Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8334–8338, August 1994
Genetics

Cytoplasmic transfer of the mtDNA nt 8993 T → G (*ATP6*) point mutation associated with Leigh syndrome into mtDNA-less cells demonstrates cosegregation with a decrease in state III respiration and ADP/O ratio

IAN TROUNCE, STEPHANIE NEILL, AND DOUGLAS C. WALLACE†

Department of Genetics and Molecular Medicine, Emory University School of Medicine, 1462 Clifton Road, Atlanta, GA 30322

Communicated by Giuseppe Attardi, May 3, 1994

ABSTRACT **A point mutation in the mtDNA-encoded *ATP6* gene (T → G at nt 8993) associated with Leigh syndrome in two pedigrees was found to decrease ADP-stimulated (state III) respiration and the ratio of ADP molecules phosphorylated to oxygen atoms reduced (ADP/O ratio) but did not affect 2,4-dinitrophenol (DNP)-uncoupled respiration, suggesting a defective mitochondrial H^+-translocating ATP synthase. Intact mitochondria isolated from patient and control lymphoblastoid cell lines were tested for state III, ADP-limited (state IV), and DNP-uncoupled respiration with various substrates. Mitochondria isolated from patient lymphoblasts harboring 95–100% of mtDNAs carrying the nt 8993 T → G mutation showed state III respiration rates 26–50% lower than controls while having normal DNP-uncoupled rates. This resulted in state III/DNP ratios of 0.52–0.70 in patient mitochondria versus 0.88–0.97 in controls. The ADP/O ratio was also decreased 30–40% in patient mitochondria. Patient lymphoblasts heteroplasmic for the nt 8993 mutation were enucleated by using Percoll gradients and the cytoplasts were fused to mtDNA-deficient (ρ^0) cells by electric shock. Cybrid clones homoplasmic for the wild-type nucleotide (T) at nt 8993 gave state III/DNP and ADP/O ratios similar to those of control cybrids, whereas cybrid clones homoplasmic for the mutant nucleotide (G) showed a 24–53% reduction in state III respiration, a state III/DNP ratio of 0.53–0.64, and a 30% decrease in the ADP/O ratio. Thus, the reduced state III respiration rates and ADP/O ratios are linked to the T → G mutation at nt 8993.**

A wide variety of clinical phenotypes have been shown to be the result of mutations in mtDNA (1, 2). mtDNA codes for 13 polypeptides required for the mitochondrial ATP-generating pathway of oxidative phosphorylation, as well as the two rRNAs and 22 tRNAs necessary for their synthesis (2). Oxidative phosphorylation encompasses five multisubunit enzymes (complexes I–V) embedded in the mitochondrial inner membrane and assembled with subunits from both the mtDNA and nuclear DNA. Complexes I–IV compose the electron transport chain which oxidizes hydrogens donated by NADH or succinate with oxygen and uses the energy released to pump protons from the mitochondrial matrix to the intermembrane space. Complex V, the mitochondrial H^+-translocating ATP synthase (H^+-ATP synthase, EC 3.6.1.34), utilizes this proton electrochemical gradient as a source of potential energy to condense ADP and P_i to ATP. Protons outside the inner membrane pass through a proton channel in the H^+-ATP synthase membrane component (F_0) and change the conformation of the enzyme's stalk and head to phosphorylate the ADP (3). Complex V is composed of 13 polypeptides, 2 of which, MTATP6 and MTATP8, participate in F_0 and are encoded by mtDNA (2).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

mtDNA missense mutations which affect the electron transport chain genes have frequently been associated with Leber hereditary optic neuropathy (LHON)(4), while those that have been reported for the H^+-ATP synthase present with neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) (5, 6) or Leigh syndrome (7–12). Most of the NARP and Leigh syndrome cases are associated with a heteroplasmic (mixture of mutant and normal mtDNAs) T → G transversion at nt 8993 (*MTATP6**NARP8993G) which converts a highly conserved leucine to an arginine in MTATP6 (ATP6 Leu^{156} → Arg) (5). A second nt 8993 mutation, T → C (*MTATP6**NARP8993C), gives a similar clinical phenotype (13).

While the association between Leigh syndrome and the nt 8993 mutation has been established in multiple families, the biochemical effect of the nt 8993 mutation remains unknown. To address this deficiency, we have examined the mitochondrial H^+-ATP synthase of Leigh syndrome patients by polarographic analysis of oxidative phosphorylation in mitochondria isolated from cultured cells. This revealed decreased state III respiration and ADP/O ratios suggestive of a proton-channel and ADP-phosphorylation defect in the H^+-ATP synthase. These defects were then linked to the nt 8993 mutation by cytoplasmic (cybrid) transfer experiments. Cybrid experiments involve enucleating donor cells which harbor a mitochondrial phenotype and fusing the cytoplasmic fragments carrying the mtDNAs to recipient cells (14, 15) which may lack resident mtDNAs (ρ^0 cells) (16). Cytoplasmic transfer of the phenotype links it to the mtDNA and has permitted demonstration that mtDNA mutations can impart chloramphenicol resistance (14, 15) and disease-related defects in mitochondrial protein synthesis (17–20). Cybrid transfer experiments using Leigh syndrome patient lymphoblasts revealed that the decreased state III respiration and ADP/O ratios were transferred when a mutant guanine was present at nt 8993, but not when there was a normal thymine.

MATERIALS AND METHODS

Patients. Members of two pedigrees harboring the *MTATP6**NARP8993G mutation were investigated. Clinical details and molecular genetic analyses of these patients have been reported previously (8, 10), and when referred to below, individuals are designated as in those reports.

Cell Lines and Culture Conditions. Epstein–Barr virus-transformed lymphoblast cell lines were established from leukocytes isolated from whole blood on Ficoll gradients. All cultures were grown in RPMI 1640 medium supplemented

Abbreviations: DNP, 2,4-dinitrophenol; FBS, fetal bovine serum; HIFBS, heat-inactivated FBS; NARP, neurogenic muscle weakness, ataxia, and retinitis pigmentosa.

†To whom reprint requests should be addressed.

with 15% heat-inactivated fetal bovine serum (HIFBS) (21), without antibiotics.

A cell line devoid of mtDNA (ρ^0 cell line) was produced by the method of King and Attardi (16). The 5-bromo-2'-deoxyuridine (BrdUrd)-resistant osteosarcoma cell line 143B TK$^-$ (ATCC CRL 8303) was grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 mg/ml), 1 mM pyruvate, 10% FBS, uridine (50 μg/ml), and ethidium bromide (50 ng/ml). After 3 weeks of growth with ethidium bromide, 10 clones were isolated and screened for mtDNA content by Southern blot hybridization of isolated cell DNA using purified HeLa mtDNA as probe (22). The clone with the lowest mtDNA levels was cultured for a further 10 weeks in the presence of ethidium bromide (50 ng/ml) and 120 subclones were isolated. These clones were screened for mtDNA by both Southern blot analysis and polymerase chain reaction (PCR) amplification of target mtDNA sequences. Twenty-one clones were found to contain no mtDNA. Selected clones were grown for several weeks without ethidium bromide and retested for mtDNA. Auxotrophy for uridine (23) and pyruvate (16) in these ρ^0 cells was demonstrated by their lack of growth in the absence of these nutrients, compared with the normal growth of the parental 143B TK$^-$ cell line under similar conditions.

Isolation of Transmitochondrial Cybrids. For each fusion, 10^7 patient lymphoblasts harvested in midlogarithmic phase were enucleated on an isopycnic Percoll gradient in the presence of cytochalasin B. A suspension of the cells in RPMI/HIFBS was mixed with an equal volume of Percoll. Cytochalasin B was added from a 2-mg/ml stock in dimethyl sulfoxide to 20 μg/ml, and the mixture was centrifuged at 44,000 $\times$ *g* for 70 min. Centrifuge temperature was controlled so that the rotor temperature was 37°C at the end of the run. The hazy band at the Percoll/medium interface was removed, diluted 10-fold with fresh complete medium, and centrifuged at 1000 $\times$ *g* for 5 min. This washed pellet of cytoplasts, karyoplasts, and intact cells was suspended in 3 ml of 0.3 M mannitol (pH 7.2), centrifuged as above, resuspended in 0.6 ml of 0.3 M mannitol, and added to a pellet of 4×10^6 freshly harvested ρ^0 cells that had been washed with 3 ml of 0.3 M mannitol. This mixture was transferred to an electrofusion slide chamber [Biotechnologies and Experimental Research, San Diego (BTX) 4543; electrode gap width, 3.2 mm; capacity, 0.7 ml].

Electrofusion was carried out with a BTX ECM 200 system by applying an ac field of 35 V (0.11 kV/cm) for 20 sec followed by two 20-μsec dc pulses of 800 V (2.5 kV/cm). The AC field was then reinstated for 3 min, beginning at 35 V and progressively lowered to 15 V. The fusion suspension was diluted slowly to 20 ml with RPMI/HIFBS and allowed to stand for 20 min at room temperature.

The fusion mixture was plated into 100-mm dishes in replicates of 2.5×10^5, 10^5, and 5×10^4 ρ^0 cells per dish in DMEM supplemented with 10% FBS and uridine (50 μg/ml). After 24 hr the medium was replaced with DMEM supplemented with 5% dialyzed FBS and BrdUrd (50 μg/ml) (select medium). This medium was replaced every 2 days. After 6–8 days, cybrid colonies appeared at a frequency of around 1 in 10^4 ρ^0 cells plated, and after 10–14 days, selected clones were ring-isolated.

Analysis of mtDNA. Total genomic DNA was isolated from frozen cells by proteinase K digestion, followed by organic extraction (24) or by anion-exchange affinity chromatography (25). Detection of the np 8993 mutation was accomplished by PCR amplification of a mtDNA fragment from nt 8829 (primer nt 8229–nt 8845) to nt 9859 (primer nt 9840–9849) followed by *Hpa* II digestion (8). Mitochondrial DNA of all cybrids was screened for the lack of the parental cell (143B TK$^-$) mtDNA by testing for the presence of an *Mbo* I restriction site present in 143B TK$^-$ mtDNA (16). Sequence analysis in our laboratory revealed the site gain to be due to an A $\rightarrow$ G transition at nt 15,937 compared with the reference Cambridge sequence (26). We have found this to be a rare polymorphism, informative in all the cybrid crosses in the present study. The region from nt 15,005 (primer nt 15,005–15,024) to nt 15,701 (primer nt 15,682–15,701) was amplified and digested with *Mbo* I. The 696-bp mtDNA fragment was cut into five fragments of 297, 195, 110, 55 and 39 bp for the 143B TK$^-$ mtDNA, but only four fragments for other mtDNAs, with the 195- and 39-bp fragments fused into a 234-bp fragment.

Mitochondria Isolation and Polarography. *Cell culture.* For isolation of mitochondria from patient lymphoblasts, cultures were expanded to 1500 ml in RPMI/10% HIFBS in single 2-liter roller bottles. Cells were harvested in midlogarithmic phase, 2 or 3 days after the final passage. For mitochondrial isolation from transmitochondrial cybrids, cultures were grown in spinner minimal essential medium with 10% FBS, high glucose (4.5 mg/ml), 1 mM L-glutamine, and 15 mM Hepes (pH 7.4) and were expanded to 2 liters in 3-liter spinner bottles. In both cases, bottles were seeded at 2×10^5 cells per ml in 500 ml, expanded at 2- to 4-day intervals (depending on the growth rate of the cell line) to half and then the full final culture volume, and harvested after a further 2 days. These cultures yielded 1–2 $\times$ 10^9 cells, around 2–4 g of wet weight.

Isolation of mitochondria. Intact mitochondria were isolated from freshly harvested cells by a modification of the digitonin isolation method of Moreadith and Fiskum (27). All procedures were carried out at 4°C. Packed cells (1×) were resuspended in 4 volumes (total, 5×) of isolation buffer (210 mM mannitol/70 mM sucrose/1 mM potassium EGTA/0.5% bovine serum albumin/5 mM Hepes, pH 7.2) and digitonin [10% (wt/vol) in dimethyl sulfoxide] was added slowly with mixing to a final concentration of 0.10 mg/ml. The cells were then checked for trypan blue exclusion. The digitonin concentration was increased in 0.05-mg/ml increments until >90% permeabilization was achieved (usually at 0.2–0.4 mg/ml). The cell suspension was then diluted to 10× with isolation buffer and centrifuged at 3000 $\times$ *g* for 5 min to remove excess detergent.

The gelatinous, permeabilized cell pellet was resuspended to 5× with isolation buffer and homogenized in a Dounce homogenizer by 15 passes with the close-fitting "A" pestle. The homogenate was diluted to 15× with isolation buffer and centrifuged at 625 $\times$ *g* for 5 min, and the supernatant was decanted and centrifuged two more times. This supernatant was then centrifuged at 10,000 $\times$ *g* for 20 min to pellet the mitochondria. A loose white pellet surrounding the dark mitochondrial pellet was allowed to pour off with the supernatant, and the remaining pellet was gently rinsed to remove more light material. The pellet was gently resuspended, diluted to 20 ml with isolation buffer, and centrifuged again at 10,000 $\times$ *g* for 20 min. The washed pellet was resuspended in 0.2 ml of isolation buffer for each gram of cells used, to give a mitochondrial suspension of around 10 mg of protein per ml. Protein was determined by the method of Lowry *et al.* (28) using bovine serum albumin as standard, with correction for the albumin in the isolation buffer by accounting for the volume contribution of the buffer by measuring the final mitochondrial suspension volume.

Polarographic assay of oxidative phosphorylation. Oxygen uptake was measured with an Instech micro oxygen electrode and 0.65-ml chamber attached to a Yellow Springs Instruments 5300 oxygraph. The reaction chamber was fitted with a magnetic stirrer and temperature was controlled at 30°C. Respiration buffer (225 mM mannitol/75 mM sucrose/20 mM KCl/10 mM Tris·HCl, pH 7.4/5 mM KH_2PO_4, pH 7.4) was preequilibrated with O_2 by shaking in a water bath at 30°C and introduced into the chamber by a syringe line. The oxygen content of isosmotic buffer at 30°C was

taken to be 480 nmol of O per ml. All subsequent additions to the chamber were made with Hamilton syringes passed through the capillary aperture on top. The following reagents were stored frozen (−80°C) in small aliquots: 26 mM ADP (pH 6.8), 65 mM ATP (pH 6.8), 0.65 M pyruvate (pH 7.2), 0.65 M glutamate (pH 7.2), 0.65 M succinate (pH 7.2), and 0.65 M malate (pH 7.2).

Mitochondria isolated from cultured cells by the digitonin method used here exhibited high specific respiratory rates with both NAD^+-linked and FAD-linked substrates, and the respiration was tightly coupled to the phosphorylation of added ADP (Fig. 1). To ensure coupling during early ADP additions, ATP had to be added to the respiration buffer, presumably to allow restoration of the mitochondrial matrix adenine nucleotide pools by the ATP–Mg/P_i carrier (29). This enhancement of coupling was observed despite the lack of added Mg^{2+} in the present assay.

While optimizing our mitochondrial isolation conditions with control cell lines, we found that suboptimal preparations showed loose coupling (failure of state III respiratory rate to slow to state IV after a small addition of ADP) or low specific rates with poor response to a second small addition of ADP. The coupling with NAD^+-linked substrates was often decreased more than with succinate in such damaged preparations. Therefore, results were not included in this study from preparations where the state III rate resulting from a second addition of ADP was significantly less than the first, or when state IV rates could not be demonstrated. Controlled exposure of cells to digitonin and rapid removal of excess digitonin are probably important for the integrity of the organelle fraction, since Moreadith and Fiskum (27) found that excessive digitonin resulted in poorly coupled isolates.

In a typical experiment, freshly isolated mitochondria (0.4 mg of mitochondrial protein) were added to the chamber containing substrate (5 mM pyruvate plus 5 mM malate, 5 mM glutamate plus 5 mM malate, or 5 mM succinate) and 0.5 mM ATP. After 2 min, 125 nmol of ADP was added to stimulate state III respiration. Usually two additions of ADP could be made before addition of uncoupler [50 μM 2,4-dinitrophenol (DNP)].

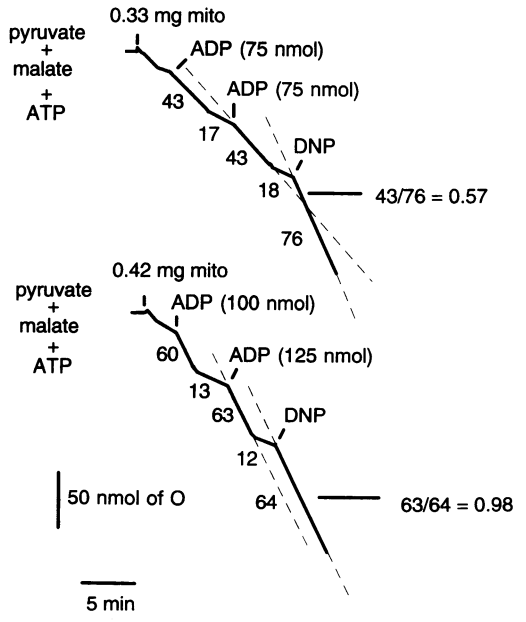

FIG. 1. Polarograph traces of intact mitochondria isolated from transmitochondrial cybrids homoplasmic for the 8993 T → G mutation (*Upper*) and homoplasmic 8993 wild type (*Lower*). Numbers to the left of each trace indicate specific respiratory rates as nmol of 0 per min per mg of mitochondrial protein (mito). DNP concentration was 50 μM. Note the greatly increased DNP-uncoupled rates compared with the ADP-stimulated (state III) rate in the mutant mitochondria.

RESULTS

Characterization of Transmitochondrial Cell Lines. Two different Leigh syndrome families bearing the T → G mutation at nt 8993 (8, 10) were cytoplasmic donors in cybrid experiments. Lymphoblastoid cell lines were enucleated, the cytoplasmic fragments were fused to ρ^0 143B TK^- cells, and cybrids were selected in medium containing BrdUrd and lacking uridine. In this selection, patient lymphoblasts which escaped enucleation and lymphoblast–ρ^0 cell hybrids were killed by the BrdUrd while residual ρ^0 cells died from lack of uridine. Cybrid clones survived and appeared at a frequency of $\approx 10^{-4}$ per ρ^0 cell plated. The genetic origin of the cybrids was confirmed by demonstrating a modal chromosome complement of 65–70, similar to the 70 seen in the ρ^0 parent; the absence of the *Mbo* I restriction site at nt 15,397, characteristic of 143B TK^- mtDNA; and the presence or absence of the *Hpa* II restriction site at nt 8993.

Two cybrid fusions were carried out for each of two families (8, 10). For the first family (10), the first cross used lymphoblasts from individual B.II-2, which appeared to be homoplasmic for the 8993 mutation. All 24 cybrids tested were also homoplasmic for the mutation. The second cross used lymphoblasts from B.II-3, which were heteroplasmic (10% mutant and 90% wild-type). Five cybrid clones were selected, 4 homoplasmic wild type and one homoplasmic mutant. For the second family (8), the first fusion used lymphoblasts from individual A.IV-2 (95% mutant) and resulted in 12 cybrids, all homoplasmic mutant. The second fusion used lymphoblasts from A.III-2 (95% wild type), and yielded 10 cybrid clones, 7 of which were homoplasmic wild type and 3 heteroplasmic mutant and wild type.

From these crosses, 5 homoplasmic mutant and 3 homoplasmic wild-type clones were chosen for studies of oxidative phosphorylation. Lymphoblasts from three unrelated healthy volunteers not carrying the 8993 mutation were used in similar fusions to isolate control cybrids.

Oxidative Phosphorylation Characteristics of Patient Lymphoblasts and Derived Cybrids. Results of polarographic measurement of oxidative phosphorylation capacity of isolated mitochondria from lymphoblasts and cybrids with and without the 8993 mutation are shown in Table 1. Compared with controls, mitochondria from two predominantly mutant patient lymphoblast cell lines (B.II-2, homoplasmic mutant, and A.IV-2, 95% mutant) showed a 30–40% reduction in state III (ADP-stimulated) rates, while the state IV (ADP-limited) and uncoupled (DNP-stimulated) rates approximated those of controls. Consequently, while in controls the ratio of state III to DNP-uncoupled rate varied in a narrow range of 0.88–0.97, in the mutant cells this ratio was decreased to 0.52–0.70 with various substrates (Table 1). Therefore, the H^+ flux of the H^+-ATP synthase appears to be severely rate-limiting for the respiratory chain in the mutant cells. Phosphorylation efficiency was also reduced in the mutant cells, with the ADP/O ratios in cells carrying the 8993 mutation 30–40% lower than in controls (Table 1).

Results from the eight independent cybrid clones studied showed that these defects cosegregated with the 8993 T → G mutation. Mitochondria isolated from three cybrid clones carrying mtDNA from the Leigh syndrome pedigrees but having the normal (wild-type) base at nt 8993 showed oxidative phosphorylation characteristics similar to three independent cybrids prepared using control mtDNA donors (Table 1). In contrast, five cybrid clones homoplasmic for the 8993 mutation, three from the first pedigree (10) and two from the second (8), showed 24–53% slower state III respiration, but normal state IV and uncoupler-stimulated rates, giving a

Table 1. Polarographic measurement of respiratory chain and phosphorylation capacity of intact mitochondria isolated from lymphoblasts and transmitochondrial cybrids with and without the mtDNA 8993 T → G mutation

Substrate(s)	Cell line (*n*)	State III respiration	State IV respiration	ADP/O ratio	DNP-uncoupled respiration	III/DNP
Pyruvate	Lymphoblasts					
+ malate	Controls (9)	164 ± 27	57 ± 19	2.55 ± 0.14	174 ± 28	0.88 ± 0.02
	8993 T → G (2)	101 ± 28	71 ± 42	1.78 ± 0.40	193 ± 35	0.52 ± 0.06
	Cybrids					
	Controls (3)	75 ± 5.6	20 ± 2.9	2.35 ± 0.09	86 ± 7.8	0.85 ± 0.01
	8993 WT (3)	70 ± 22	17 ± 2.5	2.32 ± 0.12	81 ± 30	0.88 ± 0.07
	8993 T → G (5)	44 ± 8.7	22 ± 3.9	1.70 ± 0.30	83 ± 22	0.53 ± 0.05
Glutamate	Lymphoblasts					
+ malate	Controls (9)	172 ± 29	57 ± 18	2.57 ± 0.21	187 ± 35	0.88 ± 0.02
	8993 T → G (2)	103 ± 3	58 ± 28	1.61 ± 0.72	172 ± 4	0.60 ± 0.03
	Cybrids					
	Controls (3)	77 ± 5.7	21 ± 1.2	2.41 ± 0.04	82 ± 13	0.95 ± 0.09
	8993 WT (3)	69 ± 19	17 ± 1.5	2.37 ± 0.14	86 ± 33	0.87 ± 0.07
	8993 T → G (5)	48 ± 12	22 ± 7.2	1.66 ± 0.09	87 ± 24	0.55 ± 0.04
Succinate	Lymphoblasts					
	Controls (9)	238 ± 33	89 ± 24	1.80 ± 0.13	238 ± 35	0.97 ± 0.03
	8993 T → G (2)	173 ± 20	107 ± 62	1.13 ± 0.39	249 ± 39	0.70 ± 0.03
	Cybrids					
	Controls (3)	115 ± 9.2	33 ± 0	1.52 ± 0.02	127 ± 32	0.88 ± 0.14
	8993 WT (3)	99 ± 32	30 ± 5.5	1.52 ± 0.11	112 ± 43	0.90 ± 0.07
	8993 T → G (5)	73 ± 17	30 ± 3.6	1.10 ± 0.14	116 ± 39	0.64 ± 0.07

State III (ADP-stimulated), state IV (ADP-limited), and DNP-uncoupled respiration rates are expressed as nmol of O per min per mg of mitochondrial protein. *n* for lymphoblasts refers to cell lines from different individuals, while for cybrids *n* indicates number of independent clones tested. Mean values ± SD are shown.

state III/DNP-uncoupled normal respiration ratio of 0.53–0.64, substantially lower than the 0.85–0.95 observed in control cybrids (Table 1; Fig. 1). The ADP/O ratios were also decreased 30–40% in the mitochondria of the mutant cybrids as compared to controls (Table 1). These data suggest that the ATP6 Leu^{156} → Arg substitution may result in both proton channel and ADP phosphorylation defects in the H^+-ATP synthase. The observed deficiencies would decrease state III ATP production 50–70% in homoplasmic mutant cells.

Comparison of the state III, state IV, and DNP-stimulated respiration rates of control lymphoblasts versus control and 8993-wild-type cybrids revealed that cells with lymphoblast nuclei had ≈2-fold higher respiration rates than cells with osteosarcoma nuclei, even though they had the same mtDNA. This indicates that nuclear factors control the maximum potential respiration rate, either as a product of differences in the differentiation or transformation status of the cells. However, these nuclear differences did not affect the state III/DNP or the ADP/O ratios of the two types of cells (Table 1).

DISCUSSION

In this study we have found that the maximal (state III) respiration and ADP/O ratios are decreased in patient cells homoplasmic for the mtDNA nt 8993 T → G mutation, which suggests that there is a combined defect in the proton channel and P/O coupling of the H^+-ATP synthase. This quantitative defect was linked to the 8993 mutation, since the defect was transferred to cybrids when the donor mtDNA carried the mutation but not when the mtDNA from the same cells carried the normal nucleotide nt 8993. These observations extend a previous report on the biochemical defect of the 8993 T → G mutation which reported a decreased rate of ATP production from patient lymphoblast mitochondria supplied with various substrates (30).

The specific assignment of these oxidative phosphorylation defects to the 8993 T → G mutation, together with the repeated association of retinal degeneration, NARP, and Leigh syndrome with the mutation in multiple independent families (8–12), demonstrates that these clinical symptoms are the direct product of a defect in the mitochondrial ATP synthase. Since the different clinical presentations of this mutation are associated with variable percentages of mutant mtDNAs, the phenotype must be a direct reflection of the proportional decrease in ATP synthesis. As such, this study provides direct proof that a defect in mitochondrial ATP production is all that is required to create specific neurological and neuroophthalmological symptoms such as retinitis pigmentosa and basal ganglia degeneration.

The leucine-to-arginine substitution caused by the 8993 T → G mutation occurs in the fourth membrane-spanning helix of the ATP6 polypeptide. ATP6 and subunit 9 (ATP9) are known to be major components of the proton channel of the ATP synthase (31, 32), and it has been postulated that the leucine at position 156 in ATP6 sits adjacent to a glutamate in ATP9, creating a protonation site essential for proton translocation. Substitution of a positively charged arginine for the leucine would then neutralize the negative charge of the glutamate and block the channel (30). This model is supported by extensive site-directed mutagenesis studies of the homologous *a* subunit of the *Escherichia coli* ATP synthase. These studies have identified several key residues for the proton-translocating function of F_0, including the residue equivalent to Leu^{156} of ATP6 (see ref. 32 for review). Hartzog and Cain (33) reported that ATP synthesis was abolished when this residue (Leu^{207} of subunit *a*) was replaced with arginine. This contrasts with the present findings, where considerable residual ATP synthesis was found in the presence of the ATP6 Leu^{156} → Arg mutation, suggesting that both similarities and differences must exist in the proton translocation routes of the bacterial and mammalian F_1F_0 ATP synthases.

Defects of the H^+-ATP synthase in mitochondrial diseases have been reliably reported in only a handful of reports, and the molecular defect has not been known in any previous case. Three patients described by Clark *et al.* (34), Holliday *et al.* (35), and Peterson *et al.* (36) all had features similar to

those later described by Holt *et al.* (5), now referred to as NARP. A more recent report (37) describes decreased ATP synthase activity in an infant with lactic acidosis, cardiomyopathy, and idiopathic 3-methylglutaconic aciduria. In all these reports, and in that of Schotland *et al.* (38) describing a 37-year-old woman with mild proximal muscle weakness, polarographic studies revealed findings similar to those reported here, where state III respiratory rates were reduced by 40–50% but were normalized by addition of uncoupler. In three of these reports (34, 37, 38), low F_1 ATPase activity was also found, indicating a defective nuclear-encoded subunit of the complex. The patients described by Schotland *et al.* (38) and Holme *et al.* (37) sit at the ends of the clinical spectrum of oxidative phosphorylation disease, perhaps representing a mild tissue-specific mutant versus a severe mutant with systemic expression such as 8993 T → G. The similarity of the clinical picture of the patient described by Clark *et al.* (34) to that of nt 8993 NARP patients (5–8, 10) also suggests that defects in different subunits of the H^+-ATP synthase complex can lead to similar clinical pictures.

The present findings further support the role of energy deficiency as an important pathoetiologic agent in Leigh syndrome, as evidenced from reports of the syndrome being caused by defects of the pyruvate dehydrogenase complex (39, 40) or cytochrome oxidase (41, 42). Moreover, both mtDNA mutations (discussed in this study) as well as nuclear mutations (43) have been demonstrated. Therefore defects in several oxidative phosphorylation complexes, resulting from either nuclear or cytoplasmic gene mutations, can lead to the same clinical phenotype, and the present studies show that a single mtDNA base change can cause the specific degeneration of basal ganglia seen in Leigh syndrome.

We thank Dr. John M. Shoffner for clinical evaluation of these patients, Ms. Judy Hodge Dunmore and Ms Ying-Jie Zhu for help in isolating ρ^0 cell lines, and Dr. Mike Brown for sequence analysis of 143B TK$^-$ mtDNA. This work was supported by National Institutes of Health Grants NS21328, NS30164, HL45572, and AG10130 and by a Muscular Dystrophy Foundation clinical research grant award to D.C.W. and by National Institutes of Health Clinical Research Center Grant RR00039 to Emory University.

1. Wallace, D. C. (1993) *Trends Genet.* **9**, 128–133.
2. Wallace, D. C. (1992) *Annu. Rev. Biochem.* **61**, 1175–1212.
3. Allison, W. S., Jault, J.-M., Zhou, S. & Paik, S. R. (1992) *J. Bioenerg. Biomembr.* **24**, 469–477.
4. Wallace, D. C., Lott, M. T., Shoffner, J. M. & Brown, M. D. (1993) *J. Inherited Metab. Dis.* **15**, 472–479.
5. Holt, I. J., Harding, A. E., Petty, R. K. H. & Morgan-Hughes, J. A. (1990) *Am. J. Hum. Genet.* **46**, 428–433.
6. Puddu, P., Barboni, P., Mantovani, V., Montagna, P., Cerullo, A., Bragliani, M., Molinotti, C. & Caramazza, R. (1993) *Br. J. Ophthalmol.* **77**, 84–88.
7. Tatuch, Y., Christodoulou, J., Feigenbaum, A., Clarke, J. T. R., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R. & Robinson, B. H. (1992) *Am. J. Hum. Genet.* **50**, 852–858.
8. Shoffner, J. M., Fernhoff, P. M., Krawiecki, N. S., Caplan, D. B., Holt, P. J., Koontz, D. A., Takei, Y., Newman, N. J., Ortiz, R. G., Polak, M., Ballinger, S. W., Lott, M. T. & Wallace, D. C. (1992) *Neurology* **42**, 2168–2174.
9. Sakuta, R., Goto, Y., Horai, S., Ogino, T., Yoshinaga, H., Ohtahara, S. & Nonaka, I. (1992) *Ann. Neurol.* **32**, 597–598.
10. Oritz, R. G., Newman, N. J., Shoffner, J. M., Kaufman, A. E., Koontz, D. A. & Wallace, D. C. (1993) *Arch. Ophthalmol.* **111**, 1525–1530.
11. Ciafaloni, E., Santorelli, F. M., Shanske, S., Deonna, T., Roulet, E., Janzer, C., Pescia, G. & DiMauro, S. (1993) *J. Pediatr.* **122**, 419–422.
12. Santorelli, F. M., Shanske, S., Macaya, A., DeVivo, D. C. & DiMauro, S. (1993) *Ann. Neurol.* **4**, 827–834.
13. de Vries, D. D., van Engelen, B. G. M., Gabreels, F. J. M., Ruitenbeek, W. & van Oost, B. A. (1993) *Ann. Neurol.* **34**, 410–412.
14. Bunn, C. L., Wallace, D. C. & Eisenstadt, J. D. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1681–1685.
15. Wallace, D. C., Bunn, C. L. & Eisenstadt, J. D. (1975) *J. Cell Biol.* **67**, 174–188.
16. King, M. P. & Attardi, G. (1989) *Science* **246**, 500–503.
17. Chomyn, A., Meola, G., Bresolin, N., Lai, S. T., Scarlato, G. & Attardi, G. (1991) *Mol. Cell. Biol.* **11**, 2236–2244.
18. King, M. P., Koga, Y., Davidson, M. & Schon, E. A. (1992) *Mol. Cell. Biol.* **12**, 480–490.
19. Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S. T., Nonaka, I., Angelini, C. & Attardi, G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4221–4225.
20. Hayashi, J.-I., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y.-I. & Nonaka, I. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10614–10618.
21. Wallace, D. C., Yang, J., Ye, J., Lott, M. T., Oliver, N. A. & McCarthy, J. (1986) *Am. J. Hum. Genet.* **38**, 461–481.
22. Giles, R. E., Blanc, H., Cann, H. M. & Wallace, D. C. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6715–6719.
23. Gregoire, M., Morais, R., Quilliam, M. A. & Gravel, D. (1984) *Eur. J. Biochem.* **142**, 49–55.
24. Wallace, D. C., Singh, G., Lott, M. T., Hodge, J. A., Schurr, T. G., Lezza, A. M. S., Elsas, L. J. & Nikoskelainen, E. K. (1988) *Science* **242**, 1427–1430.
25. Lott, M. T., Voljavec, A. S. & Wallace, D. C. (1990) *Am. J. Ophthalmol.* **109**, 625–631.
26. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, E. G. (1981) *Nature (London)* **290**, 457–465.
27. Moreadith, R. W. & Fiskum, G. (1984) *Anal. Biochem.* **137**, 360–367.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
29. Aprille, J. R. (1988) *FASEB J.* **2**, 2547–2556.
30. Tatuch, Y. & Robinson, B. H. (1992) *Biochem. Biophys. Res. Commun.* **192**, 124–128.
31. Cox, G. B., Fimmel, A. L., Gibson, F. & Hatch, L. (1986) *Biochim. Biophys. Acta* **849**, 62–69.
32. Fillingame, R. H. (1992) *J. Bioenerg. Biomembr.* **24**, 485–491.
33. Hartzog, P. E. & Cain, B. D. (1993) *J. Biol. Chem.* **268**, 12250–12252.
34. Clark, J. B., Hayes, D. J., Byrne, E. & Morgan-Hughes, J. A. (1983) *Biochem. Soc. Trans.* **11**, 626–627.
35. Holliday, P. L., Martens, M. E., Lee, C.-P., Barnhart, M. I. & Gilroy, J. (1984) *Ann. Neurol.* **16**, 138 (abstr.).
36. Peterson, P. L., Martens, M. E., Lee, C.-P., Hatfield, J. S., Klepach, G. L. & Gilroy, J. (1985) *Ann. Neurol.* **18**, 146 (abstr.).
37. Holme, E., Greter, J., Jacobson, C.-E., Larsson, N.-G., Lindstedt, S., Nilsson, K. O., Oldfors, A. & Tulinius, M. (1992) *Pediatr. Res.* **32**, 731–735.
38. Schotland, D. L., DiMauro, S., Bonilla, E., Scarpa, A. & Lee, C.-P. (1976) *Arch. Neurol.* **33**, 475–479.
39. Kretzschmar, H. A., DeArmond, S. J., Koch, T. K., Patel, M. S., Newth, C. J. L., Schmidt, K. A. & Packman, S. (1987) *Pediatrics* **79**, 370–373.
40. Robinson, B. H. (1988) *J. Bioenerg. Biomembr.* **20**, 313–323.
41. DiMauro, S., Bonilla, E., Zeviani, M., Nakagawa, M. & DeVivo, D. C. (1985) *Ann. Neurol.* **17**, 521–538.
42. DiMauro, S., Servidei, S., Zeviani, M., DiRocco, M., DeVivo, D. C., DiDonato, S., Uziel, G., Berry, K., Hoganson, G., Johnsen, S. D. & Johnson, P. C. (1987) *Ann. Neurol.* **22**, 498–506.
43. Miranda, A. F., Ishii, S., DiMauro, S. & Shay, J. W. (1989) *Neurology* **39**, 697–702.