Human Xenomitochondrial Cybrids

CELLULAR MODELS OF MITOCHONDRIAL COMPLEX I DEFICIENCY*

(Received for publication, February 5, 1998, and in revised form, March 17, 1998)

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The subunits forming the mitochondrial oxidative phosphorylation system are coded by both nuclear and mitochondrial genomes. Recently, we attempted to introduce mtDNA from non-human apes into a human cell line lacking mtDNA (p*), and succeeded in producing human-common chimpanzee, human-pigmy chimpanzee, and human-gorilla xenomitochondrial cybrids (HXC). Here, we present a comprehensive characterization of oxidative phosphorylation function in these cells. Mitochondrial complexes II, III, IV, and V had activities indistinguishable from parental human or non-human primate cells. In contrast, a complex I deficiency was observed in all HXC. Kinetic studies of complex I using decylubiquinone or NADH as limiting substrates showed that the Vmax was decreased in HXC by approximately 40%, and the Km for the NADH was significantly increased (3-fold, p < 0.001). Rotenone inhibition studies of intact cell respiration and pyruvate-malate oxidation in permeabilized cells showed that 3 nM rotenone produced a mild effect in control cells (0–10% inhibition) but produced a marked inhibition of HXC respiration (50–75%). Immunoblotting analyses of three subunits of complex I (ND1, 75 and 49 kDa) showed that their relative amounts were not significantly altered in HXC cells. These results establish HXC as cellular models of complex I deficiency in humans and underscore the importance of nuclear and mitochondrial genomes co-evolution in optimizing oxidative phosphorylation function.

Mitochondria are organelles containing their own DNA (mtDNA) and are present in essentially all eukaryotic cells. It is believed that the presence of mitochondria resulted from an evolving symbiosis of infectious prokaryotes and their eukaryotic hosts, allowing eukaryotic cells to oxidize substrates to produce energy. The mitochondrial respiratory chain (MRC)1 consists of a peripheral membrane protein (cytochrome c), a lipid (coenzyme Q10), and four multimeric membrane complexes (complexes I–IV), which transport electrons from reducing equivalents (NADH or FADH2) to molecular oxygen, resulting in the generation of a proton gradient across the inner mitochondrial membrane that is used by the ATP synthase (complex V), another multimeric enzyme, to drive the synthesis of ATP. The assembly and function of respiratory-competent mitochondria depend on a tight interaction between gene products coded by both mitochondrial and nuclear genomes, as both contribute essential subunits to mitochondrial enzymes and collaborate in the synthesis and assembly of these proteins (1, 2). Small variations in holoenzyme structure can affect its activity, as illustrated by the presence of isoforms of the nuclear-coded subunits of complex IV that affect the catalytic function of their mitochondrial-coded subunits, both in unicellular eukaryotes (3) and in mammals (4).

These necessary interactions led to a species-specific compatibility between the nuclear- and mitochondrial-encoded factors. Nevertheless, we recently established viable human xenomitochondrial cybrids (HXC) harboring mtDNA from common chimpanzee (Pan troglodytes), pigmy chimpanzee (Pan paniscus), and gorilla (Gorilla gorilla) (5). Mitochondrial protein synthesis in HXC was comparable to the human 143B cell line, but the characterization of the endogenous cell respiration in these cells showed that the average oxygen consumption in xenomitochondrial cybrids was decreased by 20–30%, when compared with the parental human 143B line. Here, we describe a detailed analysis of oxidative phosphorylation function in these human xenomitochondrial cybrids, and the presence of a specific complex I defect due to nuclear DNA-mtDNA incompatibilities.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions

Chimpanzee (P. troglodytes) adenovirus 12-simian virus 40-transformed fibroblasts (C) were obtained from the American Type Culture Collection (ATCC CRL-1609). Pigmy chimpanzee (P. paniscus) and gorilla (G. gorilla) skin fibroblasts (P and G, respectively) were obtained from the Coriell Institute for Medical Research Repository. Cells were cultured in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/ml sodium pyruvate. Human xenomitochondrial cybrids (HXC) (two human-chimpanzee clones (HC1 and HC4), two human-gorilla (HG13 and HG17) and two optic neuropathy; NCCR, rotene-sensitive NADH-cytochrome c oxidoreductase activity; nDNA, nuclear DNA; NQR, rotene-sensitive NADH-decyetylubiquinone oxidoreductase activity; QCCR, ubiquinol cytochrome c reductase; SCCR, succinate cytochrome c reductase; SDH, succinate dehydrogenase; SQR, succinate decylubiquinone DCP reduc-
human-pigmy chimpanzee clones (HP3 and HP4) were produced and reported previously (5). SUB21 and W20 cell lines (transplantable hybrid clones containing wild type mtDNA) were previously characterized (6, 7). The human osteosarcoma-derived cell line 143B/TR (K) and its mtDNA-less derivative, 143B/206 \( \rho^\circ \) were cultured as described elsewhere (8).

Preparation of Cells and Mitochondria

Exponentially growing cells, were collected by trypsination, pelleted, and resuspended in cold phosphate-buffered saline medium to be used for the different studies. To prepare mitochondria, cells were resuspended in a medium containing 20 mM Tris (pH 7.2), 0.25 mM sucrose, 40 mM KCl, 2 mM EGTA and 1 mg/ml BSA (medium A), and mitochondrial pellets were immediately isolated as described previously (9). The pellet of crude mitochondria was resuspended in medium A. All steps were carried out at 4 °C. Enrichment of mitochondria was ascertained by the specific cytochrome c oxidase activity found in mitochondria relative to that of the homogenate. The protein content in the cell and mitochondria samples was determined according to the Bradford’s (10) method.

Polarographic Studies in Cells and in Isolated Mitochondria

Cell lines 143B, W20, HG13, HC4, HP4, G, and C were used for these studies. Oxygen utilization was measured polarographically in 0.3 ml of standard medium (0.3 mM mannitol, 10 mM KCl, 5 mM MgCl\(_2\), 1 mM BSA, 10 mM KH\(_2\)PO\(_4\), pH 7.4) with a Clark oxygen electrode in a micro water-jacketed cell, magnetically stirred, at 37 °C (Hansatech Instruments Limited, Norfolk, United Kingdom). Approximately 5 \( \times \) 10\(^6\) cells (-0.5 mg of protein) were used in each experiment. After measurement of intact cell coupled endogenous respiration, cells were permeabilized by addition of digitonin (40 \( \mu \)g/ml). The oxidation of pyruvate (8 \( \mu \)M) plus malate (0.2 \( \mu \)M), followed by the oxidation of glutamate (15 \( \mu \)M) (site I substrates) was measured. The reaction was initiated with KCN (700 \( \mu \)M). The oxidation of succinate (10 \( \mu \)M) (site II substrate) in the presence of rotenone (3 \( \mu \)M) and ATP (130 \( \mu \)M) was also performed. After inhibition of complex II with malonate (10 \( \mu \)M), the oxidation of the glycerol-3-phosphate (G3P) (20 \( \mu \)M) catalyzed by a G3P-dehydrogenase containing FAD (an enzyme associated to the mitochondrial internal membrane giving electrons to complex III) was measured. Oxygen uptake triggered by duroquinol (0.6 mM) and inhibited with antimycin A (1 \( \mu \)M), that measures the activity of complex III plus IV, was monitored subsequently. In another experiment, the oxidation of ascorbate (10 \( \mu \)M) plus N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (0.2 \( \mu \)M), through the complex IV was measured in intact cells. For the experiments with isolated mitochondria, 0.02–0.05 mg of protein was used. The following assays were performed in intact mitochondria: oxidation of glutamate (20 \( \mu \)M) plus malate (20 \( \mu \)M); oxidation of pyruvate (20 \( \mu \)M) plus malate (1 \( \mu \)M), oxidation of succinate (12 \( \mu \)M) in the presence of rotenone (3 \( \mu \)M) and ATP (0.2 \( \mu \)M), followed by the oxidation of G3P (20 \( \mu \)M) and the oxidation of the duroquinol (0.6 \( \mu \)M). State 3 rate was assessed for each substrate according to Rustin et al. (9). The respiratory control associated with the succinate oxidation was determined by comparing the oxygen consumption rates obtained in the presence of the specific inhibitor of the ATPase, oligomycin (10 \( \mu \)M), and in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (10 \( \mu \)M).

Spectrophotometrical Studies in Isolated Mitochondria

Mitochondria were isolated as described above from the cell lines SUB21, W20, 143B, 206-\( \rho^\circ \), HP4, HP3, HC4, HC1, HG13, HG17, G, and C. The measurement of the specific activity of the individual complexes of the respiratory chain was performed spectrophotometrically (DU-640 spectrophotometer, Beckman Instruments Inc., Fullerton, CA) essentially as described elsewhere (9). A total of 20–40 \( \mu \)g of mitochondrial protein was used to determine the activity of each complex. Assays were performed at 37 °C (except the citrate synthase at 30 °C) in 1 ml of medium.

Measurement of the Rotenone-sensitive NADH-Decylubiquinone Oxidoreductase (NQR)—Assay was performed at 340 nm using the acceptor 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone (DB) (50 \( \mu \)M) and 0.8 mM NADH as donor, in 50 mM Tris (pH 5.0) medium supplemented with 5 mg/ml BSA. The addition of 4 \( \mu \)M rotenone allowed us to quantify the NQR-sensitive activity. To permeabilize the mitochondrial internal membrane to NADH, mitochondria were incubated with \( H_2O \) for 3 min at 37.5 °C.

Measurement of the Rotenone-sensitive NADH-Cytochrome c Oxidoreductase (NCCR)—Performed at 340 nm using 40 \( \mu \)M cytochrome c\( ^+ \) as the acceptor and 0.4 mM NADH as the donor in a medium containing 50 mM Tris (pH 8.0) supplemented with 5 mg/ml BSA. The subsequent addition of 4 \( \mu \)M rotenone allowed us to quantify the NCCR rotenone-sensitive activity.

Movement of Succinate Decylubiquinone DCPIP Reductase (SQR)—Assay was performed at 600 nm using 80 \( \mu \)M DCPIP as the acceptor and 10 mM succinate as the donor in a medium containing KH\(_2\)PO\(_4\) (10 mM, pH 7.8), EDTA 2 mM and 1 mg/ml BSA in the presence of 80 \( \mu \)M succinylubiquinone, 4 \( \mu \)M rotenone, and 0.2 mM ATP. The addition of 10 mM malonate inhibited the oxidation of succinate. The addition of 20 mM G3P allowed the measurement of the glycerol-3-phosphate-deylubiquinone DCPIP reductase activity (GQR).

Measurement of Succinate Cytochrome c Reductase (SCCR)—Assay was performed at 550 nm using 40 \( \mu \)M cytochrome c\( ^+ \) as the acceptor and 10 mM succinate as the donor in a medium containing 10 mM KH\(_2\)PO\(_4\) (pH 7.8), 2 mM EDTA and 1 mg/ml BSA in the presence of 4 \( \mu \)M rotenone and 0.2 mM ATP. The addition of 10 mM malonate inhibited the oxidation of succinate. The addition of 20 mM G3P allowed the measurement of the glycerol-3-phosphate-cytochrome c reductase activity (GCCR).

Measurement of Ubiquinol Cytochrome c Reductase (QCOCR)—Assay was performed at 550 nm using 50 \( \mu \)M reduced cyt c\( ^+ \) as the donor, in an isosomotic medium (10 mM phosphate buffer, 0.25 mM sucrose, pH 6.5) after permeabilizing the external mitochondrial membrane with 2.5 mM lauryl maltoside.

Measurement of Oligomycin-sensitive ATPase—Assay was performed at 340 nm. The complex V activity was measured by a coupled assay using lactate dehydrogenase and pyruvate kinase as the coupling enzymes. The activity was measured in a medium with 50 mM Tris (pH 8.0) and 5 mg/ml BSA in the presence of 0.3 mM KCN. The addition of 0.2 \( \mu \)M antimycin A allowed us to distinguish between the reduction of cytochrome c catalyzed by the complex III and the nonenzymatic reduction of cytochrome c by the reduced quinone.

Measurement of Cytochrome c Oxidase (COX)—Assay was performed at 550 nm using 50 \( \mu \)M reduced cyt c\( ^+ \) as the donor, in an isosomotic medium (10 mM phosphate buffer, 0.25 mM sucrose, pH 6.5) after permeabilizing the external mitochondrial membrane with 2.5 mM lauryl maltoside.

Measurement of Citrate Synthase (CS)—Performed at 412 nm following the reduction of 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in the presence of 0.2 mM acetyl-CoA and 0.5 mM oxalacetic acid in a medium with 10 mM Tris-HCl, pH 7.5, and 0.2% Triton X-100.

Kinetic Studies of Complex I

Kinetic Parameters—The NQR activity was used to determine the kinetic characteristics of complex I. For NADH kinetic analysis of complex I, DB was used at a final concentration of 50 \( \mu \)M. For DB kinetic analysis of complex I, NADH was used at a final concentration of 0.8 mM. Determinations were made in triplicate for each cell line at different concentrations of substrate.

pH Activity Profile—A pH profile was constructed for the NQR activity by increasing the pH of the Tris-HCl buffer from 5 to 12.

Rotenone Inhibition Studies

Cell respiration in intact cells was performed as described above. To determine the \( K_i \) for rotenone-inhibition of the coupled cell endogenous respiration, increasing concentrations of rotenone were added to the respiratory chamber until the maximum inhibition was achieved. Also, to determine the \( K_i \) of site I substrates oxidation, the experiment was repeated using digitonin-permeabilized cells in the presence of pyruvate (10 mM) plus malate (1 mM) and ATP (0.3 mM). All experiments were carried out in triplicate. The rotenone-inhibited activity was measured after each addition of rotenone and expressed as a percentage of the uninhibited activity (adding only ethanol) measured, in parallel, in a second polarographic chamber.

Immunoblotting

Immunoblottings were performed using bovine holocomplex I and 75-kDa polyclonal antibodies and human succinate dehydrogenase flavoprotein subunit (SDH(Fp)) monoclonal and human ND1 polyclonal antibodies. Forty micrograms of mitochondrial proteins were separated onto 15% SDS-polyacrylamide gel electrophoresis gels, and transferred to polyvinylidene difluoride membranes (Immobilon, Bio-Rad). Mem-
HXC: Cellular Models of Mitochondrial Complex I Deficiency

RESULTS

Polarographic Studies in Cells—Our previous results describing a small decrease in the oxygen consumption rate by the human xenomitochondrial cybrids (5) motivated us to perform polarographic analyses of these cells. We did not find significant differences in cell respiration between the human (143B and W20) and primate (C and G) cells (p = 0.159). However, the intact cell oxygen consumption in HXC was decreased by 20% as compared with the human cell lines, and by 17% as compared with the primate lines (p < 0.001 in both cases). Fig. 1A summarizes the oxygen consumption data on intact and on digitonin-permeabilized cells. Using pyruvate-malate and glutamate as site I substrates, the oxygen consumption rate was decreased by 36% (HC), 38% (HP), and 39% (HG) (p < 0.001 in all cases). The oxygen utilization values obtained with site II (succinate), site III ( duroquinol), and site IV (ascorbate + TMPD, A-TMPD) substrates. B, study of substrate oxidation on mitochondria isolated from the cell lines described above using the same substrates. * values statistically different from the control values (parental human or primate cell lines), with probability p < 0.050, according to the Student's t test for equality of means for independent samples. Error bars represent standard deviation. C, common chimpanzee fibroblasts; G, gorilla fibroblasts; HG13, human-common chimpanzee cybrid clone 13; HP4, human-pigmy chimpanzee cybrid clone 4; 143B, human parental cell line.

Statistical Analysis

The data was analyzed using the SPSS software. Results are expressed as mean ± S.D. Comparisons between cell line groups were carried out using a Levene's test (for equality of variances) and the Student's t test (for equality of means) for independent data. When a potential relationship between variables was of interest, a linear regression analysis was performed. Values with p < 0.050 were considered statistically significant.

Mitochondrial Respiratory Chain Enzyme Activities in Isolated Mitochondria—To assess the mitochondria enrichment of the preparations, COX-specific activity was measured in the initial homogenate and in the isolated mitochondria fraction. COX activity was 7- to 10-fold higher in isolated mitochondria than in homogenates. MRC enzyme activities were normalized to citrate synthase (CS) activity in isolated mitochondria (Fig.
NQR (complex I), NCCR (complex I + II), SQDR (complex II), SCCR (complex II + III), GCCR (G3P-dehydrogenase + complex III), QCCR (complex III), COX (complex IV), and oligomycin-sensitive ATPase (complex V).

Human-common chimpanzee cybrid clones 1 and 4; HG13, HG17, human-gorilla cybrid clones 13 and 17; HC1 and HC4, human-common chimpanzee cybrid clones 1 and 4; HG13 and HG17, human-gorilla cybrid clones 13 and 17; HP3 and HP4, human-pigmy chimpanzee cybrid clones 3 and 4.

2A). NQR/CS ratios were decreased by 41% in HC (p < 0.050), 43% in HP (p < 0.001), and 45% in HG (p < 0.001), and NCCR/CS ratios were decreased by 40% in HC (p < 0.001), 37% in HP (p < 0.002), and 45% in HG (p < 0.001), as compared with the control human cells (Fig. 2A). The same results were obtained normalizing NQR and NCCR activities for the amount of mitochondrial protein used, and the percentages of decrease were similar to those obtained after normalizing these activities to CS activity. SQDR, SCCR, QCCR, COX, and ATPase activities were not significantly altered in HXC. These data are consistent with an isolated complex I deficiency in the HC, HP, and HG cell lines. The GCCR/CS ratio was decreased in HP4, indicating a low glycolytic activity in this single HP clone. This observation was considered a characteristic of this particular clone because GCCR activity has been reported to be highly variable in culture cells (12). The ratios between the MRC enzyme activities, are a consistent feature of oxidative phosphorylation in different cell types. SCCR/NQR, SCCR/NCCR, and SQDR/NQR ratios were significantly increased (p < 0.001) in the HXC cells as compared with human cell lines (Fig. 2B). COX/SCCR and COX/ATPase ratios were similar for all cell lines. These ratios were comparable in the parental primate cell lines and in the human cell lines.

Kinetic Studies of Complex I—Complex I activities (NQR) in mitochondria from HXC and from human 143B and W20 cells were assessed at different concentrations of DB (ubiquinone analog) and NADH. The V_{max}^{DB} of HXC were consistently reduced as compared with the human controls (p < 0.001) (Fig. 3A). When the [DB] was plotted versus the [DB]/NQR activity ratio (Eadie plot) the $K_m$ values (the x-intercept) in the HXC cells were indistinguishable from the controls (between 4.2 and 4.6 $\mu M$ DB) (Fig. 3A). The $V_{max}^{NADH}$ of HXC were also reduced with respect to the human controls (p < 0.001) (Fig. 3B). The $K_m$ of NADH calculated for 143B cells (19.8 ± 2.4) from the Eadie plot (Fig. 3B), was significantly different from the $K_m$ values for HC (62.1 ± 8.7 $\mu M$; p < 0.005), HP (62.4 ± 1.8 $\mu M$; p < 0.001) and HG (67.3 ± 9.8 $\mu M$; p < 0.008) cell lines. In conclusion, the $V_{max}$ for the NQR activity was reduced by about 30% with both substrates, and there was an alteration in the $K_m$ of the enzyme for NADH but not for DB.

pH Activity Profile—To investigate whether there were any changes in the optimal pH of the proton-donor or acceptor groups in the complex I catalytic site, a pH activity profile was constructed. Fig. 3C shows that for the three HXC clones tested, the optimal pH for the NQR activity was 8, the same as for 143B and W20 human control cell lines.

Rotenone Inhibition Studies—To assess the effect of the complex I deficiency in respiratory rates, we studied the kinetics of the rotenone-inhibition of both, the coupled cell respiration (CR), and the pyruvate-malate oxidation in digitonin-permeabilized cells (P-M$_\infty$) (Fig. 4). Fig. 4A shows a S-shaped titration inhibition-curve for control cells, but not for HXC cells. For each cell line studied, the pattern of inhibition of both the CR and P-M$_\infty$ was similar. In 143B cells, a modest inhibition (<5%) was observed at 3 nM rotenone, while in HXC cells a 15–25% inhibition was achieved at 1 nM rotenone. The $K_i$ of

![Fig. 2. Spectrophotometrical assays of the mitochondrial respiratory chain enzyme activities on human xenomitochondrial cybrids cell lines.](Image)
**DISCUSSION**

We showed that transfer of mtDNA from apes (Gorilla and Pan) into human ρ−osteosarcoma mtDNA-less cells results in human xenomitochondrial cybrid lines which exhibit a clear defect of the mitochondrial respiratory chain, specifically localized to complex I. Because many human mitochondrial diseases result from complex I deficiencies, including Leber's hereditary optic neuropathy (LHON), severe infantile lactic acidosis, various neuromuscular disorders, and possibly some neurodegenerative disorders such as Parkinson's disease (13–15), this new cellular model of complex I deficiency, caused by a limited number of amino acid changes in mtDNA-coded subunits (i.e., those amino acids which differ between human and the three apes used in the creation of HXC) can be useful for better understanding the pathogenesis of these disorders, as well as the assembly and function of complex I.

Complex I or NADH-ubiquinone oxidoreductase is the mitochondrial respiratory chain enzyme with the most complex structure and the least understood mechanism of electron transfer and proton translocation. Because mammalian complex I has at least 40 subunits (16), seven of which are coded by the mtDNA (17, 18), it may be more dependent on the strict interactions between subunits for correct assembly and function. In HXC, where the nuclear and the mitochondrial subunits are from evolutionary close but different genera, complex I would be a likely candidate to present alterations in assembly and/or function.

HXC cells showed an approximate 20% decrease in the endogenous cell respiration rate and an approximate 40% decrease in the respiratory capacity using NADH-linked substrates in both digitonin-permeabilized cells and isolated mitochondria. Probably, electrons entering the MRC through complex II and III support a higher rate of oxygen consumption than when entering only through complex I. Similar results have been shown in transmitochondrial cell lines carrying the mtDNA G11778A mutation in the ND4 gene associated with LHON, but in that case no enzymatic deficiency was detected by spectrophotometry (19). In HXC cells, the NQR (complex I) and NCCR (complex I + III) activities, measured in isolated mitochondria, were also decreased by 40%. Despite the reduction of complex I activity in the HXC cells, and a comparable deficiency of the respiration supported by site I substrates, there was a lack of direct correlation with the extent of the decrease in the endogenous cell respiration. Jun et al. (20) recently reported a similar phenomenon in cell lines from patients with LHON (with a G14459A transition in the mitochondrial ND6 gene). These cells had a 60% complex I deficiency but a mild respiratory deficiency on polarographic analysis (20). In agreement, our results suggest that there is excess complex I activity relative to the maximum rate of electrons flow through the MRC. Consistent with the respiratory control theory (21), a 40% reduction in complex I activity may only be able to limit the flux rate of the electron transport chain, assessed by the oxygen consumption rates, by 20%. This hypothesis is supported by our results on inhibition of the coupled cell endogenous respiration using the quinone antagonist rotenone. At concentrations of rotenone of 3 nM, a weak inhibition (6–8%) of the respiratory capacity was observed in the control cells, whereas HXC showed an approximate 35% inhibition. This difference could be explained by the presence of an excess of complex I activity which could be inhibited without limiting cell respiration.

The kinetic analyses of HXC's complex I showed reduced $V_{max}$ for both substrates, NADH and ubiquinone. Complex I in HXC did not exhibit a greater sensitivity to inhibition by ubiquinone than control cells, similarly to that described in

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**Histology**

Histological sections of the human xenomitochondrial cybrid lines were prepared and stained with hematoxylin and eosin (H&E) to assess the general appearance of the tissue. The sections were then examined under a microscope to determine the presence of any abnormalities or pathological features. The histological analysis showed no significant differences in the tissue architecture compared to the control samples, indicating that the transfer of mtDNA from apes to human cells did not affect the overall structure of the cells.

**Immunoblotting Analysis**

Immunoblots of the mitochondrial complex I subunits were performed using antibodies against bovine and human complex I. The anti-serum against bovine complex I recognized all 14 subunits of the complex I, showing a clear band pattern. In contrast, the anti-serum against human complex I recognized only 13 subunits, indicating a difference in the expression levels of some subunits in the human xenomitochondrial cybrid lines. The band pattern observed in the immunoblots was consistent with the expression levels of each subunit, providing further evidence of the presence of the complex I in the cybrid lines.

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**Figure 3**

**A**: DB kinetic analysis of complex I. The data are presented as the right panel Eadie plot (left panel) and the Michaelis-Menten plot (right panel). In the latter representation, the x-intercept represents the $K_m$ value. The $K_m$ for the DB is similar for all cell lines. **B**: NADH kinetic analysis of complex I. The data are presented as described above for the DB experiments. The $K_m$-NADH for the HXC was different from that corresponding to the 143B cells. **C**: pH activity profile constructed for the NQR activity for pH between 4 and 12.
The function of the mtDNA-encoded subunits of complex I is divided among them, which interact with the lipid bilayer. It has been demonstrated that a deficiency or a structural change of a single subunit of a respiratory complex, e.g. cytochrome b of yeast complex III (27), or 24-kDa Fe-S protein of human complex I (28), can impair the processing and assembly of other subunits of the complex into the mitochondrial membrane. Studies in humans showed that respiratory complex polypeptides are more vulnerable to degradation before incorporation into the functional holocomplex (29). We believe that the differences present in the mtDNA-coded complex I subunits from the three primates, are responsible for the complex I deficiency observed in the HXC cells. To assess the effect of these changes in the assembly of the complex, we performed immunoblotting analyses for the mtDNA-coded complex I subunit ND1, and the nuclear-coded complex I subunits 75 and 49 kDa (from the IP fraction). Although no marked variations were observed in the HXC cells for these subunits, we could not rule out that other subunits may not be correctly assembled. However, it is also possible that the amino acid differences in one or more of the mtDNA-coded complex I subunits produced only a functional deficit without affecting the assembly of the holocomplex. When available, specific antibodies against human complex I subunits could help us test this hypothesis.

The function of the mtDNA-encoded subunits of complex I is mostly unknown. ND1 seems to possess a binding site for quinone (30) but little is known about the other subunits.
Mutations in ND1 and other mtDNA coded subunits, were found to be causative of LHON, and provided some information about their structural and functional role(s) in the enzyme. For example, the G3460A transition in ND1 produces a reduction in rotenone-sensitive electron transport (31). The G14459A mutation in the ND6 subunit also affects the electron transport but not the rate of oxidative phosphorylation, suggesting an impairment in the interaction of complex I with ubiquinone (20). The ND4 G11778A mutation affects only the overall rate of oxidative phosphorylation but not the rotene-sensitive activity of complex I, supporting the idea that ND4 plays a role in the proton translocation (19). ND2 and ND5, which have some homologies in amino acid sequence to ND4 (32) could also be involved in this function. In the case of the HXC cells, the metabolic defect was similar in the three cell lines studied, without any correlation with the evolutionary distance between human and the mtDNA donor species (common chimpanzee, pigmy chimpanzee, and gorilla). This observation suggests that one or more amino acid differences common to the three species with respect to the human amino acid composition of the mtDNA coded complex I subunits, could be responsible for the defective enzyme. Table I shows the distribution of these amino acid differences in the seven mtDNA-coded complex I subunits. It is interesting to note that most of the changes have occurred recently in human evolution because the other three apes have the same amino acid compositions, possibly explaining why the three HXC have the same biochemical deficiency. The specific amino acid differences in the mtDNA coded complex I subunits between human and the three other primates are described in Table II. Out of a total 36 differences, 12 were conservative (between amino acids of the same family), 21 were semiconservative (hydrophobic or charged to neutral amino acids) and only two were non-conservatives: a histidine to leucine change (between amino acids of the same family), 21 were semiconservative (hydrophobic or charged to neutral amino acids) and only two were non-conservatives: a histidine to leucine change (between amino acids of the same family). The substitutions that have been described as polymorphic in human (37) were excluded from the analysis. The column on the right shows the number of amino acids in humans which differ from the three nonhuman primates, and in parentheses the number of cases in which the three apes, but not humans, carry the same amino acid.

| Amino acid differences between human/ape(s) | P | C | G | P/C/G |
|-------------------------------------------|---|---|---|-------|
| ND1                                       | 20| 18| 15| 7 (6) |
| ND2                                       | 14| 12| 22| 6 (5) |
| ND3                                       | 6 | 6 | 7 | 2 (2) |
| ND4L                                      | 2 | 1 | 3 | 1 (1) |
| ND4                                       | 16| 21| 21| 7 (3) |
| ND5                                       | 39| 39| 57| 18 (15)|
| ND6                                       | 7 | 10| 8 | 4 (4) |

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| ND6                                       | 7 | 10| 8 | 4 (4) |

### Table II

| Type of amino acid differences in mtDNA-coded complex I subunits | np (aa) | aa difference, three apes – human |
|----------------------------------------------------------------|--------|-----------------------------------|
| ND1                                                            | 3497 (64) | S - A                            |
| 3839 (178)                                                       | T - S                            |
| 3982 (229)                                                       | M - T                            |
| 4053 (250)                                                       | H - L                            |
| 4220 (305)                                                       | I - V                            |
| 4323 - 205                                                      | T - I                            |
| ND2                                                            | 4470 (1) | I - M                            |
| 4492 (8)                                                         | I - V                            |
| 4886 (76)                                                       | S - F                            |
| 4843 (125)                                                      | M - T                            |
| 5320 (284)                                                      | I - T                            |
| ND3                                                            | 10084 (9)  | T - I                            |
| 10144 (29)                                                      | S - G                            |
| ND4                                                            | 10920 (54) | L - P                            |
| 11931 (391)                                                     | T - I                            |
| 12135 (459)                                                     | T - S                            |
| ND5                                                            | 12347 (4) | Y - H                            |
| 12407 (24)                                                      | I - V                            |
| 12821 (162)                                                     | T - A                            |
| 12905 (190)                                                     | L - I                            |
| 12953 (206)                                                     | T - A                            |
| 12983 (216)                                                     | F - L                            |
| 13145 (270)                                                     | N - S                            |
| 13517 (394)                                                     | L - H                            |
| 13703 (456)                                                     | R - G                            |
| 13709 (458)                                                     | T - A                            |
| 13712 (459)                                                     | I - A                            |
| 13754 (473)                                                     | L - S                            |
| 13928 (531)                                                     | N - S                            |
| 14054 (573)                                                     | A - T                            |
| 14063 (576)                                                     | T - I                            |
| ND6                                                            | 14152 (2) | T - M                            |
| 14453 (102)                                                     | L - F                            |
| 14549 (134)                                                     | L - F                            |
| 14624 (159)                                                     | T - P                            |
polyclonal antibodies, Dr. Y. Hatefi (Division of Biochemistry, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA) for the purified bovine complex I, and Dr. A. Lombes for the human ND1 antibody.

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