components of the respiratory chain, including seven subunits of NADH:ubiquinone reductase (complex I), cytochrome *b* of ubiquinol:cytochrome *c* reductase (complex III), subunits I, II and III of cytochrome oxidase (complex IV) and subunits 6 and A6L of ATPase (complex V) [reviewed by Wallace (1992)]. All other mitochondrial proteins, including those involved with the maintenance, replication, transcription and translation of the mitochondrial genome, are encoded by nuclear genes.

To study the role of each genome in mitochondrial function and biogenesis, several groups have investigated respiratory-deficient mutants created in both lower and higher eukaryotes (Sun et al., 1975; Ditta et al., 1976; Tzagoloff and Dieckmann, 1990). Respiratory-defective mutants with cytoplasmically inherited mutations, as well as mutants with defects in nuclear genes, have been identified. These mutants can be roughly divided into three groups: (i) mutations in specific genes encoding enzymes of intermediary metabolism; (ii) mutations which alter the components of the mitochondrial import machinery; and (iii) altered factors involved in the maintenance or expression of mtDNA. These mutants have provided considerable insight into the mechanisms involved in the maintenance of mitochondrial function (Tzagoloff and Myers, 1986; Attardi and Schatz, 1988).

Cell lines derived from patients with mitochondrial disease provide an opportunity for one to conduct similar studies using human cells. Respiratory-deficient human cell lines have been identified with both cytoplasmic- and nuclear-controlled defects. Respiratory-defective-cell cultures have been established with various mitochondrial DNA (mtDNA) abnormalities, including a point mutation in the mitochondrial tRNA^{Leu} gene from patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes ('MELAS') (Kobayashi et al., 1991; King et al., 1992), a point mutation in the mitochondrial tRNA^{Lys} gene from patients with myoclonic epilepsy and ragged red fibres ('MERRF') syndrome (Chomyn et al., 1991) and cell lines with deletions in mtDNA from patients with chronic progressive external ophthalmoplegia (Hayashi et al., 1991). Respiratory-defective cell lines with deficiencies of nuclear-encoded mitochondrial proteins have also been identified (Robinson et al., 1986; Glerum et al., 1989). Recently, two human diseases have been associated with primary nuclear mutations which control the stability of the mitochondrial genome. Firstly, several families have been described with an autosomal dominantly inherited mitochondrial myopathy characterized by multiple deletions of variable length in muscle mtDNA (Zeviani et al., 1989; Zeviani et al., 1990; Servidei et al., 1991). However, no myoblast cell lines have yet been reported from these patients. Secondly, a disease associated with severe depletion of mtDNA in affected tissues has recently been shown to be under nuclear control (Moraes et al., 1991; Tritschler et al., 1992; Bodnar et al., 1993). In the present study we have characterized fibroblast cell lines exhibiting mtDNA depletion derived from two siblings (referred to as patients 1 and 2) with mitochondrial disease. Previous experiments fusing cells from patient 1 to human-derived *rho*^{0} cells have shown that the mtDNA depletion is due to a defect in the nuclear genome and not the mtDNA (Bodnar et al., 1993). Here we show that the mtDNA in these fibroblasts was unstable and was eliminated from the cells over successive generations in culture. These fibroblast cell lines are reminiscent of a group of *petite* mutants of the yeast *Saccharomyces cerevisiae*, with unstable mtDNA due to defects in mitochondrial transcription or translation (Myers et al., 1985; Tzagoloff and Dieckmann, 1990). However, unlike their yeast counterparts, the mtDNA in these cells can be expressed, suggesting that the defective component is involved directly in the replication or maintenance of mtDNA.

Abbreviations used: mtDNA, mitochondrial DNA; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; ECL, Enhanced Chemiluminescence.

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MATERIALS AND METHODS

Cell lines and growth media

Fibroblast cell lines from the affected infants (patients 1 and 2), mother and father were previously established by standard methods (Martin, 1973). The clinical details of patients 1 and 2 have been previously described (Leonard et al., 1991; Clayton et al., 1986). Two control fibroblast cell lines (161BR and 171BR) were acquired from the European Collection of Animal Cell Cultures and the other controls were obtained from skin biopsies of healthy individuals as described by Martin (1973).

Cells were normally grown in supplemented growth medium consisting of Dulbecco's modified Eagle medium (DMEM) containing 25 mM glucose, supplemented with 10% fetal-calf serum (FCS), 50 units/ml penicillin, 50 μg/ml streptomycin, 0.2 mM uridine and 1 mM sodium pyruvate. Selective growth medium was used for the growth curves and consisted of DMEM containing 25 mM glucose, 5% dialysed FCS, 50 units/ml penicillin and 50 μg/ml streptomycin.

Isolation of mitochondria and enzyme assays

Mitochondrial fractions were prepared from $(1–2) \times 10^7$ cells collected at confluency, essentially as previously described (Rickwood et al., 1987), except that the homogenization buffer was 10 mM Tris/HCl (pH 7.4)/0.25 M sucrose/1 mM EDTA.

Rotenone-sensitive NADH:coenzyme Q_1 reductase (complex I) activity was measured by the method of Krige et al. (1992). Succinate:cytochrome *c* reductase (complex II and III) and cytochrome oxidase (complex IV) activities were determined by the procedures of King (1967) and Wharton and Tzagoloff (1967) respectively. Citrate synthase was measured by the method of Coore et al. (1971). Protein concentrations were determined by the procedure of Lowry et al. (1951), with BSA as standard.

Southern-blot and PCR analysis of DNA

Total DNA was isolated from cultured cells by the procedure described by Hauswirth et al. (1987). For Southern-blot analysis of mtDNA, 3 μg of total DNA was digested with the restriction endonuclease *Pvu*II, electrophoresed through 0.8%-agarose gels and transferred to nylon membranes as previously described (Sambrook et al., 1989). mtDNA for the probe was isolated from human placenta by the protocol of Welter et al. (1989). The nuclear DNA probe was a 5.8 kb fragment of 18 S rDNA. This fragment was cloned into the *Eco*RI site of the Bluescript vector. The mtDNA and nuclear DNA probes were isolated from agarose gels using the Gene Clean Kit (Bio 101 Inc.) and were labelled with fluorescein by the Random Primer Labelling Kit (Amersham). The nylon filters were prehybridized, hybridized and developed according to the Enhanced Chemiluminescence (ECL) procedure (Amersham, International).

The PCR amplification reaction contained 10 mM Tris/HCl (pH 9.0 at 25 °C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 1 μM of each primer. For amplification of mtDNA, 1–100 ng of total DNA was used and the primers were M4 [nucleotides (nt) 2928–2,947] and M5 (nt 3558–3539), which generated a 630 bp fragment. For amplification of nuclear DNA, 250 ng of total DNA and primers (G3 and G4) were used. These primers hybridize to the P450-*CYP2D6* gene (Smith et al. 1992). Primer G3 is located at nt 2616–2636, and G4 is located at nt 2885–2867 of this gene, generating a 269 bp product. The reaction mixture was initially incubated at 94 °C for 4 min prior to the addition of 2.5 units of *Taq* DNA polymerase (Promega Corp.). PCR was performed for 30 cycles of: 1 min at 92 °C; 1 min at 55 °C (60 °C for the nuclear primers); and 1 min at 72 °C. For quantification, the PCR was performed on a dilution series of DNA. The reactions were spiked with 2.5 μCi of [α-^{32}P]dCTP (3000 Ci/mmol). Following amplification the products were separated on 1.2%-agarose gels. Gel slices containing bands of interest were excised and the radioactivity was eluted using Solvable [du Pont (U.K.)] prior to liquid-scintillation counting.

Western-blot analysis

Proteins (30 μg per sample) were separated by SDS/PAGE on 14%-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose using the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The blots were incubated with antibodies against the 49 kDa subunit of complex I (Haines et al., 1992), the 24 kDa subunit of complex I or succinate dehydrogenase. The blots were incubated with these antibodies for 1 h at a 1:100 dilution and developed using Protein G–alkaline phosphatase conjugate as previously described (Towbin et al., 1979).

Mitochondrial protein synthesis

Mitochondrial protein synthesis in cultured fibroblasts was evaluated essentially as described by Beattie and Sen (1987). Confluent 100 mm dishes of control fibroblasts and fibroblasts of patient 2 were incubated in growth medium containing [^{35}S]-methionine (50 μCi/ml, 1217 Ci/mmol) for 4 h in the presence of either 100 μg/ml emetine (a cytosolic translation inhibitor) or 100 μg/ml emetine and 200 μg/ml chloramphenicol (a mitochondrial translation inhibitor). One confluent dish of control cells and three dishes of patient 2's cells were used for each of these conditions. The cells were washed with PBS and incubated for 1 h in normal growth medium. The cells were collected by scraping, washed with PBS and pelleted in a Microfuge at 10500 $g_{av.}$ for 5 min. Mitochondrial fractions were prepared as described above and dissolved in sample buffer. Labelled proteins were separated on an 18%-urea/SDS/polyacrylamide gel (Kadenbach et al 1983), followed by fluorography (Chamberlain, 1979).

RESULTS

Respiratory-chain function in fibroblasts from controls, patient 1 and patient 2

Mitochondria were isolated from control fibroblasts and fibroblasts of mother, father and patients 1 and 2 and assayed for respiratory-chain-enzyme activities as well as for citrate synthase activity (Table 1). The parents' mitochondrial enzyme activities did not differ from control values; however, mitochondria isolated from the fibroblasts from patients 1 and 2 showed a specific decrease in all respiratory-chain enzyme activities. Mitochondria from patient 1's fibroblasts had a greater-than-95% reduction of activities of NADH:ubiquinone reductase (complex I), succinate cytochrome *c* reductase (complex II/III) and cytochrome oxidase (complex IV), whereas the activity of the matrix enzyme citrate synthase was normal. Fibroblasts from patient 2 showed a greater than 60% reduction in the activities of all respiratory-chain enzymes, while citrate synthase activity was within control levels. The respiratory-chain-enzyme activities for the fibroblasts from the controls, mother and father were stable between passage numbers. For fibroblasts of patient 2, however, the activity of the respiratory-chain enzymes decreased progressively in mitochondria isolated from cells at increasing passage number (results not shown). In addition, for both patients 1 and 2 we observed a decrease in activities from earlier experiments conducted at earlier passage numbers (Leonard et

Table 1 Enzyme analyses of mitochondria isolated from human fibroblast cell lines

The activities of rotenone-sensitive NADH:coenzyme Q_1 reductase (CxI), succinate cytochrome *c* reductase (CxII/III) and citrate synthase (CitSyn) are expressed as nmol/min per mg of protein, while cytochrome oxidase (CxIV) activity is expressed as the first-order rate constant, *k* (min/mg). Abbreviations: ND, not detectable; PN, passage number.

Enzyme	Control fibroblasts ($n = 6$)*	Mother's fibroblasts ($n = 3$)	Father's fibroblasts ($n = 3$)	Patient 1 fibroblasts ($n = 3$)	Patient 2 fibroblasts† ($n = 3$)
CxI	25.6±5.8	24.3±1.7	30.8±5.4	0.62±1.07	10.1±4.3
CxII/III	58.4±14.8	43.6±7.8	59.3±8.5	ND	12.8±11.3
CxIV	3.07±0.90	2.89±0.21	2.39±0.26	0.05±0.02	0.87±0.31
CitSyn	187±49	176±16	144±12	162±51	228±39
PN...	4→16	6→10	5→9	7→14	6→10

* The values reported for controls represent the average for six independent control fibroblast lines.
† In this cell line it was evident that the activities of CxI,CxII/III and CxIV decreased with increasing passage number.

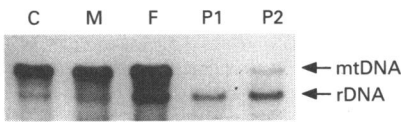

Figure 1 Southern-blot analysis of fibroblast DNA

Total DNA was extracted from control fibroblasts (C), and fibroblasts from mother (M), father (F), patient 1 (P1), and patient 2 (P2). A 3 μg portion of DNA was digested with *Pvu*II, electrophoresed through a 0.8%-agarose gel and transferred to a nylon membrane. The blot was simultaneously hybridized with fluorescein-labelled human mtDNA and a fragment of the 18 S rRNA gene and developed according to the ECL procedure (Amersham). This Southern blot was conducted using DNA extracted from cell cultures at the following passage numbers: C, passage number 8; M, passage number 6; F, passage number 5; P1, passage number 7; and P2, passage number 6.

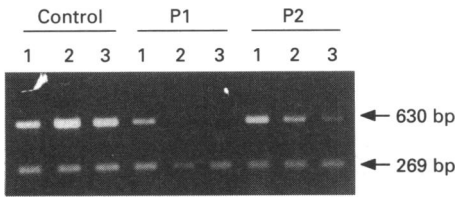

Figure 2 PCR analysis of fibroblast DNA isolated from cells at increasing passage numbers

Total DNA was isolated from control fibroblasts (C) and fibroblasts of patient 1 (P1) and patient 2 (P2) at increasing passage numbers. PCR was performed using primers specific to mtDNA which generated a 630 bp fragment. For this reaction 1 ng of control DNA, 100 ng of DNA from P1 and 5 ng of DNA from P2 were used. The nuclear DNA fragment (269 bp) was generated using primers which hybridize to the P450-*CYP2D6* gene (Smith et al., 1992) and 250 ng of total DNA. The nuclear and mitochondrial PCR reactions were performed independently, and 10 μl of each reaction mixture (from the same DNA samples) were combined prior to electrophoresis through a 1.2%-agarose gel. The numbers 1, 2 and 3 represent increasing passage numbers incremented by 3 passages each time, starting at passage numbers 5, 7 and 6, for control, P1 and P2 respectively.

al., 1991; Clayton et al., 1986). When the cells from patients 1 and 2 were received for the current experiments, the exact passage numbers of the cultures were not known. For comparison of the data within the present paper the number of passages since obtaining the cells are stated.

Citrate synthase activity in whole-cell homogenates was used to assess the mitochondrial content of the cells from patient 1 and controls. In patient 1 the citrate synthase activity was both stable with increasing passage number (47.3, 47.5 and 48.6 nmol/min per mg of protein for passage numbers 7, 10 and 14 respectively) and similar to control values (53.4±10.8 nmol/min per mg). This suggests mitochondrial content in these cells was both normal and stable with passage number.

Analysis of mtDNA

The biochemical data suggested that the defect in the fibroblasts of patients 1 and 2 specifically affected the respiratory chain, which contains both nuclear- and mtDNA-encoded subunits. To analyse mtDNA, total DNA isolated from each cell line was digested with *Pvu*II and transferred to nylon membranes. The blot was simultaneously hybridized with a mtDNA probe (16.6 kb) and a fragment of nuclear encoded 18 S rDNA (12.0 kb) (Figure 1). This nuclear probe serves as a control to compensate for unequal amounts of DNA loaded in each lane of the gel. The Southern blot revealed a severe reduction in mtDNA in fibroblasts from both patients 1 and 2. Quantification was performed using PCR on a dilution series of these DNA samples (results not shown). These results showed that there was only 0.9±0.3% ($n = 3$, passage number 7) and 16.2±1.4% ($n = 3$, passage number 6) of control mtDNA levels in fibroblasts from patient 1 and patient 2 respectively. As the respiratory-chain-enzyme activity decreased with increasing passage number of fibroblasts, we investigated whether the levels of mtDNA were stable over time in culture. PCR analysis was performed on DNA samples prepared from cells at increasing passage numbers (Figure 2). 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 269 bp (Smith et al., 1992). Figure 2 shows that while the level of nuclear DNA remained constant, mtDNA levels in fibroblasts from both patient 1 and 2 decreased with increasing passage number. This decrease in mtDNA was not seen in DNA samples from the father's, mother's or control fibroblasts investigated at comparable passage number (only one control is shown).

Growth requirements of mtDNA-depleted cells

Mammalian cells that have been artificially depleted of mtDNA (*rho*^{0} cells) by treatment with ethidium bromide are dependent on pyruvate and uridine for growth (King and Attardi, 1989). We observed that this was also true for fibroblasts from patient 1 and

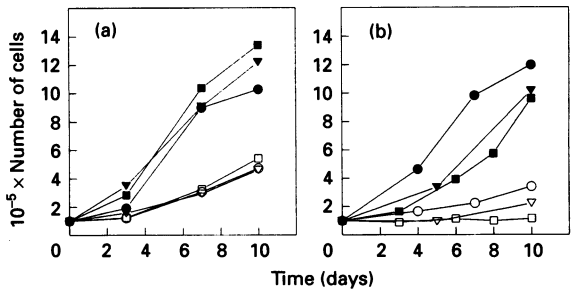

Figure 3 Growth curves of control fibroblasts and fibroblasts from patient 2 in medium with and without pyruvate and uridine

Control cells (1×10^5) and cells of patient 2 were plated in a series of 10 cm-diameter tissue culture dishes and grown in medium lacking pyruvate and uridine (open symbols) or medium containing pyruvate and uridine (closed symbols). At indicated times the cells were trypsin-treated and their numbers counted. (**a**) Control fibroblasts at passage 8 (●), passage 10 (▼) and passage 12 (■). (**b**) Fibroblasts from patient 2 at passage 5 (○), passage 9 (▽) and passage 10 (□).

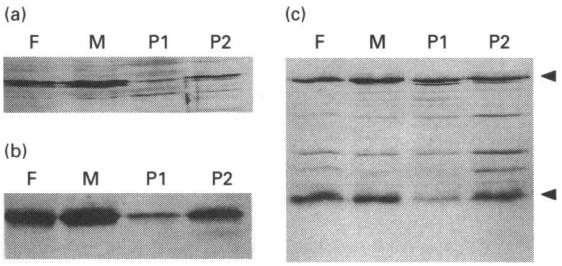

Figure 4 Western-blot analysis of nuclear-encoded mitochondrial proteins

Mitochondria isolated from fibroblasts of the father (F), mother (M), patient 1 (P1) and patient 2 (P2) (at passage numbers 5, 10, 7 and 6 respectively) were electrophoresed through 14%-polyacrylamide gels (30 μg of protein for each sample) and transferred to nitrocellulose. Western blotting was performed using antibodies against the 49 kDa subunit of complex I (**a**), the 24 kDa subunit of complex I (**b**) and succinate dehydrogenase (**c**). The 27 kDa and 70 kDa subunits of succinate dehydrogenase are indicated by the arrowheads.

patient 2 with decreased levels of mtDNA. Growth curves of patient 2 showed that, as the passage number increased and the mtDNA level decreased, cell growth slowed down markedly (Figure 3b, open symbols), whereas growth in the presence of pyruvate and uridine was relatively normal (Figure 3b, closed symbols). Growth curves of control fibroblasts did not show this effect with increasing passage number.

Western-blot analysis of nuclear-encoded mitochondrial proteins

In order to establish if the fibroblasts of patients 1 and 2 had normal synthesis and import of nuclear-encoded mitochondrial proteins, Western blots were performed using antibodies against nuclear-encoded subunits of complex I (subunits 24 kDa and 49 kDa) and the 27 kDa and 70 kDa subunits of succinate dehydrogenase (Figure 4). The Western blots were conducted on mitochondria isolated from patient 1 containing approx. 1% mtDNA (passage number 7) and patient 2 containing approx. 16% mtDNA (passage number 6). Mitochondria from both patient 1 and patient 2's fibroblasts had reduced levels of the 24 kDa and 49 kDa subunits of complex I. Cells from patient 1 had less of these subunits than patient 2, which is consistent with the lower levels of mtDNA in these cells. There was also a noticeable reduction in the 27 kDa subunit of succinate dehydrogenase in the cells of patient 1, whereas the 72 kDa subunit of patient 1 and both subunits of patient 2 were not greatly affected.

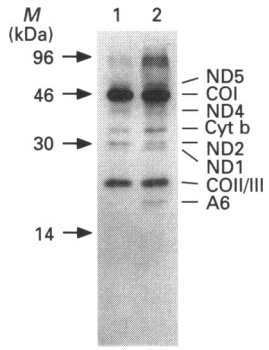

Figure 5 Mitochondrial protein synthesis in control fibroblasts and fibroblasts from patient 2

Control fibroblasts and fibroblasts of patient 2 (P2, passage number 6) were incubated with [^{35}S]methionine in the presence of emetine. After labelling, mitochondrial fractions were prepared and mitochondrial proteins separated using SDS/PAGE and detected by fluorography. Three times as much protein was loaded in lane 2 (P2) as in lane 1 (control). Mitochondrial proteins are identified according to their relative mobilities and the absence of incorporation of the label in the presence of chloramphenicol (results not shown). Positions of molecular-mass (*M*) markers are indicated to the left. The various proteins indicated on the right are described in the text. Cyt b is cytochrome b; A6 is an ATPase subunit; ND1, ND2, ND3, ND4 and ND5 are complex I subunits; COI, COII and COIII are complex IV subunits.

Mitochondrial protein synthesis

To determine if the mitochondria with residual mtDNA could still conduct mitochondrial protein translation, a cell-labelling experiment was performed. Control cells and cells of patient 2, containing 16% mtDNA (passage number 6), were incubated with [^{35}S]methionine and either the cytosolic translation inhibitor emetine or emetine together with the mitochondrial translation inhibitor chloramphenicol. It can be seen from Figure 5 that mitochondrial protein synthesis occurred in patient 2's cells, generating a similar pattern of labelled polypeptides as the control cells. For this experiment, three times the amount of patient 2's cells were used compared with the control, accounting for the intensity of the labelled products. Although not all of the mitochondrial translation products are visible on this autoradiogram, the polypeptide pattern resembles that for cultured skin fibroblasts (Beattie and Sen, 1987). The bands are recognizable as ND5 (apparent molecular mass 51 kDa), COI (apparent molecular mass 44 kDa), ND4 (apparent molecular mass 39 kDa), cytochrome *b* (apparent molecular mass 35 kDa), ND1 and ND2 (apparent molecular mass 30 and 33 kDa) and COII/III, which co-migrate at a molecular mass of 18–20 kDa (Hare et al., 1980; Gibb and Ragan, 1990).

DISCUSSION

Recently, a disease exhibiting respiratory-chain dysfunction has been identified which involves a depletion in the level of mtDNA in affected tissues. We have characterized fibroblast cell lines with mtDNA depletion from two siblings with a clinically similar disorder. Previous experiments fusing patient 1's cells to human-derived *rho*^{0} cells have shown that the mtDNA depletion is due to a defect in the nuclear genome and not the mtDNA (Bodnar et al., 1993). The fibroblast cells showed a specific biochemical defect in respiratory-chain function as the result of decreasing

mtDNA levels. The progressive loss of mtDNA in these cells explains why earlier experiments exhibited higher levels of respiratory-chain-enzyme activity (Leonard et al., 1991; Clayton et al., 1986). The current experiments were undertaken after the patients' fibroblasts had been in culture for some time and had less than 0.9 and 16.2% of control mtDNA levels for patient 1 and patient 2 respectively. The original levels of mtDNA present in the patient's fibroblasts were unknown.

Normal citrate synthase activity in mitochondria isolated from the patient's fibroblasts implies that these matrix enzymes are synthesized and imported normally in these cells. This indicated that the mitochondrial import machinery was operating even in the virtual absence of a functional respiratory chain. This has also been reported for other *rho*^{0} cells (Tzagoloff and Myers, 1986). To investigate the synthesis and import of nuclear-encoded subunits of the respiratory chain, Western blots were performed using antibodies to the 24 kDa and 49 kDa subunits of complex I and the 27 kDa and 70 kDa subunits of succinate dehydrogenase. Western blots indicated that nuclear-encoded subunits of complex I were present in the patient's cells, albeit at reduced levels compared with controls. The apparent concentration of these subunits was less in patient 1 than in patient 2, consistent with the lower levels of mtDNA in the former. The decrease in quantities of these subunits, however, did not appear proportional to the reduction of mtDNA. The reduction in complex I subunits may relate to the stability of the individual polypeptides in the absence of the assembled complex, or there may be down-regulation of expression of the subunits in the absence of mitochondrially encoded subunits. It is not clear to what extent, if any, the mitochondrial genetic system influences nuclear genes, and there is little evidence of the existence of this type of regulation in either yeast or human cells (Tzagoloff and Myers, 1986; Attardi and Schatz, 1988). It is curious that the 27 kDa subunit of succinate dehydrogenase was reduced in patient 1's cells, as complex II does not contain mtDNA-encoded subunits. This observation suggests some type of co-ordinated regulation of components of the respiratory chain rather than degradation of the unassembled complexes.

Mitochondrial protein translation was detected in the fibroblasts of patient 2, indicating that the residual mtDNA was expressed in these cells. There are several *petite* mutants of *S. cerevisiae* which appear to be analogous to the patient's cell lines in that they are characterized by unstable mtDNA, which is eventually eliminated from the yeast (Myers et al., 1985; Greenleaf et al., 1986; Tzagoloff and Dieckmann, 1990). These *petite* mutants have been shown to have defective mitochondrial transcription or translation. mtDNA replication is primed by RNA, and mitochondrial RNA polymerase is thought to play a role in this process, explaining why a defect in RNA polymerase will prevent mtDNA replication in addition to mitochondrial transcription (Greenleaf et al., 1986). However, it is not yet known how defects in mitochondrial translation result in unstable mtDNA in yeast (Myers et al., 1985). Our cells differ from these mutants in that the residual mtDNA is expressed, suggesting that the defect in patient 1 and patient 2's cells is at the level of mtDNA replication or maintenance of the mitochondrial genome.

The basic mechanism of mammalian mtDNA replication has been elucidated; however, the components of the replication machinery are not well characterized (Clayton, 1982, 1991). In addition, little is known about the mechanisms that set mtDNA copy number in various somatic tissues or those that co-ordinate mtDNA levels with changes in organelle or cell populations (Clayton, 1982). Further characterization of the components involved will provide a detailed understanding of the process of mammalian mtDNA replication and perhaps provide some information concerning the factors regulating mtDNA copy number. The unique fibroblasts from patients 1 and 2 provide model cell lines for investigating interactions between the nuclear and mitochondrial genomes in the maintenance of mtDNA. The dependence of these cells on pyruvate and uridine for growth provides a selection mechanism for complementation of the defect which should allow characterization of the factor involved in mtDNA replication.

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