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Lack of Complex I Activity in Human Cells Carrying a Mutation in MtDNA-encoded ND4 Subunit Is Corrected by the *Saccharomyces cerevisiae* NADH-Quinone Oxidoreductase (*NDI1*) Gene*

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The gene for the single subunit, rotenone-insensitive, and flavone-sensitive internal NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* (*NDI1*) can completely restore the NADH dehydrogenase activity in mutant human cells that lack the essential mitochondrial DNA (mtDNA)-encoded subunit ND4. In particular, the *NDI1* gene was introduced into the nuclear genome of the human 143B.TK$^-$ cell line derivative C4T, which carries a homoplasmic frameshift mutation in the *ND4* gene. Two transformants with a low or high level of expression of the exogenous gene were chosen for a detailed analysis. In these cells the corresponding protein is localized in mitochondria, its NADH-binding site faces the matrix compartment as in yeast mitochondria, and in perfect correlation with its abundance restores partially or fully NADH-dependent respiration that is rotenone-insensitive, flavone-sensitive, and antimycin A-sensitive. Thus the yeast enzyme has become coupled to the downstream portion of the human respiratory chain. Furthermore, the P:O ratio with malate/glutamate-dependent respiration in the transformants is approximately two-thirds of that of the wild-type 143B.TK$^-$ cells, as expected from the lack of proton pumping activity in the yeast enzyme. Finally, whereas the original mutant cell line C4T fails to grow in medium containing galactose instead of glucose, the high *NDI1*-expressing transformant has a fully restored capacity to grow in galactose medium. The present observations substantially expand the potential of the yeast *NDI1* gene for the therapy of mitochondrial diseases involving complex I deficiency.

The mammalian respiratory NADH-ubiquinone oxidoreductase (complex I) is the largest and least understood of the respiratory enzymes, consisting in beef heart of 42 (possibly 43) subunits (1). Seven of these subunits are encoded in mitochondrial DNA (mtDNA)[1] (2, 3). These subunits have attracted considerable interest because of the diseases that have been associated with mutations in one or the other of their coding genes, in particular the various forms of Leber's hereditary optic neuropathy (LHON) (4). In contrast to the multisubunit enzyme of mammalian cells, which contains a proton translocating site and is rotenone-sensitive, the internal NADH-Q oxidoreductase of *Saccharomyces cerevisiae*, NDI1, is a simple subunit of 513 amino acid residues, including the NH_2-terminal 26-residue signal sequence for import into mitochondria, which is not proton-translocating and is rotenone-insensitive (5, 6).

The finding that the NDI1 enzyme, expressed in *Escherichia coli*, acts as a member of the bacterial respiratory chain (7) and the observation that complex I-type enzymes and NDI1-type enzymes co-exist in bacteria and plant and fungal mitochondria (8, 9) have suggested the possibility that the *NDI1* gene, introduced into the nuclear genome of mammalian cells, could be expressed, imported into mitochondria, and integrated into the host cell respiratory chain. If the host cells are complex I-deficient because of a nuclear or mtDNA mutation, functional expression of the NDI1 could be useful to correct the complex I defect. That this is indeed the case for a complex I deficiency associated with a nuclear gene mutation has recently been demonstrated by experiments showing that the *S. cerevisiae NDI1* gene, transfected into Chinese hamster cells carrying a deletion in the gene for the essential nuclear-encoded MWFE subunit, can restore the respiratory NADH dehydrogenase activity in the host cells (10). These experiments have clearly pointed to the potential value of transfection of the *NDI1* gene for the therapy of mitochondrial diseases. Subsequent observations that the *NDI1* gene can be functionally expressed in human embryonic kidney 293 cells (11) and in non-proliferating human cells (12) have supported the above conclusion.

Because most human mitochondrial diseases associated with defective complex I activity, which have been reported so far, are due to mutations in the mitochondrial genome (13), it was important to analyze the function of the yeast *NDI1* gene and its encoded protein in human cells carrying mutations in mtDNA-encoded complex I subunits. The availability of C4T, a human cell line, isolated in the laboratory, with a homoplasmic frameshift mutation in the mitochondrial *ND4* gene (14), provided a unique opportunity to investigate this problem. It has, in fact, been shown that, in C4T cells, there is no assembly of the mtDNA-encoded subunits of NADH dehydrogenase and a complete loss of its respiratory function and enzyme activity. The investigation of the behavior of the NDI1 enzyme introduced into C4T cells seemed the more necessary, as it was conceivable that the continued synthesis and accumulation of

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[1] The abbreviations used are: mtDNA, mitochondrial DNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

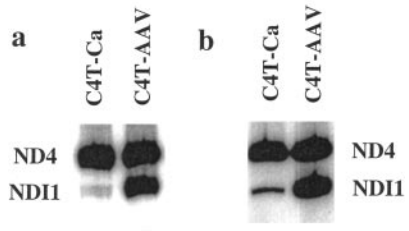


FIG. 1. **Detection of the *NDI1* gene and mRNA in transfectant cells.** *a,* PCR amplification of total cell DNA was carried out using two sets of primers specific for the human mitochondrial *ND4* gene and the yeast *NDI1* gene, and the products were analyzed by polyacrylamide gel electrophoresis. *b*, total RNA from the transfectants was subjected to RT-PCR, as detailed under "Experimental Procedures," using the same primers employed in the DNA analysis, and the products were analyzed by polyacrylamide gel electrophoresis.

the non-mutated mtDNA-encoded subunits of complex I in this cell line (14) could interfere with the correct integration of the yeast gene in the human respiratory chain, especially in view of the proposed role of ND1 in ubiquinone binding (1).

In the present work, the *NDI1* gene has been introduced into C4T cells, leading to the isolation of two transformant cell lines with low or high levels of expression of the exogenous gene. These two cell lines exhibited, respectively, near-complete and full restoration of NADH-dependent respiration. This was in both cases insensitive to rotenone and fully inhibitable by antimycin A, the latter finding indicating a coupling of the NDI1 to the downstream portion of the respiratory chain. Furthermore, measurements of P:O ratios revealed that, in transformed cells, this enzyme can modulate oxidative phosphorylation in the host cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Media—All the human cell lines used in the present work were grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS).

NDI1 Transfection—The construction of the NDI1-expressing plasmid pHook(NDI1) has been described previously (10). The rAAV-NDI1, a recombinant adeno-associated virus carrying the *NDI1* gene, was prepared by Dr. Terence R. Flotte (University of Florida, Gainesville) as reported earlier (12). The human cell line C4T, carrying in homoplasmic form a frameshift mutation in the *ND4* gene had also been previously isolated by cytoplast x cell fusion-mediated transfer of mitochondria from a rotenone-resistant mutant (C4) of the VA_2B cell line into human mtDNA-less 143B.TK$^-$ ρ°206 cells (14). In the present work, C4T cells were transfected with pHook (NDI1) by the calcium-phosphate precipitation method (15), and several transfectant cells lines, among them C4T-Ca, were isolated by screening in DMEM with 10% FBS and 0.6 mg/ml G418 (gentamycin). In another experiment, C4T cells were infected with 9×10^8 infectious units of rAAV-NDI1 virions, and several transduced clones, among them C4T-AAV, were isolated by screening in DMEM with 10% FBS and 0.1 μM rotenone. The clones C4T-Ca and C4T-AAV, with a low and, respectively, a high level of expression of NDI1, were selected for a detailed analysis.

Mitochondrial Protein Synthesis Analysis—To analyze mitochondrial protein synthesis, pulse-labeling experiments with [^{35}S]methionine were performed according to Chomyn (16). Samples of 2×10^6 cells of the desired type were plated on 10-cm Petri dishes, incubated overnight, washed with methionine-free DMEM, and then incubated for 7 min at 37 °C in 4 ml of the same medium containing 100 μg/ml of the cytosolic translational inhibitor emetine. Thereafter, [^{35}S]methionine (0.2 mCi (1175 Ci/mmol)) was added, and the cells were incubated for 30 min. The labeled cells were trypsinized, washed, and lysed in 1% SDS. Samples containing 30–50 μg of protein were electrophoresed through a 15–20% exponential SDS-polyacrylamide gradient gel (16).

NDI1 Gene and mRNA Detection by Polyacrylamide Gel Electrophoresis—The presence and amount of *NDI1* gene(s) in the C4T-Ca and C4T-AAV cells were analyzed by polyacrylamide gel electrophoresis after PCR amplification from total cell DNA. The PCR reactions were carried out by using primers NDI1-For, AGCACAGCGACAAACCAAAC and NDI1-Rev, TCAGGTTGGTATAGCTGGCT. Another set of primers was used for PCR amplification of the mitochondrial *ND4* gene: ND4-For, TGCCCAAGAACTATCAAACTCC (positions 11,305–11,326 in the Cambridge sequence, Ref. 17) and ND4-Rev, GGTTTGGATGAGAATGGCTG (positions 11,674–11,655). The PCR conditions were as previously described (18), except that denaturation was carried out at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. The ND4–4For and ND4–4Rev primers were added to the reaction mixture at the end of 6 cycles, and then another 21 cycles were carried out. Total cell RNA was isolated by the RNAzol B procedure (Tel-Test, Inc., Friendswood, TX). RNA extracted from 5×10^6 cells was treated with 30 units of RNase-free DNase I (Roche Molecular Biochemicals) at 37 °C overnight. After phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation, the reverse transcription reactions were carried out as previously described (18). Subsequently, PCR amplification was performed using the same conditions as for DNA analysis, and the products were analyzed by polyacrylamide gel electrophoresis.

Confocal Fluorescence Microscopy—Cells were grown on glass coverslips, for the last 1.5 h in the presence of 500 nM MitoTracker Green (Molecular Probes) in serum-free medium. The coverslips were washed in PBS (140 mM NaCl, 3.8 mM NaH_2PO_4, 16.2 mM Na_2HPO_4, pH 7.4) and fixed in a mixture of methanol and acetone (3:1) for 2 min at −20 °C. The coverslips were then incubated with rabbit anti-NdI1 antiserum, diluted 1:100 in PBS containing 2% horse serum for 2 h at 37 °C in a humidified chamber. After three washes in the same buffer, the coverslips were incubated with 1:100-diluted rhodamine red-X-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After three washes in PBS, the coverslips were mounted onto microscope slides in FluoroGuard Antifade Reagent (Bio-Rad), and analyzed on a Zeiss 310 laser-scanning microscope equipped with a 488 nm argon and a 543 nm helium neon lasers, using constant laser parameters.

O_2 Consumption Measurements—The medium of the cell lines to be analyzed was replaced with fresh medium the day before the measurements. Determination of the O_2 consumption rate in intact cells was carried out on 3.7–6.7 $\times$ 10^6 cells in Tris-based, Mg^{2+},Ca^{2+}-deficient (TD) buffer (0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris-HCl, pH 7.4 at 25 °C), as previously described (19). For measurements of O_2 consumption rate in digitonin-permeabilized cells (20), about 3.9 to 6.1 $\times$ 10^6 cells were resuspended in 1 ml of buffer (20 mM HEPES pH 7.1, 10 mM $MgCl_2$, 250 mM sucrose), and then 100 μg of digitonin (1 μl of a 10% solution in dimethyl sulfoxide) in 1 ml of buffer were added. After incubation for 1 min at room temperature, the cell suspension was diluted with 8 ml of buffer. The cells were rapidly pelleted and resuspended in respiration buffer (20 mm HEPES pH 7.1, 250 mM sucrose, 2 mM KP_i, 10 mM $MgCl_2$, and 1.0 mM ADP). The measurements were carried out in two chambers of an YSI Model 5300 Biological Oxygen Monitor. The substrates (adjusted to ~pH 7.0 with NaOH) and inhibitors were added with Hamilton syringes. The final concentrations were as follows: malate, 5 mM; glutamate, 5 mM; succinate, 5 mM; glycerol-3-phosphate, 5 mM; ascorbate, 10 mM; *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), 0.2 mM; NADH, 0.5 mM; rotenone, 100 nM; flavone, 0.5 mM; antimycin A, 20 nM; and KCN, 1 mM.

P:O Ratio Assays—These were carried out, by a novel *in situ* method in digitonin-permeabilized cells (21), using a Hansatech oxygraph with an adjustable chamber volume. The measurements were made on 3–7 $\times$ 10^6 cells, permeabilized with 0.005% digitonin in an assay buffer consisting of 75 mM sucrose, 5 mM KH_2PO_4, 40 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.4, 0.3 mM P^1,P^5-di(adenosine-5′) pentaphosphate (AP_5A) (Sigma), 0.32% (w/v) bovine serum albumin. Malate (5 mM) and glutamate (5 mM), or, after addition of rotenone (100 nM), succinate (5 mM) were used as respiratory substrates. 37.5–50 nmols of ADP were added, depending on the volume of the cell suspension in the chamber (0.5–1.0 ml) and on the substrate for each P:O measurement.

Growth Measurements—Multiple identical samples of 10^5 cells were grown for 7 days on 10-cm Petri dishes in 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, Ref. 22, both supplemented with 10% dialyzed FBS), and counted on a daily base. Both floating and attached cells were counted.

RESULTS

Isolation and Characterization of NDI1 Human Transfectants—C4T is a human cell line with a homoplasmic frameshift mutation in the mitochondrial *ND4* gene, in which there is no assembly of mtDNA-encoded subunits of NADH dehydrogenase and complete loss of its respiratory function and enzyme

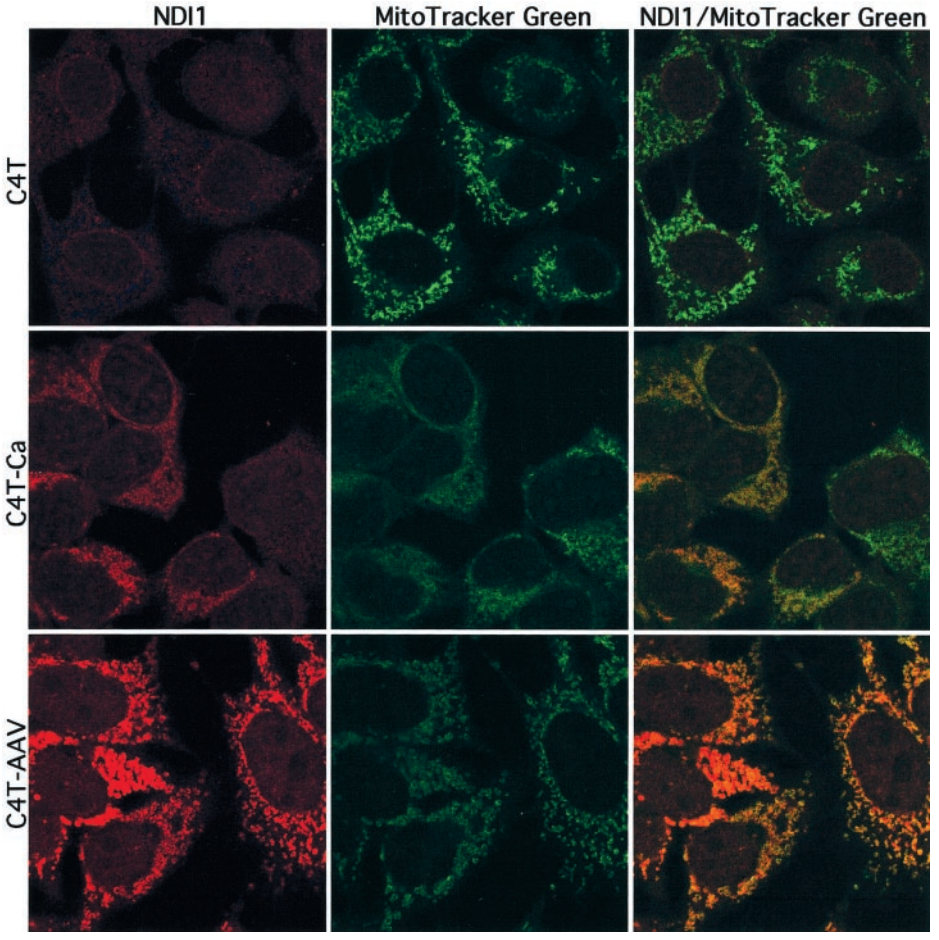


FIG. 2. **The NDI1 protein is localized in mitochondria in C4T transfected cells.** Double-labeling confocal fluorescence microscopy of C4T, C4T-Ca, and C4T-AAV cells is shown. Patterns of NDI1 (in *red*), mitochondria-specific MitoTracker Green probe and merged patterns of the same representative fields are shown. There is a low nonspecific background in C4T cells treated with anti-NDI1 antiserum.

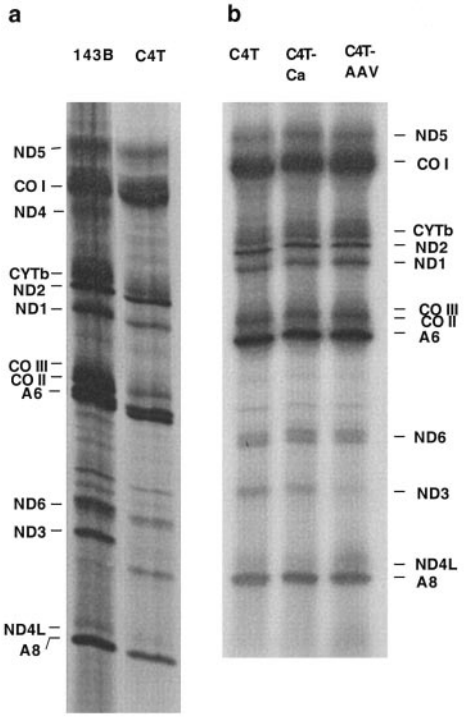


FIG. 3. **Mitochondrial protein synthesis analysis.** Electrophoretic patterns of SDS lysates from wild-type 143B.TK$^-$ cells, ND4 mutation-carrying C4T cells, and NDI1-transfected C4T-Ca and C4T-AAV cells, pulse-labeled with [^{35}S]methionine in the presence of 100 μg/ml emetine. *ND1, ND2, ND3, ND4, ND4L, ND5*, and *ND6* are subunits of NADH dehydrogenase; *CYTb* is apocytochrome *b*; *COI, COII* and *COIII* are subunits of cytochrome *c* oxidase; *A6* and *A8* are subunits of the H$^+$-ATP synthase.

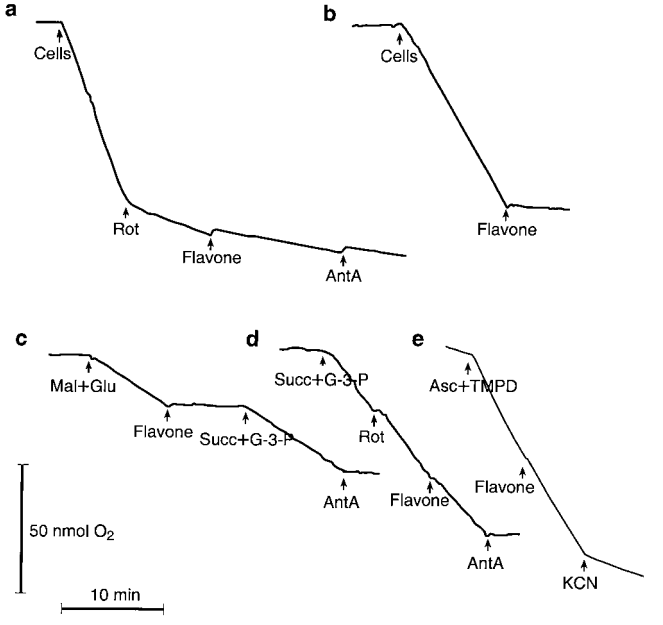


FIG. 4. **Respiration measurements in 143B.TK$^-$ cells.** Rotenone and flavone sensitivity in 143B.TK$^-$ cells of endogenous respiration, measured in intact cells (*a* and *b*), and of malate/glutamate-dependent (*c*), succinate/glycerol-3-phosphate-dependent (*d*) and TMPD/ascorbate-dependent respiration (*e*), measured in digitonin-permeabilized cells.

activity (14). C4T cells were either transfected with the plasmid pHook (NDI1) by calcium-phosphate precipitation or infected with rAAV-NDI1 virions, and two transformants, C4T-Ca and C4T-AAV, respectively, were isolated. The success of the transformation was shown by PCR amplification of a

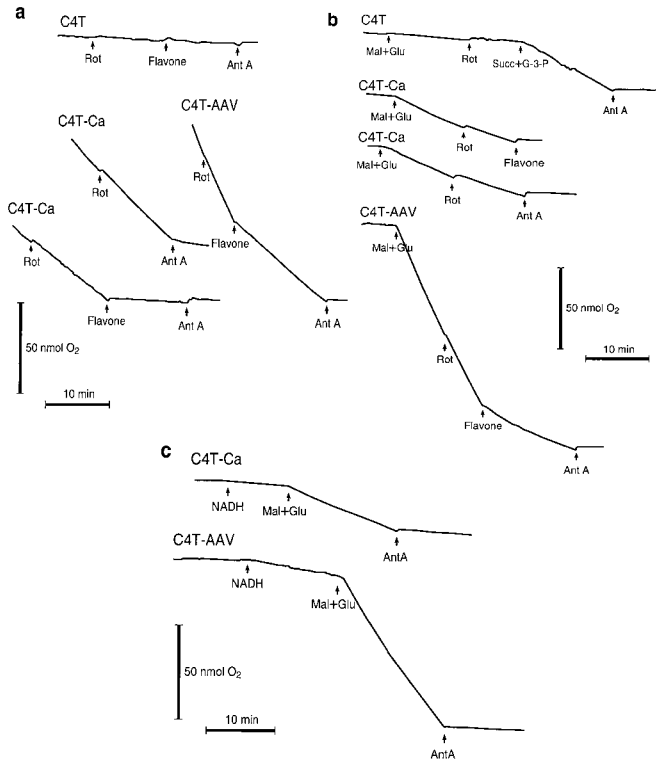


FIG. 5. **Recovery of NADH oxidase activity in C4T-Ca and C4T-AAV transformants.** *a,* endogenous respiration in intact C4T, C4T-Ca, and C4T-AAV cells; *b,* substrate-dependent respiration in cells permeabilized with digitonin; *c*, low response to NADH (0.5 mM) of C4T-Ca and C4T-AAV cells.

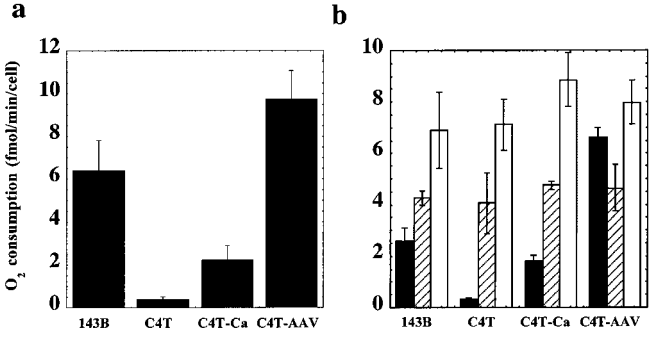
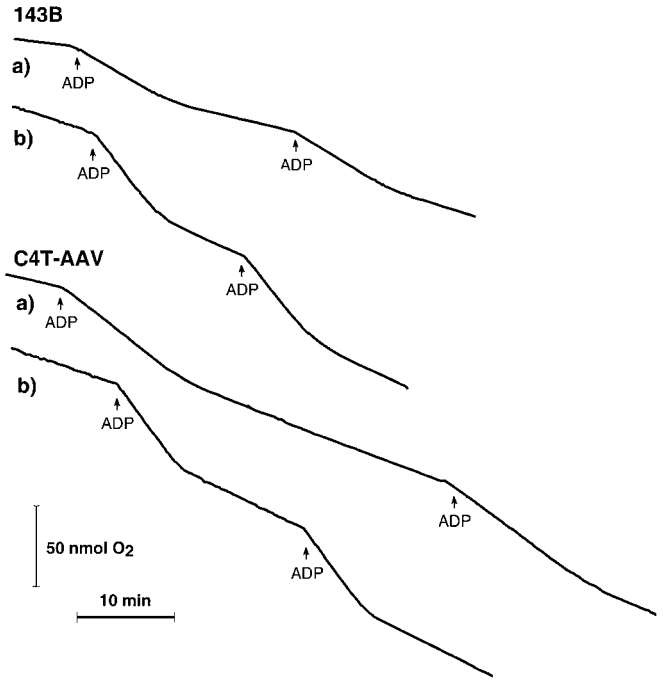


FIG. 6. **Quantitative behavior of endogenous and substrate-dependent respiration in 143TK$^-$ cells, C4T cells, and C4T transformants.** *a,* averages ± S.E. of triple measurements of endogenous respiration rate in intact 143B.TK$^-$, C4T, C4T-Ca, and C4T-AAV cells. *b,* averages ± S.E. of triple measurements of the rates of respiration dependent on malate/glutamate (*filled bars*), succinate/glycerol-3-phosphate (*hatched bars*), and TMPD/ascorbate (*open bars*) in digitonin-permeabilized 143B.TK$^-$, C4T, C4T-Ca, and C4T-AAV cells.



Cell lines	Malate/Glutamate	Succinate
143B	2.10 ± 0.07	1.35 ± 0.08
C4T-Ca	1.16	1.31 ± 0.15
C4T-AAV	1.34 ± 0.04	1.46 ± 0.01

FIG. 7. **Comparison of P:O ratios in 143B.TK$^-$ cells and NDI1-transformed C4T cells.** Malate/glutamate (*a*) and succinate (*b*) were used as respiratory substrates. The P:O ratios are summarized in the *inset table*. Each P:O is the average ± S.E. of two or three independent measurements, except for the single measurement for P:O of C4T-Ca with malate/glutamate.

NDI1 gene fragment (Fig. 1*a*). The results also showed that C4T-AAV had a higher *NDI1* gene copy number (estimated to be ~10-fold) than C4T-Ca, as a result of the higher transducing efficiency of the recombinant adeno-associated virus vector as the delivering system (12). The RNA expression levels of the *NDI1* gene were measured by reverse transcription-PCR, and the results (Fig. 1*b*) showed a higher NDI1 mRNA level in C4T-AAV than in C4T-Ca cells, presumably because of the higher *NDI1* gene copy number.

C4T-Ca and C4T-AAV cells were also analyzed by confocal immunofluorescence microscopy by using anti-NDI1 antiserum and the mitochondrial-specific fluorescent probe MitoTracker. As shown in Fig. 2, the NDI1 protein was predominantly localized in mitochondria in both transformants. C4T-AAV cells showed a higher expression level of its protein product.

Mitochondrial Protein Synthesis Analysis—To exclude the possibility of the occurrence of revertants in the original C4T cell population (23), the mitochondrial translation products were labeled with [^{35}S]methionine for 30 min in the presence of emetine. As shown in Fig. 3, *a* and *b*, there were no ND4 products in either C4T-Ca or C4T-AAV cells, as in their parent C4T cells.

Respiration Properties of Transformants—To assess the activities of the yeast NDI1 protein in human cells and to investigate whether it can restore the complex I defects in mutant cells, the respiration properties of the wild-type 143B.TK$^-$ cells, the mutant C4T cells, and the two NDI1-transfectants, C4T-Ca and C4T-AAV, were investigated in detail. To distinguish the NADH dehydrogenase activity of the mammalian complex I from that of the yeast NDI1 enzyme, the significant difference in their sensitivity to the respiration inhibitor rotenone (5) was utilized. At first, experiments were carried out with intact or digitonin-permeabilized 143B.TK$^-$ cells, to investigate the action of flavone, a drug which inhibits the yeast NDI1 (5), on the mammalian respiratory chain. As shown in Fig. 4, *a* and *b*, the endogenous respiration of intact cells was extensively inhibited by 100 nM rotenone and nearly completely by 0.5 mM flavone, the residual low respiration in the presence of the latter drug being insensitive to 20 nM antimycin A. These observations strongly suggested that flavone inhibits complex I or NADH-producing dehydrogenases. This conclusion was confirmed by experiments carried out on digitonin-permeabilized 143B.TK$^-$ cells. The malate/glutamate-driven respiration, which usually reflects the rate-limiting activity of complex I, was completely inhibited by rotenone (not shown) and flavone (Fig. 4*c*). By contrast, the succinate/glycerol-3-phosphate-

driven respiration, which usually reflects the rate-limiting activity of complex III, was nearly insensitive to flavone (Fig. 4*d*), whereas the ascorbate/TMPD-dependent respiration, which reflects the activity of complex IV (cytochrome *c* oxidase) was completely insensitive to this drug (Fig. 4*e*).

The endogenous respiration activities of intact C4T cells and their intact NDI1 transformants are shown in Fig. 5*a*. C4T cells, which carry in homoplasmic form the ND4 frameshift mutation, showed severely defective endogenous respiration. The respiration was restored in both C4T-Ca and C4T-AAV cells, although to a different extent relative to that in 143B.TK$^-$ cells, in particular, to ~35 and ~150%, respectively (Fig. 6*a*). These restoration extents corresponded to the different expression levels of the *NDI1* gene. The restored activities in the NDI1 transformants were insensitive to the complex I inhibitor rotenone, but sensitive to flavone (Fig. 5*a*), as previously shown for NDI1 in yeast (5), indicating they were because of the function of *NDI1* gene. Increasing the concentration of flavone to a saturating level did not decrease the flavone-resistant respiration in C4T-AAV cells. The high copy number of *NDI1* gene in these cells and its consequent overexpression, together with the poor solubility of this drug, provide an explanation for ~30% of the endogenous respiration being insensitive to flavone in this cell line (Fig. 5*a*).

To assess further the respiration activities in the transformed cells, the malate-glutamate-dependent respiration, the succinate/glycerol-3-phosphate-dependent respiration, and the TMPD/ascorbate-dependent respiration were measured. The oxygraphic tracings for malate/glutamate-dependent respiration are shown in Fig. 5*b*. In C4T-Ca cells, NDI1 restored ~70% of the NADH-dependent respiration rate, relative to that in 143B.TK$^-$ cells (Fig. 6*b*). Interestingly, C4T-AAV cells showed a much higher NADH-dependent respiration rate than the wild-type 143B.TK$^-$ cells (Figs. 5*b* and 6*b*). NADH stimulated only slightly the respiration of digitonin-permeabilized transformants, in particular, to an extent estimated to correspond to ~14% of the malate/glutamate-dependent respiration in C4T-Ca cells and ~11% in C4T-AAV cells (Fig. 5*c*). These observations indicated that the NADH-binding site of the expressed NDI1 faces the matrix compartment, as in yeast mitochondria. The slight stimulation of respiration by NADH in these experiments may be because of the activity of the rotenone-insensitive NADH-cytochrome *c* reductase, which is associated with the outer mitochondrial membrane (24). Fig. 6 summarizes the quantitative behavior of the endogenous respiration (*a*) and substrate-dependent respiration (*b*) in 143B.TK$^-$ cells, C4T cells, and the two C4T transformants.

P:O Ratio Assays—The lack of a proton-translocating site in the yeast NDI1 led to the prediction that if the enzyme introduced into C4T cells did transfer electrons from NADH to ubiquinone, the P:O ratio coupled to NADH oxidation would be lower in the C4T-Ca and C4T-AAV transformants compared with the control (143B.TK$^-$) ratio, whereas the P:O ratio coupled to succinate oxidation would be identical in the transformants and the control. This proved indeed to be the case. As shown in Fig. 7, in 143B.TK$^-$ cells, the P:O ratios with malate/glutamate and succinate were, respectively, ~2.1 and ~1.35. By contrast, the P:O ratios with malate/glutamate and succinate were, respectively, 1.16–1.34 and 1.31–1.46 in the transformants. These experiments did indeed show that the respiration restored by NDI1 in defective C4T cells was coupled with ATP synthesis, confirming the results previously obtained in normal embryonic kidney 293 cells transfected with the *NDI1* gene (11).

Growth Behavior of Transformants—Mammalian cells rely on both oxidative phosphorylation and glycolysis to generate

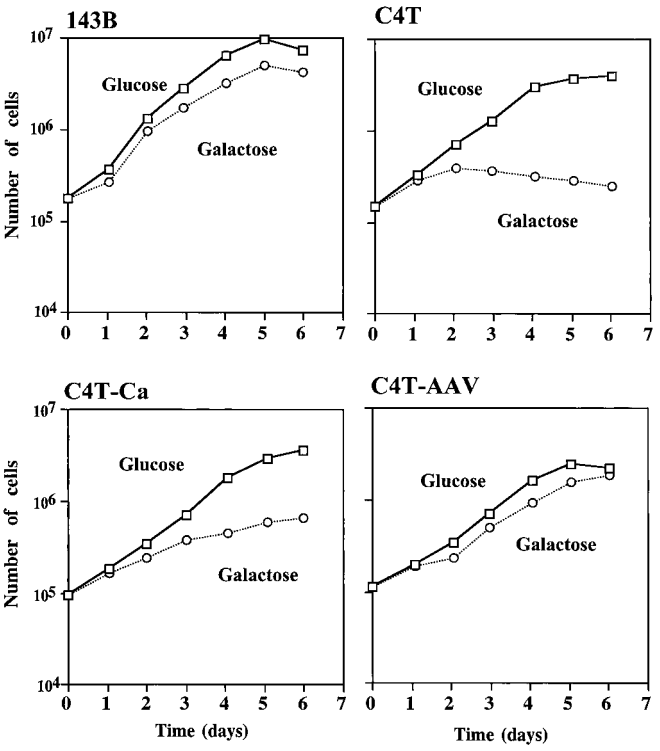


FIG. 8. **Growth curves of 143B.TK$^-$, C4T, C4T-Ca, and C4T-AAV cells in glucose-containing DMEM and in galactose-containing DMEM.** Cells were plated on multiple 10-cm plates at 10^5 per plate and counted on a daily basis for 7 days.

ATP. However, they cannot utilize galactose efficiently in the glycolytic pathway. Consequently, in medium containing galactose instead of glucose, they are forced to rely predominantly on oxidative phosphorylation as a source of ATP. As shown in Fig. 8, whereas the wild-type 143B.TK$^-$ cells could grow well in both glucose- and galactose-containing medium, the mutant C4T cells grew well in glucose medium, but failed to grow in galactose medium, with the cells becoming progressively detached from the plate after 3 days. C4T-AAV cells showed in galactose medium a totally recovered growth capacity, which was comparable with that of wild-type 143B.TK$^-$ cells. By contrast, C4T-Ca cells exhibited only a partial restoration, presumably because of the limited expression level of the *NDI1* gene(s), with a considerable decrease in growth rate after the third day, but no significant cell detachment.

DISCUSSION

The present work has extended in a significant way the previous observations indicating that the single polypeptide, rotenone-insensitive internal NADH-quinone oxidoreductase of *S. cerevisiae* (NDI1) can restore the NADH oxidoreductase activity of complex I-deficient Chinese hamster cells carrying a deletion in the gene for an essential nuclear-encoded subunit. In fact, it has been clearly shown here that the *NDI1* gene, either transfected by the calcium-phosphate precipitation method to reach a low copy number, or transduced with a virus vector to reach a high copy number into human C4T cells, carrying a mitochondrial *ND4* gene mutation, was expressed appropriately in the nucleus and the cytosol of the chosen host cells. The corresponding protein was, thereafter, imported correctly into mitochondria, its NADH-binding site facing the matrix compartment, as in yeast mitochondria, and restored an NADH-quinone oxidoreductase activity that was rotenone-insensitive, flavone-sensitive, and antimycin A-sensitive. The latter result indicated that the yeast enzyme was integrated with the downstream portion of the human respiratory chain.

Furthermore, as expected from the substitution of the proton-translocating complex I by a non-proton-translocating enzyme, the P:O ratios associated with NADH oxidation were lower in the transformed cells (~1.16–1.34) compared with the value of ~2.1 found in 143B.TK$^-$ cells, whereas the P:O ratios coupled to succinate oxidation in the transformants (1.31–1.46) were similar to the value found in 143B.TK$^-$ cells (~1.35). This important result indicated that the respiration supported by NDI1 was appropriately coupled to ATP synthesis. The reestablishment of oxidative phosphorylation in the C4T transformants was shown by their reacquisition of the capacity to grow under conditions where their glycolytic activity was strongly limited, *i.e.* in medium containing galactose instead of glucose.

The rate of malate/glutamate-dependent respiration, relative to the rates of succinate or TMPD/ascorbate-driven respiration, in the transformant C4T-Ca appeared to be somewhat lower than in 143B.TK− cells, whereas it was much higher in the transformant C4T-AAV. These differences, presumably, reflected the different level of expression of the *NDI1* gene in the two transformants, indicating an independent regulation of NADH-quinone oxidoreductase activity. However, a comparison of the endogenous respiration rates and the malate/glutamate-dependent respiration rates in the two transformed cell lines with the corresponding rates in the parental 143B.TK$^-$ cells suggested that the NDI1 activity was rate-limiting for respiration in the two transformants, as complex I activity usually is in 143B.TK$^-$ cells (19, 20).

The results obtained in the present work have extended to complex I deficiencies associated with mtDNA mutations the complementing capacity of the yeast NDI1, providing at the same time important insights into the regulation of its integration in the human respiratory chain. The potential usefulness of the yeast *NDI1* gene as a therapeutic tool for diseases involving complex I defects has, therefore, been confirmed, and its scope, amplified.

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