MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 771–777 Vol. 16, No. 3
0270-7306/96/$04.00+0

Use of Transmitochondrial Cybrids To Assign a Complex I Defect to the Mitochondrial DNA-Encoded NADH Dehydrogenase Subunit 6 Gene Mutation at Nucleotide Pair 14459 That Causes Leber Hereditary Optic Neuropathy and Dystonia

ALBERT S. JUN, IAN A. TROUNCE, MICHAEL D. BROWN, JOHN M. SHOFFNER, AND DOUGLAS C. WALLACE*

Department of Genetics and Molecular Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

Received 29 June 1995/Returned for modification 17 August 1995/Accepted 30 November 1995

A heteroplasmic G-to-A transition at nucleotide pair (np) 14459 within the mitochondrial DNA (mtDNA)-encoded NADH dehydrogenase subunit 6 (ND6) gene has been identified as the cause of Leber hereditary optic neuropathy (LHON) and/or pediatric-onset dystonia in three unrelated families. This ND6 np 14459 mutation changes a moderately conserved alanine to a valine at amino acid position 72 of the ND6 protein. Enzymologic analysis of mitochondrial NADH dehydrogenase (complex I) with submitochondrial particles isolated from Epstein-Barr virus-transformed lymphoblasts revealed a 60% reduction ($P < 0.005$) of complex I-specific activity in patient cell lines compared with controls, with no differences in enzymatic activity for complexes II plus III, III, and IV. This biochemical defect was assigned to the ND6 np 14459 mutation by using transmitochondrial cybrids in which patient Epstein-Barr virus-transformed lymphoblast cell lines were enucleated and the cytoplasts were fused to a mtDNA-deficient (ρ^0) lymphoblastoid recipient cell line. Cybrids harboring the np 14459 mutation exhibited a 39% reduction ($P < 0.02$) in complex I-specific activity relative to wild-type cybrid lines but normal activity for the other complexes. Kinetic analysis of the np 14459 mutant complex I revealed that the V_{max} of the enzyme was reduced while the K_m remained the same as that of wild type. Furthermore, specific activity was inhibited by increasing concentrations of the reduced coenzyme Q analog decylubiquinol. These observations suggest that the np 14459 mutation may alter the coenzyme Q-binding site of complex I.

Leber hereditary optic neuropathy (LHON) and pediatric-onset dystonia have been associated with a heteroplasmic G-to-A transition at nucleotide pair (np) 14459 within the mitochondrial DNA (mtDNA)-encoded ND6 gene (24). This mutation has been identified in three independent pedigrees presenting with LHON plus dystonia (24, 36), LHON alone, or dystonia alone (46). Each of these families had a different background mtDNA haplotype. Consequently, the np 14459 mutation must have arisen three independent times and must be the cause of these clinical phenotypes.

The np 14459 mutation changes a moderately conserved alanine to a valine at amino acid 72 within the most evolutionarily conserved "span C" of the ND6 polypeptide (14). The associated LHON is an adult-onset acute or subacute central vision loss (8, 55), while the pediatric-onset neurodegenerative disorder is characterized by a severe, generalized movement disorder (dystonia) with a mean age of onset under 5 years old and is associated with basal ganglion lucencies, short stature, various degrees of dementia, and bulbar and corticospinal tract dysfunction (36, 46).

Mitochondria generate ATP for the cell via oxidative phosphorylation (OXPHOS), an enzyme pathway consisting of five multisubunit enzyme complexes located within the mitochondrial inner membrane. This pathway is subdivided into the electron transport chain, consisting of complexes I through IV and the ATP synthase, complex V. Electrons enter the electron transport chain from NADH via complex I (NADH:ubiquinone oxidoreductase [EC 1.6.5.3]) or from succinate to complex II (succinate:ubiquinone oxidoreductase [EC 1.3.5.1]). They pass through ubiquinone to complex III (ubiquinol:ferricytochrome *c* oxidoreductase [EC 1.10.2.2]), then through cytochrome *c* to complex IV (ferrocytochrome *c*:oxygen oxidoreductase [EC 1.9.3.1]), and finally to oxygen to generate water. Complexes I, III, and IV couple electron transport to the extrusion of protons from the mitochondrial matrix to the intermembrane space. The resulting proton electrochemical gradient across the inner membrane provides the potential energy necessary for the condensation of ADP and P_i to ATP by complex V (ATP synthase [EC 3.6.1.34]) (47, 55, 56).

Complexes I, III, IV, and V are assembled from both nuclear DNA- and mtDNA-encoded gene products. The human mtDNA is a 16,569-np double-stranded, circular molecule encoding a total of 13 polypeptides including seven subunits (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) of complex I, one subunit (cytochrome *b*) of complex III, three subunits (COI, COII, and COIII) of complex IV, and two subunits (ATPases 6 and 8) of complex V. Complex II is composed entirely of nuclear DNA-encoded products. The mtDNA also encodes the 12S and 16S rRNAs and 22 tRNAs required for mitochondrial protein translation (1, 47, 55–57, 59).

Mutations of the mtDNA have been shown to cause a wide variety of human degenerative diseases, with the central nervous system, skeletal and cardiac muscle, kidneys, and endocrine organs being most commonly affected (47, 56, 59). Biochemical deficiencies have been associated with a variety of pathogenic mtDNA point mutations (44, 47). For LHON, reductions in complex I activity have been demonstrated for mutations at np 11778 (27, 32, 49), np 3460 (32, 49), and np 4160 plus np 14484 (39). However, evidence suggesting a bio-

* Corresponding author. Mailing address: Department of Genetics and Molecular Medicine, 1462 Clifton Rd., NE, Rm. 446, Atlanta, GA 30322. Phone: (404) 727-5624. Fax: (404) 727-3949.

TABLE 1. OXPHOS enzyme specific activity of control and patient EBV-transformed lymphoblast cell lines

Cell line	Genotype of np 14459[a]	Sp act (nmol of substrate/min/mg of mitochondrial protein) of[b]:					Complex I/CS ratio[c]
		Complex I	Complex II+III	Complex III	Complex IV	CS	
Control (n = 10)[d]	100% WT	55 ± 17	234 ± 70	441 ± 64	530 ± 238	502 ± 152	0.11 ± 0.02
Patient (n = 5)[e]	95–100% mutant	22 ± 8* (P = 0.0027)	215 ± 66	500 ± 141	543 ± 187	481 ± 114	0.05 ± 0.01* (P = 0.0007)

[a] The genotype was determined for four control lines and five patient lines at the time of mitochondrial isolation. For patients, four of five cell lines demonstrated 0% wild-type (WT) mtDNA and one demonstrated residual (<5%) wild-type mtDNA.
[b] Asterisks represent statistical significance ($P < 0.05$) by the two-tailed, Mann-Whitney U test for unpaired samples.
[c] Complex I specific activity divided by CS specific activity as a normalization for total mitochondrial protein, determined for individual mitochondrial isolates.
[d] n is the number of cell lines, each from a different individual. Values listed represent means ± standard deviations (SDs) of single mitochondrial isolates performed for each control cell line.
[e] n is the number of cell lines, each from a different individual. All three np 14459-positive families are represented. Mean values of OXPHOS enzyme activity were determined for each cell line by using multiple independent mitochondrial isolates. Mean values for individual cell lines were used to calculate overall patient group means ± SDs listed above.

chemical mechanism for the complex I enzymatic defects seen in LHON is still lacking. A complex I defect has been found in cultured cells harboring the mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) np 3243 mutation (21), and a complex V defect involving inhibition of the proton channel and altered ADP-to-O ratio has been associated with the Leigh's syndrome np 8993 mutation (52). Defects in OXPHOS also have been associated with a number of neurodegenerative movement disorders (2, 45) including Parkinson's disease (5–7, 35, 38, 41–43, 48), multiple-system atrophy (7), Huntington's disease (37), and idiopathic dystonia (3).

The mtDNA origin of OXPHOS defects can be demonstrated by the cytoplasmic hybrid (cybrid) transfer technique (58). In these studies, patient cells are enucleated and the mitochondria and mtDNA-containing cytoplasmic fragments are fused to recipient cells that have been cured of their mtDNAs (ρ^0 cells) through long-term treatment with ethidium bromide (25). Transfer of a biochemical OXPHOS defect together with a mtDNA mutation has been shown for the np 3243 MELAS mutation (26), the np 8344 myoclonic epilepsy and ragged red fibers (MERRF) mutation (12, 13), the mtDNA deletion mutations of chronic progressive external ophthalmoplegia (17), and the np 8993 mutation of Leigh's syndrome (52).

In the present study, we report a biochemical defect associated with the LHON-and-dystonia np 14459 mutation. OXPHOS enzymology of mitochondria isolated from Epstein-Barr virus (EBV)-transformed lymphoblast cell lines revealed a 60% reduction ($P < 0.005$) in complex I-specific activity in patients with respect to controls. Enucleation of the patient lymphoblasts and fusion with ρ^0 recipient cells followed by enzymologic analysis revealed that the defect was transferred with the np 14459 mutant mtDNA. Further biochemical analysis of the mutant enzyme revealed a reduced V_{max} and evidence of product inhibition. This study represents the most extensive biochemical characterization of a mtDNA mutation causing LHON.

MATERIALS AND METHODS

Cell lines and culture conditions. Lymphoblast cell lines were established by EBV transformation of leukocytes isolated from whole blood by using Ficoll-Hypaque gradients (60). Patient lymphoblast lines were established from family 1, a five-generation Native American kindred presenting with both LHON and dystonia (36); family 2, a two-generation African American pedigree expressing only LHON; and family 3, a singleton European American case expressing only dystonia (46). One patient lymphoblast line was established from blood leukocytes of a heteroplasmic (~25 to 50% wild-type mtDNA) individual from each of the three pedigrees. Two additional patient lymphoblast lines were established from homoplasmic mutant individuals. Genotyping of five patient lines revealed that four lines had 100% mutant mtDNA while one line had <5% residual wild-type mtDNA (Table 1). The observed segregation to higher levels of mutant mtDNA and the indistinguishable growth characteristics between wild-type and mutant lines indicate that the np 14459 mutation does not adversely affect the growth of cultured cells. The tendency toward higher levels of mutant mtDNA in cultured cells has been observed previously for the MELAS $tRNA^{Leu(UUR)}$ np 3243 mutation (15, 62).

Control cell lines were established from clinically normal men (n = 8) and women (n = 6) (age range, 22 to 45 years). Genotyping of four of the control lines confirmed that all contained 100% wild-type mtDNA.

All EBV-transformed lymphoblast cell lines were maintained in RPMI 1640 medium (Bio-Whittaker, Walkersville, Md.) supplemented with 15% (vol/vol) heat-inactivated fetal bovine serum (Gibco-BRL Life Technologies, Grand Island, N.Y.), 4 mg of glucose per ml, 50 μg of uridine per ml, and 1 mM pyruvate (GUP). EBV-transformed lymphoblast cell lines were maintained in culture for 10 to 20 population doublings at the time of mitochondrial isolation.

The WAL2A-ρ^0 line was cured of its mtDNA by long-term exposure of the parental line WAL2A to low levels of ethidium bromide (51). WAL2A is an $HPRT^-$ clone of the diploid lymphoblast cell line WI-L2 (29, 58) and is grown in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 2 mg of glucose per ml, and 1 μg of 6-thioguanine per ml (6TG). The WAL2A-ρ^0 line is dependent on pyruvate and uridine for growth because of the absence of a functioning respiratory chain (16, 25, 51) and is grown in GUP medium containing 1 μg of 6TG per ml. Cybrid lines were passaged between 5 and 10 population doublings prior to mitochondrial isolation (51).

mtDNA genotyping. Chelex 100 (Cetus, Emeryville, Calif.) (61) was used to isolate total genomic DNA from approximately 10^6 EBV-transformed lymphoblasts or cybrids sampled from roller-bottle cultures at the time of mitochondrial isolation. The mutant and wild-type sequences at np 14459 in the ND6 gene were detected by PCR amplification of mtDNA, digestion with *Mae*III endonuclease (Boehringer-Mannheim), agarose gel electrophoresis, and ethidium bromide visualization (24). Mutant and wild-type fragments were quantitated by standard densitometric methods.

Mitochondrial isolation, polarography, and OXPHOS enzymology. The procedures for the isolation of intact mitochondria, polarographic analysis, and OXPHOS enzymology of submitochondrial particles are described in detail elsewhere (51, 63). Protein concentrations of mitochondrial isolates were determined by the method of Lowry et al. (30) with bovine serum albumin as a standard. EBV-transformed lymphoblast and cybrid mitochondrial protein concentrations ranged from 5 to 15 mg/ml.

For polarographic analysis of all mitochondrial preparations, two runs were performed for each substrate set (malate plus pyruvate, malate plus glutamate, and succinate), with two additions of ADP and an addition of the OXPHOS uncoupler 2,4-dinitrophenol to conclude the run. All runs were performed with 250 to 500 μg of mitochondrial protein. For each ADP addition, 125 nmol of ADP was used for complex I substrate combinations and 75 nmol of ADP was used for the complex II substrate, succinate. The respiratory control ratio for each mitochondrial isolate was defined as the mean state III respiration rate divided by the mean state IV respiration rate, and all isolates included in this study had respiratory control ratios of >2 as a minimum standard of mitochondrial integrity.

OXPHOS enzyme activity of sonicated mitochondria was measured spectrophotometrically with a dual-beam UV-visual spectrophotometer (model DW-2000; SLM-Aminco, Urbana, Ill.). Complex I was assayed with 30 μg of mitochondrial protein, 30 μM NADH, and 10 μM decylubiquinone (DB), a coenzyme Q_{10} analog. Enzyme activity was monitored by the reduction of DB at 272 nm, with all activities assayed in triplicate. Rotenone-sensitive complex I activity was also determined and consistently made up 90 to 100% of total activity. For complex II plus III assays, 15 μg of mitochondrial protein was used and activity was assayed in duplicate. For complex III assays, 7.5 μg of mito-

TABLE 2. OXPHOS enzyme specific activity of control and patient transmitochondrial cybrids

Cell line	Genotype of np 14459[a]	Sp act (nmol of substrate/min/mg of mitochondrial protein) of[b]:					Complex I/CS ratio
		Complex I	Complex II+III	Complex III	Complex IV	CS	
Control ($n = 6$)[c]	100% WT	41 ± 10	162 ± 52	823 ± 308	396 ± 72	362 ± 78	0.12 ± 0.02
Patient ($n = 5$)[d]	95–100% mutant	25 ± 9* ($P = 0.0173$)	159 ± 31	786 ± 77	435 ± 109	466 ± 66	0.05 ± 0.01* ($P = 0.0087$)

[a] Genotypes were determined for six control lines and five patient lines at the time of mitochondrial isolation. For patients, one of five cell lines demonstrated 0% wild-type (WT) mtDNA and four demonstrated residual (<5%) wild-type mtDNA.

[b] Asterisks represent statistical significance ($P < 0.05$) by the two-tailed, Mann-Whitney U test for unpaired samples.

[c] n is the number of cell lines, each from a different individual. Values listed represent means ± SDs of single mitochondrial isolates performed for each control cell line.

[d] n is the number of cell lines, each from a different individual. All three np 14459-positive families are represented. Mean values of OXPHOS enzyme activity were determined for each cell line by using two independent mitochondrial isolates. Mean values for individual cell lines were used to calculate the overall patient cybrid mean ± SD values.

chondrial protein was used and activity was assayed in triplicate. Antimycin A-sensitive complex III activity made up 75 to 100% of total cytochrome *c* reducing activity. For complex IV assays, 7.5 μg of mitochondrial protein was used and the activity was assayed in duplicate. For citrate synthase (CS) assays, 15 μg of mitochondrial protein was used and the activity was assayed in duplicate.

Kinetic analysis of complex I. EBV-transformed lymphoblast mitochondria from five control and five patient cell lines (representing all three affected families) were isolated and assayed as described above with the following modifications. For DB kinetic analysis of complex I, NADH was used at a final concentration of 30 μM. For NADH kinetic analysis of complex I, DB was used at a final concentration of 5 μM. Determinations were made in duplicate for each cell line at different concentrations of substrate.

Product inhibition studies were performed with final concentrations of 5 μM DB and 20 μM NADH. The reaction mix was incubated with specified concentrations of the reduced ubiquinol analog DBH_2, and determinations were made in duplicate for each cell line at different DBH_2 concentrations. DBH_2 was prepared from 1.2 mM DB in ethanol by the addition of potassium borohydride (Sigma, St. Louis, Mo.) and 1/5 volume of 0.1 N HCl in a microcentrifuge tube. Particulate potassium borohydride was separated from DBH_2 by microcentrifugation, the supernatant was removed, and 1/10 volume of 1 N HCl was added.

The oxidation rate of DBH_2 by complex III in the absence of cytochrome *c* was determined to be negligible by preincubating submitochondrial particles with 0 μM DB and 5 μM DBH_2 under standard complex I assay conditions and measuring the change in A_{272} for 2 min. K_m and V_{max} values were estimated for control and patient complex I activities by using Enzfitter version 1.0.

Statistical analysis. *P* values were calculated by the Mann-Whitney, unpaired, two-tailed test (Instat for Macintosh, version 2.03; Graphpad Software, Inc.).

RESULTS

Construction of transmitochondrial cybrid cell lines. Transmitochondrial cybrids were prepared by enucleating EBV-transformed lymphoblastoid cell lines from patients with the ND6 np 14459 mutation and controls and fusing the mitochondrion-containing cytoplasts with the mtDNA-deficient (ρ^0) lymphoblastoid cell line WAL2A-ρ^0. Cybrids were isolated from the electrofusion of 10^7 WAL2A-ρ^0 cells and 2×10^7 enucleated cytoplasts by inoculation into 75 ml of RPMI 1640 medium supplemented with 10% (vol/vol) dialyzed fetal bovine serum (Gibco-BRL Life Technologies), 2 mg of glucose per ml, and 1 μg of 6TG per ml. The 6TG selected for the WAL2A-ρ^0 nucleus and against the cytoplasmic donor cells and the absence of uridine and pyruvate selected against the WAL2A-ρ^0 parent and for cybrids with functional mitochondria. Rapid growth of the cybrid cultures was observed 20 to 28 days postfusion.

Cybrid cell lines were isolated from six independent control fusions and five independent patient fusions. Patient cybrid lines included two cybrid lines from family 1 with a Native American mtDNA background (24), two cybrid lines from family 2 with an African mtDNA background, and one cybrid line from family 3 with a European mtDNA background (46). All of the patient cybrids were >95% mutant at np 14459.

OXPHOS enzymology of patient and control lymphoblast and cybrid cell lines. To determine if the np 14459 mutation was associated with an OXPHOS defect, enzymatic assays for complexes I, II plus III, III, and IV and CS were performed for 10 control cell lines and 5 patient cell lines representing all three np 14459-positive families. The mean complex I specific activity for patient lymphoblast mitochondria was reduced by 60% ($P = 0.0027$) compared with controls (Table 1), with both patient and control complex I activities being inhibited by greater than 95% with 3.8 μM rotenone. By contrast, the mean specific activities for complex II plus III, III, and IV and CS assays were indistinguishable among groups.

Calculation of the ratio of complex I to CS specific activities also revealed a highly significant 55% reduction ($P = 0.0007$) in patients compared with controls (Table 1). Thus, reduction in both the mean complex I specific activity and mean complex I/CS ratio demonstrates that the np 14459 mutation is associated with a complex I defect.

To confirm that the complex I defect was the result of the np 14459 mutation and not the product of a nuclear gene defect, six control cybrid lines and five patient cybrid lines were assayed for OXPHOS enzyme activity. The mean complex I specific activity in mutant cybrids was reduced by 39% ($P < 0.02$) compared with wild-type cybrid lines, and the mean complex I/CS ratio was reduced in mutant cybrids by 58% ($P < 0.01$) (Table 2). Both groups exhibited complex I activity which was highly sensitive to rotenone inhibition. The mean specific activities for complexes II plus III, III, and IV and CS were not altered (Table 2). Thus, the complex I defect observed in the patient donor lymphoblast cell lines was inherited by the transmitochondrial cybrids, linking the complex I enzymatic defect to the np 14459 mutation in the context of three distinct mtDNA haplotypes.

Polarographic analysis of patient and control lymphoblast and cybrid cell lines. To further characterize the np 14459-associated biochemical defect, polarographic analysis was performed on patient and control lymphoblast mitochondria with substrates specific for both complex I and complex II (Table 3). While a trend for reduced state III and uncoupled respiration rates was observed for the patient group with both sets of complex I substrates, statistical comparisons revealed no significant differences between patient and control groups for state III respiration rate, state IV respiration rate, ADP/O ratio, and uncoupled respiration rate.

The complex I-linked respiratory rates for each mitochondrial isolate were internally standardized by calculating the

TABLE 3. Polarographic analysis of control and patient EBV-transformed lymphoblast cell lines

Substrate(s)	Cell line[a]	Respiration (nmol of O/min/mg of mitochondrial protein)		ADP/O ratio	Uncoupled respiration (nmol of O/min/mg of protein)	State III ratio (site I/site II)[b]
		State III	State IV			
Pyruvate + malate	Control (n = 12)	131 ± 43	44 ± 13	2.40 ± 0.13	153 ± 52	0.79 ± 0.03
	Patient (n = 6)	113 ± 14	41 ± 8	2.35 ± 0.19	136 ± 16	0.71 ± 0.04* (P = 0.0004)
Glutamate + malate	Control (n = 12)	136 ± 44	42 ± 13	2.37 ± 0.16	153 ± 54	0.81 ± 0.05
	Patient (n = 6)	116 ± 13	40 ± 7	2.33 ± 0.21	138 ± 13	0.74 ± 0.04* (P = 0.0047)
Succinate	Control (n = 12)	166 ± 50	59 ± 16	1.60 ± 0.13	193 ± 60	
	Patient (n = 6)	159 ± 22	59 ± 9	1.57 ± 0.15	191 ± 22	

[a] n is the number of cell lines, each from a different individual. For control cell lines, values represent means ± SDs of single mitochondrial isolates performed for each control cell line. Genotyping of eight control lines revealed 100% wild-type mtDNA. For patient cell lines, all three np 14459-positive families are represented. Mean values for individual cell lines were determined for multiple independent mitochondrial isolates and were used to calculate the overall patient group mean ± SD. Genotyping revealed five cell lines with 0% wild-type mtDNA and one cell line with residual (<5%) wild-type mtDNA.

[b] Represents state III (ADP-limited) respiration rate with complex (site) I substrates (either pyruvate plus malate or glutamate plus malate) divided by state III respiration rate with complex (site) II substrate (succinate). The ratio was determined for each mitochondrial isolate. Asterisks represent statistical significance (P < 0.05) by the two-tailed, Mann-Whitney U test for unpaired samples.

ratio of state III respiration rates for the complex I-linked substrates (either pyruvate plus malate or glutamate plus malate) to the state III rate for the complex II-linked substrate (succinate) (Table 3). Patient lymphoblasts demonstrated a 10% reduction ($P < 0.001$) when pyruvate plus malate was used as the complex I-linked substrate and a 9% reduction ($P < 0.005$) when glutamate plus malate was used as the complex I-linked substrate (Table 3). This suggests that the np 14459 mutation causes a subtle defect in complex I-linked respiration.

To determine if these respiration defects were linked to the np 14459 mutation, polarographic analysis was performed with transmitochondrial cybrids. A slight trend of increased mean state III, state IV, and uncoupled respiration rates and of ADP/O ratios was observed in patient cybrids compared with controls for all substrates used (Table 4). However, the ratio of complex I-linked to complex II-linked state III respiration was virtually identical among groups (Table 4). The only statistically significant difference was a 4% increase in mean patient ADP/O ratios with glutamate plus malate as the substrate relative to controls ($P = 0.03$), although, because of ADP/O ratio fluctuation, this increase was probably an artifact. Thus, the ND6 np 14459 mutation does not appear to exert a detectable effect on cybrid respiration.

Kinetic analysis and product inhibition of complex I. Having linked a partial complex I defect to the ND6 np 14459 mutation, we sought to further characterize the nature of the enzymatic inhibition. The mean complex I activities of np 14459 wild-type and mutant EBV-transformed lymphoblast mitochondria were assessed at different concentrations of the ubiquinone analog DB and NADH (Fig. 1). At 1 and 3 μM DB, control and patient mean complex I activities were indistinguishable ($P > 0.05$ for both concentrations) (Fig. 1a). At 5 μM DB, patient complex I activity was reduced by 32% compared with controls ($P < 0.05$). Above 5 μM DB, patient complex I activity demonstrated a consistent decline, resulting in 56% ($P < 0.01$) and 68% ($P < 0.01$) reductions compared with controls at 7 and 10 μM DB, respectively. The observed differences between controls and patients at 10 μM DB correlate well with the 55 and 58% reductions in normalized complex I activities noted above for EBV-lymphoblast and transmitochondrial cybrid cell lines (Tables 1 and 2).

Because patient complex I activity exhibited a peak at 5 μM DB, NADH kinetics for control and patient cell lines were

TABLE 4. Polarographic analysis of control and patient transmitochondrial cybrids

Substrate(s)	Cell line[a]	Respiration (nmol of O/min/mg of mitochondrial protein)		ADP/O ratio	Uncoupled respiration (nmol of O/min/mg of protein)	State III ratio (site I/site II)
		State III	State IV			
Pyruvate + malate	Control (n = 6)	93 ± 20	34 ± 9	2.29 ± 0.25	101 ± 20	0.81 ± 0.02
	Patient (n = 5)	105 ± 14	38 ± 9	2.39 ± 0.24	125 ± 11	0.80 ± 0.03
Glutamate + malate	Control (n = 6)	94 ± 20	33 ± 10	2.23 ± 0.23	115 ± 26	0.82 ± 0.01
	Patient (n = 5)	108 ± 13	38 ± 9	2.31 ± 0.23* (P = 0.0303)[b]	130 ± 17	0.83 ± 0.03
Succinate	Control (n = 6)	115 ± 26	45 ± 12	1.58 ± 0.18	137 ± 29	
	Patient (n = 5)	131 ± 16	49 ± 11	1.64 ± 0.12	169 ± 16	

[a] n is the number of cell lines, each from a different individual. For control cell lines, values represent means ± SDs of single mitochondrial isolates performed for each control cell line. Genotyping of six control lines revealed 100% wild-type mtDNA. For patient cell lines, all three np 14459-positive families are represented. Mean values for individual cell lines were determined for multiple independent mitochondrial isolates and were used to calculate the overall patient group mean ± SD. Genotyping revealed one cell line with 0% wild-type mtDNA and four cell lines with residual (<5%) wild-type mtDNA.

[b] Asterisk represents statistical significance ($P < 0.05$) by the two-tailed, Mann-Whitney U test for unpaired samples.

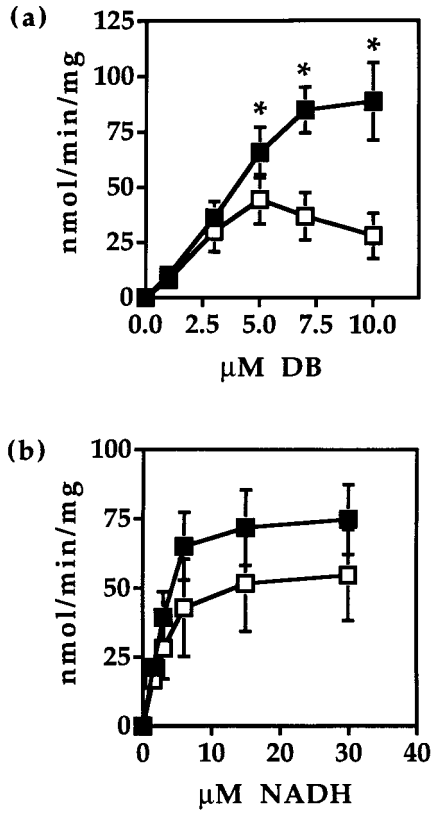

FIG. 1. DB (a) and NADH (b) kinetic analysis of complex I from EBV-transformed lymphoblast mitochondria. All determinations used 20 μM NADH or 5 μM DB. Error bars represent ±1 SD. Asterisks denote $P < 0.05$. Symbols: ■, controls ($n = 5$); □, patients ($n = 5$).

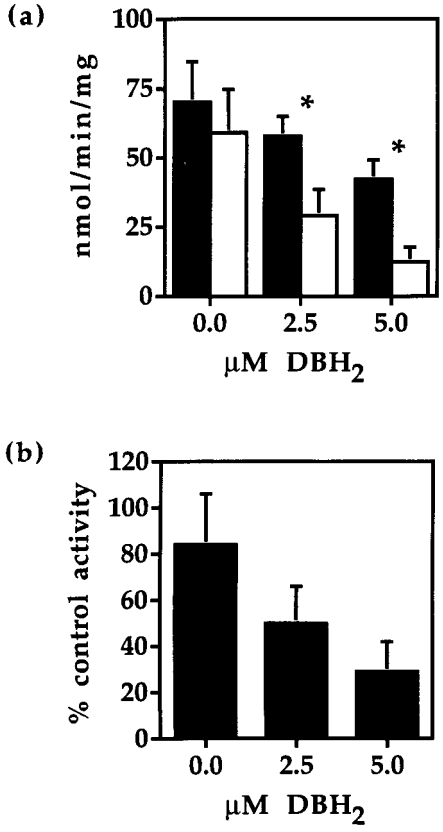

FIG. 2. Product inhibition of complex I from EBV-transformed lymphoblast mitochondria. (a) Complex I activities were assessed after preincubation of samples with 5 μM DB and the specified concentrations of DBH_2. Error bars represent ±1 SD of mean complex I activities. Asterisks denote $P < 0.05$. Symbols: ■, controls ($n = 5$); □, patients ($n = 5$). (b) Mean patient complex I activities divided by mean control complex I activities determined at the same concentration of DBH_2. Error bars represent standard deviations of patient complex I activities divided by mean control complex I activities determined at the same concentration of DBH_2.

assessed at this DB concentration. At all NADH concentrations used in this study (1.5, 3.0, 6.0, 15.0, and 30.0 μM NADH), no statistically significant differences were observed between patient and control complex I activities, despite a consistent trend for reduced complex I activity in patients of 22 to 34% (Fig. 1b).

Calculation of the complex I kinetic parameters with NADH as a substrate for patient cell lines gave a V_{max} of 62.9 ± 7.3 nmol/min/mg of protein and a K_m of 3.5 ± 1.4 μM, while for control cell lines, the V_{max} was 86.8 ± 5.9 nmol/min/mg of protein and the K_m was 3.2 ± 0.8 μM. Thus, at 5 μM DB, the np 14459 mutation produces a 28% reduction in complex I V_{max} without changing the K_m of the enzyme for NADH.

The inhibition of mutant complex I by DB concentrations between 5 and 10 μM suggested a defect in the coenzyme Q-binding site. To determine if this was due to product inhibition, complex I activities were determined for patient and control EBV-lymphoblast mitochondria preincubated with 5.0 μM DB, to which different concentrations of DBH_2 were added (Fig. 2a). Preincubation without addition of DBH_2 produced a 16% reduction in mean patient complex I activity (59.2 nmol/min/mg) compared with mean control complex I activity (70.2 nmol/min/mg) ($P > 0.05$). Preincubation with 2.5 μM DBH_2 produced a 50% reduction (patient activity at 29.1 nmol/min/mg versus control activity at 57.8 nmol/min/mg [$P < 0.01$]), and preincubation with 5.0 μM DBH_2 produced a 71% reduction (patient activity at 12.4 nmol/min/mg versus control activity at 42.2 nmol/min/mg [$P < 0.01$]). Thus, increasing concentrations of DBH_2 resulted in substantial inhibition of patient complex I activity compared with controls. These data suggest that the np 14459 mutation in vivo may alter the interaction between complex I and ubiquinone and ubiquinol in the mitochondrial inner membrane.

DISCUSSION

A marked reduction in respiratory complex I specific activity was detected in EBV-transformed lymphoblast cell lines harboring the ND6 np 14459 mutation associated with LHON and dystonia. Cybrid transfer experiments demonstrated that the complex I defect cosegregated with the np 14459 mutation carried on three different background mtDNAs. Thus, the np 14459 mutation produces a biochemical deficiency in complex I activity and the associated clinical phenotype.

Mean complex I activity was reduced by 60% ($P < 0.005$) in patient lymphoblasts and by 39% ($P < 0.02$) in patient cybrids. The levels of complex I impairment for patient lymphoblasts and cybrids are indistinguishable after normalization for CS activity, with the complex I/CS ratio showing a 55% reduction ($P < 0.001$) in lymphoblasts and a 58% reduction ($P < 0.01$) in cybrids.

Despite the reduction of complex I enzymatic activity associated with the np 14459 mutation, there was an absence of a comparable respiratory deficiency on polarographic analysis.

This is intriguing, since it suggests that there is excess complex I activity relative to the maximum flux rate of electrons through the electron transport chain. Hence, a 40 to 60% reduction in complex I activity is not sufficient to limit the flux rate of the electron transport chain in EBV-transformed lymphoblasts, consistent with respiratory control theory (28, 33).

LHON together with severe neurological symptoms including movement disorders was also associated with a complex I defect and a combination of the np 4160 and np 14484 mutations in a large Australian kindred (20, 22, 23, 31, 39, 54). The seemingly specific association of LHON, movement disorders, and a complex I biochemical defect observed for both the ND6 np 14459 mutation and the ND6 np 14484 plus ND1 np 4160 mutations raises the possibility that optic nerve and basal ganglion neurons are acutely sensitive to the pathogenic effects of complex I defects. Possibly, additional ND6 mutations also contribute to the etiology of LHON and/or dystonia with basal ganglion lucencies (4, 9, 10, 34).

The np 14459 ND6 mutation substitutes a valine for an alanine at codon 72 within the most evolutionarily conserved region of the ND6 polypeptide (14). Since the identification of ND6 as a subunit of complex I (11), very little has been learned about its structural and functional role(s) in the enzyme. On the basis of sequence information, the ND6 protein is thought to possess four or five transmembrane regions (14) and probably resides in the hydrophobic portion of complex I.

The observation that complex I containing the np 14459 mutant ND6 subunit exhibits greater sensitivity to inhibition by ubiquinone and ubiquinol analogs than control complex I is intriguing. Details of the mechanism by which complex I reduces ubiquinone are currently a topic of much speculation (40, 53). This enzyme is thought to utilize two protein-bound quinones with different redox potentials (53). At least one complex I subunit from bovine heart (50) and one from *Neurospora crassa* (18, 19) have been characterized with ubiquinone-binding properties. Neither of these resembles the ND6 protein, and it remains possible that ND6 also is involved in the interaction of complex I with ubiquinone, possibly contributing to the coenzyme Q-binding site(s). Similar kinetic and product inhibition studies applied to the LHON mutation in the ND6 gene at np 14484 could support this hypothesis.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants NS21328, HL45572, and NS30164 and a Muscular Dystrophy Foundation Clinical Grant awarded to D.C.W.

We thank Dale Edmondson for his assistance with the complex I kinetics experiments.

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