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Respiration and Growth Defects in Transmitochondrial Cell Lines Carrying the 11778 Mutation Associated with Leber's Hereditary Optic Neuropathy*

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Mitochondrial DNA from two genetically unrelated patients carrying the mutation at position 11778 that causes Leber's hereditary optic neuropathy has been transferred with mitochondria into human mtDNA-less $\rho^0$206 cells. As analyzed in several transmitochondrial cell lines thus obtained, the mutation, which is in the gene encoding subunit ND4 of the respiratory chain NADH dehydrogenase (ND), did not affect the synthesis, size, or stability of ND4, nor its incorporation into the enzyme complex. However, NADH dehydrogenase-dependent respiration, as measured in digitonin-permeabilized cells, was specifically decreased by approximately 40% in cells carrying the mutation. This decrease, which was significant at the 99.99% confidence level, was correlated with a significantly reduced ability of the mutant cells to grow in a medium containing galactose instead of glucose, indicating a clear impairment in their oxidative phosphorylation capacity. On the contrary, no decrease in rotenone-sensitive NADH dehydrogenase activity, using a water-soluble ubiquinone analogue as electron acceptor, was detected in disrupted mitochondrial membranes. This is the first cellular model exhibiting in a foreign nuclear background mitochondrial DNA-linked biochemical defects underlying the optic neuropathy phenotype.

Leber's hereditary optical neuropathy (LHON)[1] has been associated with several mitochondrial DNA (mtDNA) mutations affecting in most cases mtDNA-encoded subunits of the respiratory chain NADH dehydrogenase (NADH:coenzyme Q oxidoreductase; E.C. 1.6.99.3; complex I). Four mutations, one in the ND4 gene at position 11778 (1), one in the ND1 gene at position 3460 (2, 3), one in the ND6 gene at position 14484 (4), and one in the cytochrome *b* gene at position 15257 (5, 6), are considered primary mutations, in that they can cause the disease in the absence of other mutations. There are, in addition, mild LHON mutations at less highly conserved positions in the ND genes, and also in the cytochrome *c* oxidase subunit genes, which appear to act synergistically with each other or with primary mutations in producing the disease phenotype (7). Mutations at position 4160 of the ND1 gene (8) or at position 14459 of the ND6 gene (9) have been found to be associated with syndromes including, besides the typical LHON defects, alterations of the central nervous system.

The 11778 mutation accounts for at least 50% of the European, North American, and Australian LHON cases (1, 10, 11), but the identification of a clear biochemical effect associated with the mutation has been elusive. The G to A transition causes a conserved arginine to be replaced by a histidine in ND4. Larsson *et al.* (12) reported a reduction of about 50% in the malate/glutamate- or pyruvate/malate-dependent respiration rate in intact mitochondria from muscle biopsy material from patients with the 11778 mutation, while succinate-dependent respiration appeared to be normal. However, they failed to detect a reduction in NADH-cytochrome *c* oxidoreductase enzyme activity in disrupted mitochondria. This result was surprising because malate/glutamate- or pyruvate/malate-driven respiration depends on respiratory chain NADH dehydrogenase activity. Other investigators, using immortalized lymphoblasts from patients carrying the 11778 mutation (13), have also reported a decrease in the rate of rotenone-sensitive oxidation of NAD-linked substrates by intact mitochondria, but no decrease in rotenone-sensitive NADH:2,6-dichlorophenolindophenol oxidoreductase activity in disrupted mitochondrial membranes. The absence of a decrease in NADH:2,3-dimethoxy-5-methyl-6-*n*-undecyl-*p*-benzoquinone (UBQ) oxidoreductase activity was also reported for mitochondrial membranes from platelets (14).

Another intriguing aspect of LHON is the low penetrance of the disease, which has pointed to the possible role of a nuclear factor(s) in the manifestation of the disease phenotype. Only about 30% of males and 10% of females carrying the 11778 mutation actually suffer visual loss (11). The predominance of males has led to the hypothesis of an X-chromosome-linked factor being involved in the disease (11, 15, 16). It should be noted that at least two genes for complex I have been assigned to the X-chromosome in rodents (17).

We have transferred mitochondria from patients carrying the 11778 mutation in their mtDNA into the mtDNA-less cell line $\rho^0$206 (18) to determine whether the biochemical defects found in patients' cells would be manifested also in the absence of the patients' nuclear background. The $\rho^0$206 nuclear background supports normal rates of respiration in transmitochon-

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[1] The abbreviations used are: LHON, Leber's hereditary optic neuropathy; mtDNA, mitochondrial DNA; UBQ, 2,3-dimethoxy-5-methyl-6-*n*-undecyl-*p*-benzoquinone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CO II, cytochrome *c* oxidase subunit II; DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; Q, coenzyme Q, ubiquinone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; ND, subunit of NADH dehydrogenase.

drial cell lines constructed with cytoplasts from normal cells (19). By contrast, in this "normal" nuclear background, the 11778 mutation caused a statistically significant reduction in the rate of glutamate/malate-driven respiration, but not in the rate of succinate/glycerol-3-phosphate (glycerol-3-P)-driven respiration. We have also shown that, under conditions in which cells are forced to rely almost exclusively on oxidative phosphorylation to produce ATP, *i.e.* in medium containing galactose instead of glucose, the mutation significantly reduced the growth rate.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Skin fibroblast cultures Le1 (cell line TC89-1973 of the Genetics Core, Mental Retardation Research Center, The Kennedy Institute, Baltimore, MD) and Le2 were established from LHON patients 1 and 2 by standard procedures. Patient 1, a male, has had longstanding (possibly lifelong) poor vision. When first examined clinically at age 35, he exhibited a mild impairment of visual acuity, with paracentral scotomas and reduced color vision. Three years later, his visual acuity and color vision had significantly improved, although there was persistence of paracentral scotomas and diffuse optic atrophy, as revealed by ophthalmoscopy. He is a member of a large pedigree with matrilineal transmission of LHON, in which some members have experienced the typical acute loss of vision normally seen in this disease, and others, like patient 1, have had long standing poor vision. Patient 2, also a male, suffered acute loss of vision at age 21, had no known family history of LHON, and his vision did not recover. Patients 1 and 2 were 49 and 33 years old, respectively, when skin biopsies and blood samples were taken. The GM03651C and GM03652A fibroblast strains, derived from presumably normal individuals, were obtained from the NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ).

All fibroblast strains were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The osteosarcoma-derived cell line 143B.TK$^-$ (ATCC CRL 8303), hereafter referred to as 143B, and $\rho^0$206, the ρ^0 cell line used in this study, were cultured as described previously (18). pT3 (19) is a transmitochondrial cell line constructed with enucleated myoblasts from a myoclonic epilepsy and ragged red fiber syndrome patient, but lacking the mitochondrial tRNA^{Lys} gene mutation causing the disease. pT3 was maintained in DMEM supplemented with 5 or 10% FBS and 100 μg of 5-bromodeoxyuridine/ml.

Mitochondria Transfer into ρ^o206 Cells—Mitochondria-mediated transformation of $\rho^0$206 cells with fibroblasts was carried out as described previously (18), by fusing fibroblasts enucleated with cytochalasin B with $\rho^0$206 cells in the presence of 40% polyethylene glycol 1500 (BDH). Mitochondrial transformants were selected in DMEM containing 100 μg of 5-bromodeoxyuridine/ml and supplemented with 10% dialyzed FBS, or, in the case of C1.C11, in DMEM supplemented with 5% dialyzed FBS. This clone was subsequently shown to be resistant to 5-bromodeoxyuridine.

Growth Measurements—These were made by plating samples of 10^5 cells on 10-cm Petri dishes in 10 ml of the appropriate medium (DMEM, which contains 4.5 mg of glucose/ml and 0.11 mg of pyruvate/ml, or DMEM lacking glucose and containing 0.9 mg of galactose/ml and 0.5 mg of pyruvate/ml (20), both supplemented with 10% dialyzed FBS), incubating them at 37 °C for 7 days and performing cell counts at daily intervals.

[^{35}S]Methionine Labeling and Immunoprecipitation of Mitochondrial Translation Products—Cells were labeled for 1 h with [^{35}S]methionine (1000 Ci/mmol; 220 μCi/ml) in the presence of 100 μg of emetine/ml, as described previously (19). For immunoprecipitation experiments, cells were labeled and chased as follows. 10^6 cells were exposed to 40 μg of chloramphenicol/ml for 22 h, then washed and labeled for 2 h with 1.7 mCi of [^{35}S]methionine in 8 ml of DMEM lacking methionine and containing 100 μg of cycloheximide/ml. Following the pulse, the cells were washed, and grown further for 18 h in the presence of unlabeled methionine and in the absence of any inhibitor. The cells were then harvested by trypsinization and pooled with $\sim 4 \times 10^7$ carrier cells for the isolation of mitochondria. Reference is made to previous work for the methods used to immunoprecipitate complex I with antibodies directed against the 49-kDa subunit (21) or the cytochrome *c* oxidase complex with antibodies directed against the COOH-terminal undecapeptide of subunit II (CO II; 22) from a Triton X-100 lysate of mitochondria.

DNA Analysis—Total DNA was extracted from peripheral blood cells of the patients and from fibroblast cultures derived from skin biopsies and subjected to polymerase chain reaction (PCR) amplification of a portion of the ND4 gene, using primers between nucleotide positions 11429 and 11448 and between positions 11910 and 11929. The DNA amplified from blood cells was sequenced by the dideoxy chain termination method (23) and found to exhibit the G to A transition at position 11778. The PCR products were checked for heteroplasmy by digestion with *Sfa*NI and *Mae*III (24). The G to A mutation abolishes the *Sfa*NI site normally present and creates a *Mae*III site. DNA was also extracted from Le1- and Le2-derived transmitochondrial cell lines, and the region between nucleotide positions 11575 and 11915 was amplified by PCR using 20-nucleotide long primers. The PCR products were incubated with *Sfa*NI. pBluescript II KS+ plasmid DNA, which contains four *Sfa*NI sites, was added to a portion of the digestion mixture to verify that all *Sfa*NI sites had been cut completely. The reaction mixtures were then analyzed by electrophoresis in a 1.5% agarose gel.

Quantification of mtDNA in total DNA samples from the ρ^0 cell transformants and from 143B cells (isolated with an Applied Biosystems 340 DNA extractor) was carried out by slot blot hybridization, using as a probe plasmid pTZ18/K4, containing the human mtDNA fragment between positions 41 and 2578, labeled with ^{32}P by random priming (25). To correct for possible quantitative variations among samples, the same membrane was incubated with a ^{32}P-labeled nuclear DNA probe, *i.e.* a plasmid containing a 498-base pair portion of 28 S rDNA (26). Quantification of the hybridization signals was performed by analyzing the blots with a PhosphorImager (Molecular Dynamics).

Oxygen Consumption in Intact and Digitonin-permeabilized Cells—Total oxygen consumption rates in intact cells were measured as described (18), except that the medium in the chamber contained 5% dialyzed calf serum, instead of 5% dialyzed FBS. Oxygen consumption measurements in digitonin-permeabilized cells were done as previously detailed (27).

Enzyme Assays—For these assays, the mitochondrial fraction was isolated from ~2 ml of packed cells as described previously (28), resuspended at 3 mg of protein/ml in cold 10 mM Tris-HCl, pH 6.7 (25 °C), 0.25 M sucrose, and sonicated in an ice-water bath for 60 s in six 10-s pulses separated by intervals of at least 30 s. The rate of transfer of electrons from NADH to 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (decylubiquinone; DB) was assayed as previously detailed (29). In particular, this activity was measured, at a protein concentration of approximately 10 μg/ml, in buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM KCN. The reaction mixture, containing 20 μM NADH, was preincubated at 30 °C for 4 min, and the reaction was then started by the addition of DB (ϵ = 14.0 mM$^{-1}\cdot$cm^{-1} at 278 nm) to 2 μM. The oxidation of NADH was monitored at 340 mM minus 400 nm (ϵ = 6.3 mM$^{-1}\cdot$cm^{-1}). The NADH:DB oxidoreductase activity was >95% sensitive to 400 nM rotenone.

Cytochrome *c* oxidase activity was assayed, using the total sonicated mitochondrial fraction, as described previously (30), with 31 μM horse heart cytochrome *c* in a 1-ml reaction volume containing 6–10 μg of protein. Digitonin (Serva) was added to the sonicated mitochondrial fraction to 0.6% at least 15 min before the assay (31). Only the KCN-sensitive activity was measured.

Protein concentration was determined by the Lowry method (32) in the samples used for the NADH:DB oxidoreductase assay, and by the method of Bradford (33) in the samples used for the cytochrome *c* oxidase assay.

RESULTS

Presence of 11778 ND4 Mutation in Cells from LHON Patients and in ρ^0 Transformants Constructed with LHON Patient-derived Fibroblasts—The mtDNA mutation at position 11778 was detected in PCR-amplified DNA from peripheral blood cells from patients 1 and 2 by DNA sequencing. The mutation was present in homoplasmic form in both patients, as verified by the disappearance of a *Sfa*NI site and the specific appearance of a *Mae*III site in the patient's blood cells and in fibroblast cultures, Le1 and Le2, derived from patients 1 and 2, respectively. The DNA from the fibroblasts and blood of patients 1 and 2 did not exhibit any other known LHON-associated primary or secondary mutations, in particular those at positions 3394, 3460, 4160, 4216, 4917, 5244, 7444, 9438, 9804, 13708, 14484, 15257, and 15812.

The mitochondria from the Le1 and Le2 fibroblast strains were transferred to the human mtDNA-less line $\rho^0$206 by cy-

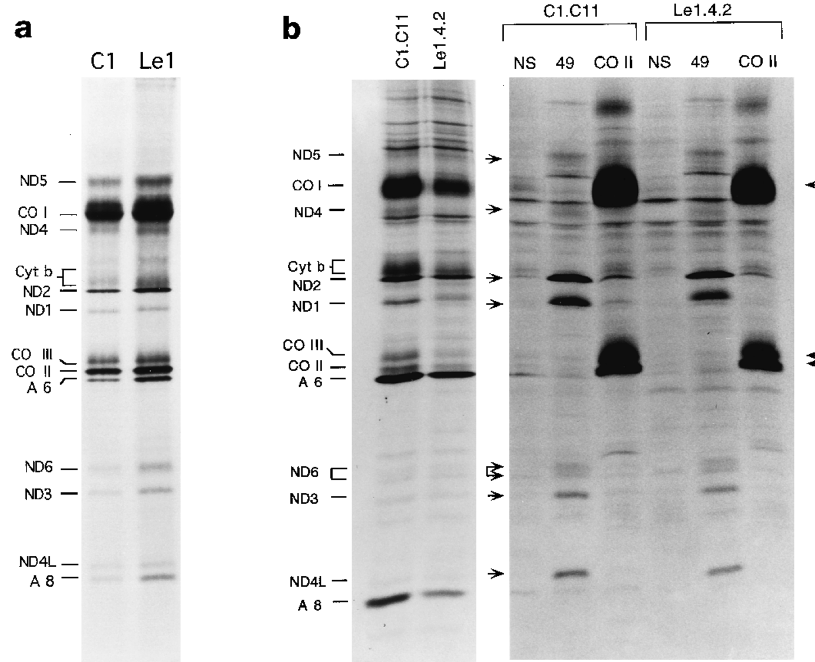

FIG. 1. **Electrophoretic patterns of [^{35}S]methionine pulse-labeled mitochondrial translation products from control and LHON fibroblasts (*a*), and immunoprecipitates obtained by incubating samples of Triton X-100 lysates from a control and a LHON ρ^0 transformant with antibodies against the bovine 49-kDa subunit of complex I or the human CO II subunit (*b*).** Proteins in both panels were run on an exponential gradient polyacrylamide-SDS gel (34) and exposed for fluorography (35). *a*, cells were labeled with [^{35}S]methionine for 1 h in the presence of emetine and immediately harvested. *C1*, fibroblast strain GM03651C; *Le1*, fibroblast strain derived from patient 1; ND1, ND2, ND3, ND4, ND4L, ND5, and ND6, subunits of the respiratory chain NADH dehydrogenase; CO I, CO II, and CO III, subunits of cytochrome *c* oxidase; A6 and A8, subunits of the H^+-ATPase; Cyt b, cytochrome *b*. *b*, the first two lanes show the patterns of labeled proteins in ρ^0 transformants C1.C11 and Le1.4.2 after exposure to [^{35}S]methionine for 2 h in the presence of cycloheximide, followed by a chase for 18 h in medium containing unlabeled methionine and no inhibitors. Proteins are identified as in *panel a*. The following six lanes show the results of immunoprecipitation experiments carried out with 0.5% Triton X-100 lysates from the C1.C11 or Le1.4.2 transformant and normal serum antibodies (*NS*), or antibodies against the purified 49-kDa subunit of bovine complex I (49; Ref. 36), or antibodies against the COOH-terminal undecapeptide of cytochrome *c* oxidase subunit II (CO II; Ref. 22). *Arrows* at *left* indicate ND subunits immunoprecipitated by the 49-kDa specific antibodies. The indicated polypeptides are not present in the NS control or in the CO II control. *Arrows* at *right* indicate subunits precipitated by the CO II-specific antibodies.

toplast × ρ^0 cell fusion (18). Of several mitochondrial transformant clones isolated, four, *i.e.* Le1.3.1, Le1.4.2, Le2.F10, and Le2.H4, were subjected to molecular and biochemical analysis. These were all homoplasmic for the 11778 mutation (data not shown). Control cell lines included mitochondrial transformant C1.C11, derived from fibroblast strain GM03651C; transformants C2.C1, C2.H1, and C2.A8, derived from fibroblast strain GM03652A; normal transformant pT3, derived from a myoblast strain established from a myoclonic epilepsy and ragged red fiber syndrome patient, and the 143B cell line. None of the controls carried the 11778 mutation, as detected by PCR and *Sfa*NI digestion. Quantification of mtDNA in the transmitochondrial cell lines showed that their mtDNA copy number varied between 40 and 150% of that of 143B cells (9100 mtDNA molecules per cell (18)), without any significant difference between the transformants derived from the two patients carrying the 11778 mutation and the control transformants.

Lack of Effect of the 11778 Mutation on the Rate of Synthesis of ND4 Subunit and Assembly of Complex I—Experiments were carried out to test whether the 11778 mutation in the ND4 gene affects the synthesis or stability of the ND4 subunit or the assembly of complex I. Fig. 1*a* shows the electrophoretic patterns, after a 1-h pulse labeling with [^{35}S]methionine in the presence of the cytosolic protein synthesis inhibitor emetine, of the mitochondrial translation products of the fibroblast culture, Le1, derived from patient 1 and of a control fibroblast culture, GM03651C. The ND4 polypeptide exhibits the same electrophoretic mobility and is labeled to the same relative extent in the two cultures, as determined by densitometric analysis of appropriate exposures of the autoradiogram.

Previous work (27) had pointed to a role of the ND4 subunit in the assembly of complex I. In the present investigation, to determine whether the 11778 mutation affects the assembly of complex I, antibodies against the 49-kDa subunit of complex I, a nuclear-encoded subunit, which are able to immunoprecipitate the entire complex I from a Triton X-100 lysate of mitochondria (21), were used. For this experiment, mitochondrial transformant cells Le1.4.2 were labeled for 2 h with [^{35}S]methionine in the presence of cycloheximide, a reversible inhibitor of cytosolic protein synthesis, and then chased for 18 h in medium lacking inhibitors. Fig. 1*b* shows that the ND4 subunit, as well as the other mtDNA-encoded subunits of the respiratory chain NADH dehydrogenase, are incorporated into this complex in cells carrying the 11778 mutation to the same extent as in the wild-type C1.C11 transformant cells.

Respiration Capacity of ρ^0 Cell Transformants Carrying the 11778 ND4 Mutation—Fig. 2 shows the total O_2 consumption rate in the intact ρ^0 transformant cells containing the 11778 mutation, in the control transformants, and in 143B cells. There is appreciable variation in O_2 consumption rate both among the control transformants and among the mutant transformants. In particular, two of the three control transformants derived from fibroblast strain GM03652A, *i.e.* C2.C1 and C2.H1, exhibited a significantly lower respiration rate than the third transformant, C2.A8, derived from the same cell line. The variability in respiration activity among transformants carry-

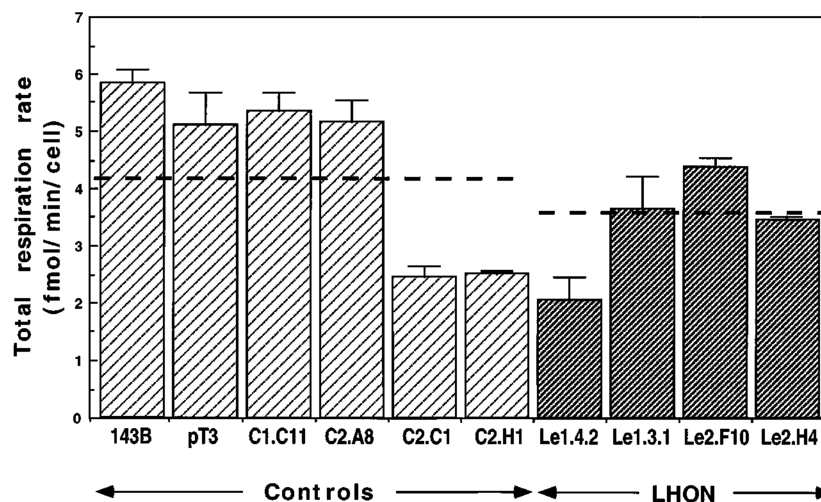

FIG. 2. **Total respiration rate of ρ^0 cell transformants.** Respiration rates were measured on approximately 5×10^6 cells. *Error bars* represent two standard errors of the mean. The data for 143B and pT3 were taken from Chomyn *et al.* (19).

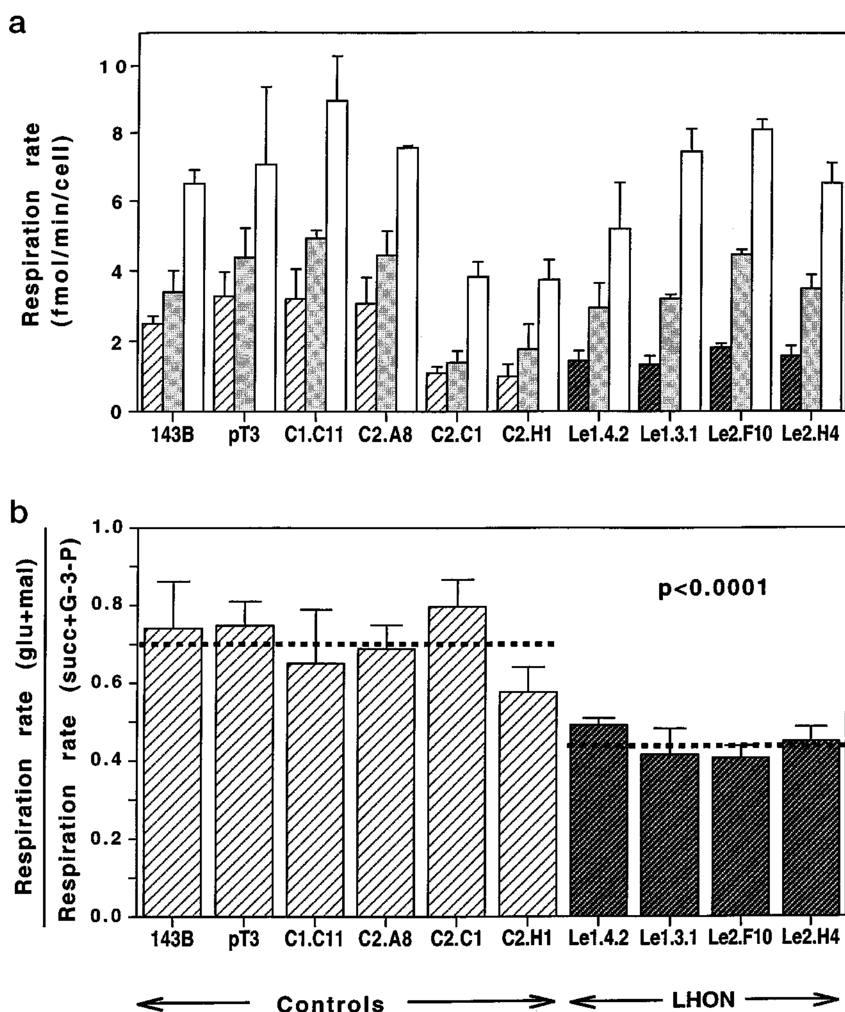

FIG. 3. **Polarography assays on digitonin-permeabilized ρ^0 cell transformants, carried out using different substrates.** *a,* the *hatched bars* represent glutamate/malate-driven respiration, the *shaded bars*, succinate/glycerol-3-P-driven respiration, and the *white bars,* TMPD/ascorbate-driven respiration, determined on 0.5–1 $\times$ 10^7 cells. *b*, for each cell line, glutamate/malate-driven respiration was normalized to succinate/glycerol-3-P-driven respiration. The *dashed horizontal lines* represent the means for the controls and the LHON transformants. *p*, probability, according to the Student's *t*-test, that the two groups of samples belong to two populations having the same mean was calculated using Microsoft Excel software. The data for 143B were taken from Hofhaus and Attardi (27). *Error bars* represent two S.E. of the mean.

ing the same mtDNA, a phenomenon which had been previously observed (37), was not correlated with any difference in either growth rate (see below) or mtDNA content of the transformants. Despite the variability just mentioned, it is clear that the average respiratory activity of the cell lines carrying the 11778 mutation is decreased relative to that of the control cell lines; however, the difference is not statistically significant ($p = 0.15$).

Because the 11778 mutation occurs in a gene coding for a subunit of the respiratory chain NADH dehydrogenase, it was expected that the primary defect accounting for the decrease in respiration would be in the respiratory NADH dehydrogenase. To obtain evidence on this point, the activity of the individual components of the mitochondrial respiratory chain was measured by a polarographic analysis of digitonin-permeabilized cells. Cells were treated with the detergent, thereby washing out the endogenous substrates (38), and then, the rates at which the cells consumed oxygen in response to different substrates were measured (27, 38, 39). With malate and glutamate as substrates, the corresponding dehydrogenases generate

TABLE I

Measurement of NADH:DB oxidoreductase activity in the sonicated mitochondrial fraction from 11778 mutation carrying and control cells

Enzyme activities are expressed in nmol · min^{-1} · (mg protein)$^{-1}$. The NADH:DB oxidoreductase activity was measured in triplicate, and the cytochrome *c* oxidase activity, in triplicate or quadruplicate. Numbers in parentheses represent two standard errors of the mean. The values for NADH:DB activity represent the total activity; however, more than 95% of this activity was rotenone-sensitive.

	Controls		LHON	
	C1.C11	C2.A8	Le1.4.2	Le2.F10
NADH:DB	60.5 (0.92)	23.7 (0.81)	26.6 (1.10)	26.0 (0.40)
Cytochrome *c* oxidase	230 (13.9)	126 (0.65)	81 (2.0)	126 (2.2)
NADH:DB / Cytochrome *c* oxidase	0.26 (0.016)	0.19 (0.007)	0.33 (0.016)	0.21 (0.005)

TABLE II

Comparison of growth and respiration properties of 11778 mutation carrying and control cells

The doubling time of different cell lines in DMEM was determined from growth curves. The extent of growth of the different cell lines in medium containing galactose as the only sugar, and in medium containing glucose at 100 h after seeding was determined from growth curves. Malate/glutamate-driven respiration and succinate/glycerol 3-phosphate-driven respiration rates were measured in triplicate (Fig. 3*a*) and are expressed as fmol · min^{-1} · $cell^{-1}$.

Cell line	Doubling time in DMEM	No. of cells in galactose at 100 h / No. of cells in glucose at 100 h	Malate/glutamate respiration	Succinate/glycerol-3-P respiration
Controls				
143B	16.8	0.20	2.5	3.4
pT3	18.3	0.36	3.3	4.4
C1.C11	21.6	0.33	3.2	4.9
C2.A8	30.9	0.25	3.1	4.4
C2.C1	19.3	0.15	1.1	1.4
Mean	21.4	0.26	2.6	3.7
LHON				
Le1.4.2	28.2	0.12	1.4	2.9
Le2.F10	22.6[a]	0.14[a]	1.8	4.4
Le2.H4	20.5	0.14	1.6	3.5
Mean	23.8	0.13	1.6	3.6
p[b]	0.42	0.014	0.004	0.89
Correlation analysis				*r*
Galactose/glucose cell number ratio *versus* malate/glutamate respiration				0.92
Galactose/glucose cell number ratio *versus* succinate/glycerol-3-P respiration				0.62

[a] Average of two values.

[b] Probability that the control data set and the LHON data set derive from two populations having the same mean (Student's *t* test).

NADH in the mitochondrial matrix, which is oxidized by the NADH:ubiquinone oxidoreductase (complex I). The electrons from NADH are transferred via the ubiquinol:cytochrome *c* oxidoreductase (complex III) and the cytochrome *c*:O_2 oxidoreductase (complex IV) to oxygen. Using succinate and glycerol 3-phosphate as substrates, the corresponding dehydrogenases cause a direct reduction of ubiquinone, by-passing complex I. The respiration observed with these substrates is dependent only on complexes III and IV. (The reduction of ubiquinone by succinate dehydrogenase and glycerol-3-P dehydrogenase in combination is not rate-limiting because the addition of either succinate or glycerol-3-P alone to a suspension of permeabilized 143B cells allows at least 80% of the respiration rate that is supported by both substrates in combination.[2]) The reagents *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate reduce intracellular cytochrome *c*, thereby allowing respiration due to complex IV activity alone to be measured. The substrate-dependent respiration rates reflect the activities of the individual complexes of the respiratory chain, because complex IV activity is usually higher than complex III activity, which in turn is usually higher than complex I activity.

Fig. 3*a* shows that the malate/glutamate-driven respiration rates in the 11778 mutation-carrying cell lines are clearly lower than those of the control cell lines, whereas the succinate/glycerol-3-P- and the TMPD/ascorbate-driven respiration rates are comparable to those of the controls. This observation is consistent with the occurrence of a specific defect in the respiratory chain at the level of complex I. Control transformants C2.C1 and C2.H1, which exhibit an unusually low oxygen consumption rate (Fig. 2), also show a low malate/glutamate-driven respiration. However, the succinate/glycerol-3-P- and TMPD/ascorbate-driven respiration rates in these cell lines are also reduced, a result which points to an overall reduction in mitochondrial respiratory enzyme activity. Fig. 3*b* shows the glutamate/malate-driven respiration rates of the various cell lines, which reflect their complex I activity, normalized to their succinate/glycerol-3-P-driven respiration rates, which reflect their complex III activity. It is clear that the complex I-dependent respiration rate in the cell lines carrying the 11778 mutation is specifically decreased when compared to the control cell lines, the difference being statistically very significant.

Enzyme Activities—The NADH dehydrogenase activity was measured in the isolated mitochondrial fraction from two control and two LHON transformants, using as electron acceptor DB, which is a soluble analogue of the endogenous acceptor, ubiquinone. Cytochrome *c* oxidase activity was also measured in the same preparations in order to correct the NADH:DB oxidoreductase activities for differences in mitochondrial membrane content among the crude mitochondrial preparations isolated from different clones. As shown in Table I, the values for NADH:DB oxidoreductase activity varied somewhat, but when the activities were normalized to cytochrome *c* oxidase activity, there was no consistent difference between mutant and wild type transformants ($p = 0.60$).

Growth Properties of ND4 11778 Mutation-carrying ρ^0 *Cell Transformants and Control Cells*—Table II shows that the growth rate in glucose-containing medium (DMEM) of the 11778 mutation-carrying cell lines is not significantly different

[2] G. Hofhaus, unpublished data.

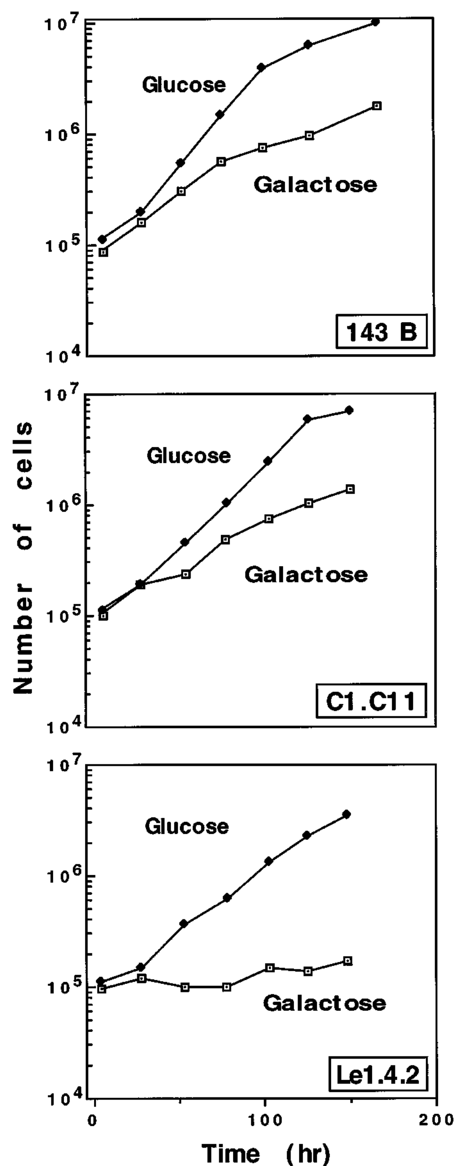

FIG. 4. **Growth curves of 143B, C1.C11, and Le1.4.2.** *Glucose*, growth curve for cells grown in DMEM containing 4.5 mg of glucose/ml and 0.11 mg of pyruvate/ml; *galactose*, growth curve for cells grown in DMEM lacking glucose and containing instead 0.9 mg of galactose/ml and 0.5 mg of pyruvate/ml.

from that measured in the control cell lines. In order to investigate whether the complex I defect detected in the ρ^0 cell transformants carrying the 11778 mutation affected their growth capacity in a selective medium, these transformants were tested for growth in a medium in which galactose replaced glucose. In this type of medium, cells deficient in respiratory function (20, 40–42) are greatly curtailed in their growth rate. Fig. 4 compares the growth curves in galactose medium and glucose medium of the transformant Le1.4.2, carrying the 11778 mutation, with those of control cells, 143B and C1.C11. Clearly the cells carrying the mutation grew only at a minimal rate in the galactose medium, whereas control cells showed a substantial growth rate in this medium, although reduced as compared to their growth in glucose-containing medium.

The growth curves in the two media were determined for several mutant and control cell lines. Table II shows the ratios of the number of cells in the galactose medium to the number of cells in the glucose medium after a 100-h incubation, as determined from the growth curves. It is clear that the extent of growth allowed by the galactose medium relative to that in glucose medium was reduced from an average of 26% in the control cell lines to an average of 13% in the mutant cell lines, the difference being statistically significant at the 99% confidence level. Table II shows also that the ratio of growth in galactose to growth in glucose of the various cell lines correlates, with a high level of significance (r = 0.92), with the malate/glutamate-dependent respiration rate, but not with the succinate/glycerol-3-P-driven respiration rate (r = 0.62). The correlation holds also for the transformant C2.C1 that grows relatively poorly in galactose medium and has a very low malate/glutamate-dependent respiration rate.

DISCUSSION

The central result reported here is the demonstration that specific biochemical defects associated with the 11778 LHON mutation could be transferred with mitochondria into $\rho^0$206 mtDNA-less cells, thereby establishing a cellular model for this disease in a foreign nuclear background. This result is particularly significant, because the tissue specificity, low penetrance, and gender bias of LHON have strongly suggested that a nuclear factor(s) plays a role in the manifestation of the disease. It is not known whether the $\rho^0$206 nuclear background carries the putative change in gene content or activity that contributes to the phenotype in affected individuals. The frequency of the X-linked allele that has been suggested to be essential for the manifestation of the pathogenetic effects of the 11778 ND4 mutation (16) has been estimated to be ~0.08 (11, 15). Further investigations using the $\rho^0$206 cellular model described here and similar models constructed with genetically different ρ^0 cell recipients, as well as extending the analysis to asymptomatic individuals carrying the 11778 ND4 mutation in homoplasmic form, should be able to distinguish the effects of the mtDNA mutation from those of the putative nuclear factor(s), and may lead to the identification of this factor.

In the present work, all the ρ^0 transformants carrying the 11778 mutation exhibited a clearly defective phenotype, which could unambiguously be associated with the mutation. In particular, they showed a very significant decrease in NADH dehydrogenase-dependent respiration after digitonin permeabilization. However, we were not able to detect a reduction in NADH dehydrogenase activity in sonicated mitochondria from the transformants using NADH and a water-soluble ubiquinone analogue in a photometric assay. These results are in agreement with those obtained by Majander and colleagues (13) and Larsson *et al.* (12) who used similar assays to ours on intact and disrupted mitochondria from individuals with the 11778 mutation. Vergani *et al.* (43), who have recently reported the transfer into $\rho^0$206 cells of mitochondria from patients' cells carrying the 11778 ND4 mutation, also failed to observe a decrease in NADH:Q oxidoreductase activity, as measured in total cell sonicates of the transformants. However, these authors did not carry out any polarographic measurements on permeabilized transformant cells.

As to the basis for the apparent discrepancy between the data from the direct and those from the indirect assays in our experiments and in those by Larsson *et al.* (12) and Majander *et al.* (13), it should be noted that, in the assays on digitonin-permeabilized cells or on isolated intact mitochondria, the NADH is possibly delivered directly to the binding site by substrate dehydrogenases, which have been suggested to be closely associated with complex I (44). The 11778 ND4 mutation could conceivably interfere with the interaction of complex I with NAD-linked dehydrogenases, and, therefore, with the channeling of NADH to complex I in the putative multienzyme complex (13), and thereby cause a decrease in enzymatic activity. This effect would not be observed in enzymatic assays in

which an excess of NADH, rather than an oxidizable substrate, is added to the mitochondrial fraction.

Another factor to take into consideration is the use of hydrophilic analogues of ubiquinone in the enzyme assays. If the diffusion of these substrates into the membrane is the rate-limiting step (45), these assays would not measure the full activity of the wild-type enzyme. As a consequence, decreases in the activity of the enzyme may not be easily detectable.

Degli Esposti *et al.* (14) have reported that, in mitochondria from individuals carrying the 11778 mutation, the NADH:Q reductase has a decreased sensitivity to the specific inhibitor rotenone, as tested in an assay with the ubiquinone analogue UBQ, as well as altered K_m values for this analogue and another analogue, ubiquinone-2. On the basis of these findings, they have proposed that the binding site for ubisemiquinone is altered, and, as a consequence, the destabilized ubisemiquinone has an increased tendency to dismutate, with a resulting loss in the energy conserving function of complex I. More information on the topology of complex I and the position and role of ND4 in it may be required for a full understanding of the basis of the discrepancy between the results of the direct spectrophotometric assays and those of the indirect assays of complex I activity.

In addition to detecting a decrease in NADH dehydrogenase-dependent respiration, we have been able to demonstrate for the first time a specific growth impairment in cells carrying a LHON mutation in a medium containing galactose in place of glucose. In glucose-containing medium, cultured wild-type mammalian cells are known to derive 45–60% of their ATP from glycolysis (46, 47); therefore, in this medium, even respiration-deficient cells can grow, although possibly at a reduced rate, relying predominantly or exclusively on glycolysis for the production of ATP. On the other hand, galactose is metabolized through the glycolytic pathway only very slowly (47); therefore, in galactose-containing medium, respiration-deficient cells would be expected to be affected in their growth rate. According to this interpretation, the decrease in respiratory efficiency of the LHON transformants reduces their rate of ATP production by oxidative phosphorylation to an extent sufficient to affect their growth capacity in galactose-containing medium. It should be mentioned that Vergani *et al.* (43) reported a decrease in growth rate of cells carrying the 11778 mutation in standard glucose-containing medium, that was not observed in our transformants.

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