The EMBO Journal vol.12 no.8 pp.3043-3048, 1993

Lack of assembly of mitochondrial DNA-encoded subunits of respiratory NADH dehydrogenase and loss of enzyme activity in a human cell mutant lacking the mitochondrial *ND4* gene product

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Communicated by G.Schatz

In most eukaryotic cells, the respiratory chain NADH dehydrogenase (Complex I) is a multimeric enzyme under dual (nuclear and mitochondrial) genetic control. Several genes encoding subunits of this enzyme have been identified in the mitochondrial genome from various organisms, but the functions of these subunits are in most part unknown. We describe here a human cell line in which the enzyme lacks the mtDNA-encoded subunit ND4 due to a frameshift mutation in the gene. In this cell line, the other mtDNA-encoded subunits fail to assemble, while at least some of the nuclear-encoded subunits involved in the redox reactions appear to be assembled normally. In fact, while there is a complete loss of NADH:Q_1 oxidoreductase activity, the NADH:$Fe(CN)_6$ oxidoreductase activity is normal. These observations provide the first clear evidence that the *ND4* gene product is essential for Complex I activity and give some insights into the function and the structural relationship of this polypeptide to the rest of the enzyme. They are also significant for understanding the pathogenetic mechanism of the *ND4* gene mutation associated with Leber's hereditary optic neuropathy.

Key words: Complex I/cybrid/frameshift mutation/mtDNA-less (ϱ°) cell/polarography

Introduction

The mitochondrial NADH dehydrogenase (Complex I) (Weiss *et al.*, 1991), the largest enzyme complex of the respiratory chain, consists of ~40 subunits in the mammalian enzyme (Walker, 1992) and ~30 in the *Neurospora crassa* enzyme (Weiss *et al.*, 1991), of which seven are encoded in mitochondrial DNA (mtDNA) (Chomyn *et al.*, 1985, 1986; Weiss *et al.*, 1991). In *Neurospora*, the enzyme has been shown to have an overall L-shaped structure (Hofhaus *et al.*, 1991), and it is likely that this model applies also to the mammalian enzyme. One arm of the 'L' is buried in the inner mitochondrial membrane and contains all mtDNA-encoded subunits, while the other arm protrudes into the matrix and contains most of the prosthetic groups that are involved in the redox reactions (Hofhaus *et al.*, 1991). With the exception of the *ND1* gene product, nothing is known about the functions of the mtDNA-encoded subunits, despite the knowledge of their primary structure (Anderson *et al.*, 1981). The ND1 subunit is known to bind rotenone, a specific inhibitor of Complex I (Earley *et al.*, 1987), and to interact with ubiquinone (Friedrich *et al.*, 1990). Furthermore, it has been reported that the *ND1* gene product binds *N,N'*-dicyclohexylcarbodiimide (DCCD) (Yagi and Hatefi, 1988), suggesting that it may be involved in proton translocation.

Further progress in our understanding of the functional role of the mtDNA-encoded subunits of Complex I will depend on the availability of mutants. In this context, the discovery of natural mtDNA mutations affecting the ND4 or ND1 subunit of NADH dehydrogenase, with resulting Complex I deficiency, in patients affected by Leber's hereditary optic neuropathy (Wallace, 1992) has opened one way for investigating the mutation-induced structural and functional changes in these subunits either directly in the patients' cells or after transfer of the patients' mitochondria into human mtDNA-less (ϱ°) cells (King and Attardi, 1989; Chomyn *et al.*, 1991). A complementary approach would involve the isolation of artificial mtDNA mutants affected in subunits of NADH dehydrogenase from human cell lines. We describe here the first example of *in vitro* isolation of this class of mutants. In the course of investigations on rotenone-resistant mutants of the human cell line VA_2B (Mitchell *et al.*, 1975), we found one mutant (C4) that exhibited a complete loss of NADH-dependent respiration. The respiration defect could be transferred to a human ϱ° cell line with the mitochondria from the C4 mutant. Protein synthesis, immunoprecipitation and DNA sequencing experiments indicated that the defect resulted from the absence of the mtDNA-encoded subunit ND4, due to a frameshift mutation in the corresponding gene.

Results and Discussion

Isolation of a human cell mutant with a defect in NADH-dependent respiration

Among several VA_2B clones capable of growing in the presence of 1 μM rotenone, one (C4) was analyzed in detail. Surprisingly, the rotenone-sensitive O_2 consumption of this mutant, measured in intact cells (King and Attardi, 1989), was found to be much lower than that of VA_2B cells (<0.5 fmol/min/cell compared with 2.5 fmol/min/cell). In order to pinpoint the block in the respiratory chain of C4 cells, an approach involving digitonin-permeabilized cells and different substrates and inhibitors (Granger and Lehninger, 1982) was used. In this approach, with malate and glutamate as substrates, the corresponding dehydrogenases generate NADH, which is oxidized in three discrete steps by the NADH:ubiquinone oxidoreductase or NADH dehydrogenase (Complex I), the ubiquinol:cytochrome *c* oxidoreductase (Complex III) and the cytochrome *c* oxidase (Complex IV). The overall reaction is measured by the O_2 consumption in the terminal step:

$$\text{NADH} \xrightarrow{\text{Complex I}} \text{Ubiquinone} \xrightarrow{\text{Complex III}} \text{cytochrome } c \xrightarrow{\text{Complex IV}} O_2$$

Using succinate and glycerol-3-phosphate as substrates, the corresponding dehydrogenases cause a direct reduction of ubiquinone, bypassing Complex I. Similarly, using *N,N,N'N'*-tetramethyl-*p*-phenylenediamine (TMPD) and

ascorbate, cytochrome *c* is reduced and the activity of Complex IV alone is measured. A comparison of the slopes obtained with different substrates allows the determination of the rate limiting step in the respiratory chain. It appears from Figure 1 that the lack of respiration in the mutant C4 is due to a defect in NADH-dependent respiration, and therefore, presumably, in Complex I. In fact, bypassing Complex I in the mutant with either succinate + glycerol-3-phosphate or TMPD + ascorbate results in a much faster O_2 consumption. Therefore, a defect in Complex III or Complex IV cannot account for the block in respiration with NADH-linked substrates. Furthermore, the absolute rates of O_2 consumption with succinate + glycerol-3-phosphate or TMPD + ascorbate in the mutant are comparable with those in VA_2B cells, suggesting that the terminal part of the respiratory chain is functionally normal in the mutant.

The Complex I defect is transferred to a ρ° cell line with the mitochondria from the C4 mutant

The defect in NADH-dependent respiration in C4 could be due to a mutation in either a nuclear gene or a mitochondrial gene for a subunit of Complex I. The mtDNA-less (ρ°) 143B.206 cell line (King and Attardi, 1989) offered a very convenient approach to distinguish between these two possibilities. Mitochondria from C4 mutant cells were transferred into ρ° cells by fusion with cytoplasts (enucleated cells) derived from the C4 mutant cells (King and Attardi, 1989), thus producing the transformant cell line C4T, which contains the mtDNA of the mutant, but in a different nuclear background. As shown in Figure 2, the NADH-dependent respiration defect was transferred with the mtDNA. By contrast, the transformant C4T was not able to grow in the presence of 1 μM rotenone, a finding which suggested that a nuclear gene mutation was responsible for the rotenone resistance of the original C4 mutant. Therefore, the respiratory defect in C4 that was being investigated here, appeared to be due to a mutation unrelated to that producing the rotenone resistance. As will be reported elsewhere (G.Hofhaus and G.Attardi, in preparation), other VA_2B clones capable of growing in the presence of 1 μM rotenone have recently been shown to exhibit a defect in NADH-dependent respiration. As in the case of C4, this defect was transferred with the mtDNA into ρ° cells, whereas the resistance to rotenone was not. A reasonable interpretation of these results is that, in the isolated VA_2B clones, which were resistant to 1 μM rotenone due to a nuclear mutation affecting presumably the cell permeability to the drug, a secondary deleterious mutation in one of the mtDNA-encoded subunits of Complex I made the clones defective in this enzyme activity also. Their growth became independent of respiration, and, therefore, resistant to any small amounts of rotenone leaking through the cell membrane. Also presented in Figure 2 is a control experiment showing that a transformant obtained by the transfer of mitochondria from HeLa cells into ρ°206 cells had a normal NADH-dependent respiration.

The finding that the NADH-dependent respiration defect in the C4 cell line and its C4T derivative was associated with a mtDNA mutation tended to exclude the possibility that this defect involved the transport of the chosen substrates into the mitochondria or the activity of the corresponding dehydrogenases, rather than Complex I activity. In order to verify this conclusion, the NADH:Q_1 oxidoreductase activity was measured in partially purified mitochondrial membranes from the C4T cell line. Table I indeed shows that also this activity is completely absent in C4T, whereas the NADH:$Fe(CN)_6$ oxidoreductase activity in this transformant is equal to that in VA_2B cells. The latter observation suggests that at least the nuclear-encoded portion of the enzyme that can be isolated as the flavoprotein fragment (Galante and Hatefi, 1979), and which is capable of carrying the NADH:$Fe(CN)_6$ oxidoreductase reaction, is assembled and associated with the membrane in the transformant.

The C4T cell line exhibits a frameshift mutation in the mitochondrial ND4 gene

In order to obtain some indications as to the possible site of the mtDNA mutation responsible for the Complex I defect

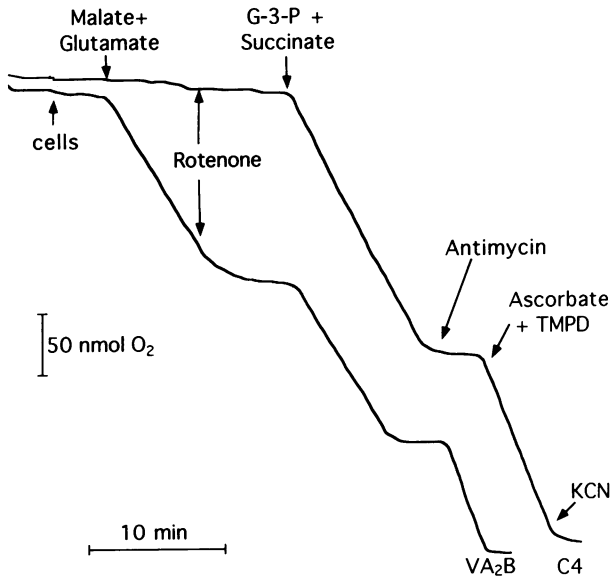

Fig. 1. O_2 consumption by digitonin-permeabilized (Granger and Lehninger, 1982) C4 and VA_2B cells in the presence of different substrates and inhibitors.

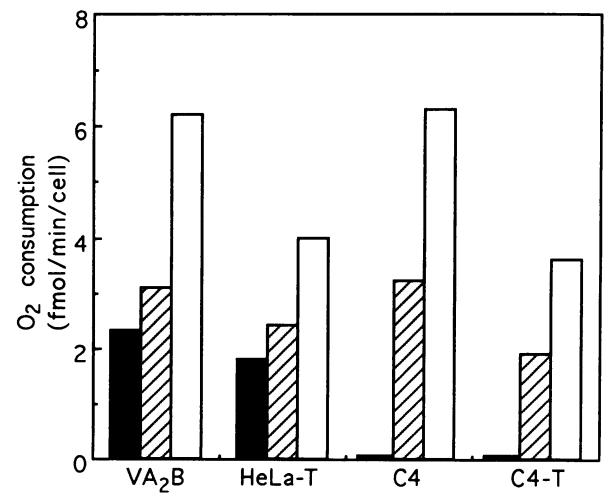

Fig. 2. Activities of the enzymes of the mitochondrial respiratory chain in the mutant clone C4 and the corresponding ρ° cell transformant C4T, VA_2B cells and a HeLa ρ° cell transformant. Using ~9.5 × 10^6 digitonin-permeabilized cells, activities of the various components of the respiratory chain were determined as malate/glutamate (filled bars), glycero-3-phosphate/succinate (hatched bars) and ascorbate/TMPD (open bars) dependent respiration.

in the C4 and C4T cell lines, the mitochondrial translation products of the C4T transformant and of the VA_2B cell line were specifically labeled for 2 h with [^{35}S]methionine in the presence of emetine, to block cytoplasmic protein synthesis (Chomyn *et al.*, 1991). The electrophoretic patterns for C4T and VA_2B cells were found to be identical, except for the complete absence of the *ND4* gene product in C4T. Also after short pulses (20 and 40 min), there was no trace of the ND4 subunit in the electrophoretic pattern from this transformant; furthermore, there were no additional band(s) that could correspond to a truncated polypeptide(s).

The polymerase chain reaction (PCR)-amplified *ND4* gene of the transformant was sequenced by the chain termination method (Sanger *et al.*, 1977) and the sequence was compared with that of the *ND4* gene from the parental VA_2B cell line. An additional C residue was found in a row of six C residues at positions 10947–10952 in the Cambridge sequence (Anderson *et al.*, 1981) (Figure 4). The insertion appeared to be homoplasmic or near-homoplasmic. Since the template utilized for PCR contained $>10^7$ mtDNA molecules, the latter observation substantially excluded a PCR artefact, as confirmed by the sequencing of the product amplified in an independent reaction. The C insertion caused a frameshift that created a stop codon ~150 bp downstream. This frameshift mutation appeared, therefore, to be the cause of the premature termination of translation and of the disappearance of the *ND4* gene product in the C4T transformant. The expected truncated polypeptide would have been 113 amino acids long and would have had a calculated molecular mass of ~12.9 kDa. The absence of a polypeptide of this size among the mitochondrial translation products of C4T (Figure 3), even in the short [^{35}S]methionine pulse labeling experiments, points to its rapid degradation.

The mtDNA-encoded subunits are not assembled into Complex I in the C4T cell line

In order to investigate whether the assembly of the other mtDNA-encoded subunits of Complex I was affected in the C4T transformant, antibodies against the nuclear-encoded 49 kDa subunit of the bovine enzyme, which is part of the matrix arm, were used to precipitate the whole human Complex I (Chomyn *et al.*, 1986). In this experiment, only those mtDNA-encoded subunits that were assembled into the Complex I would be expected to be coprecipitated.

The mtDNA-encoded subunits of C4T cells and, as a control, those of VA_2B cells were labeled for 2 h with [^{35}S]methionine in the presence of cycloheximide (to inhibit cytoplasmic protein synthesis reversibly), and chased for 16 h in unlabeled medium in the absence of inhibitors, in order to allow their incorporation into Complex I. As shown in Figure 5, the electrophoretic pattern of an SDS mitochondrial lysate from VA_2B cells pulse–chased as described above revealed all the mitochondrial translation products, with a few extra bands in the high mol. wt region

Table I. Measurement of the NADH:Q_1 and NADH:$Fe(CN)_6$ oxidoreductase activities in mitochondrial membranes isolated from C4T and VA_2B cells

	VA_2B	C4T
NADH:Q_1 (nmol/min/mg)	5.9 ± 0.2	n.d.
NADH: $Fe(CN)_6$ (nmol/min/mg)	418 ± 8	411 ± 16

The determinations were made in triplicate (NADH:Q_1 oxidoreductase activity) or duplicate [NADH:$Fe(CN)_6$ oxidoreductase activity]. n.d.: not detectable.

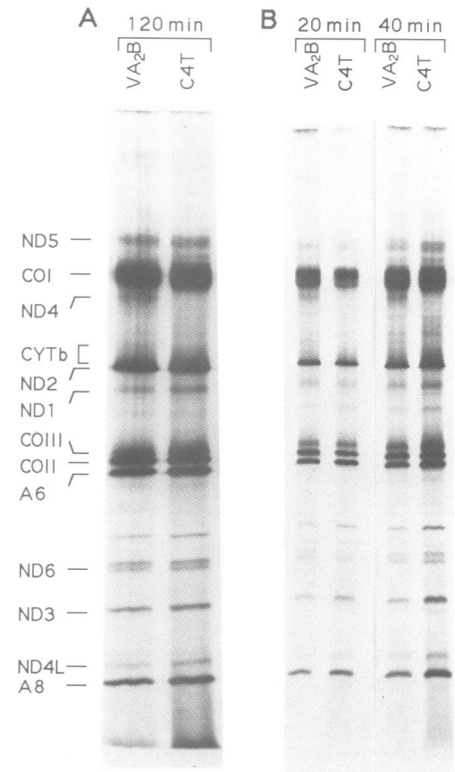

Fig. 3. Absence of the *ND4* gene product from the newly synthesized mitochondrial translation products of C4T cells. Shown are the fluorograms, after polyacrylamide gradient gel electrophoresis, of samples of total cell lysates from VA_2B and C4T cells exposed to [^{35}S]methionine for the indicated times in the presence of 100 μg/ml emetine. Identification of the individual mitochondrial translation products was made according to Chomyn *et al.* (1991).

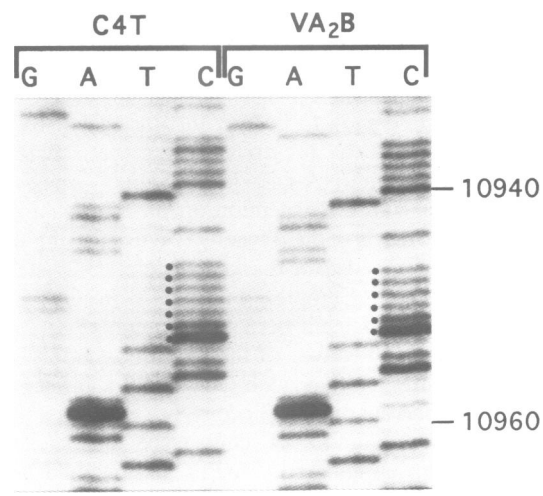

Fig. 4. Portion of the sequence of the *ND4* gene from VA_2B and C4T cells (between positions 10 932 and 10 965), showing the insertion of a C residue in the C4T sequence. The single-stranded PCR product corresponding to the coding (light) strand was used as a template for sequencing by the chain termination method, and the sequence

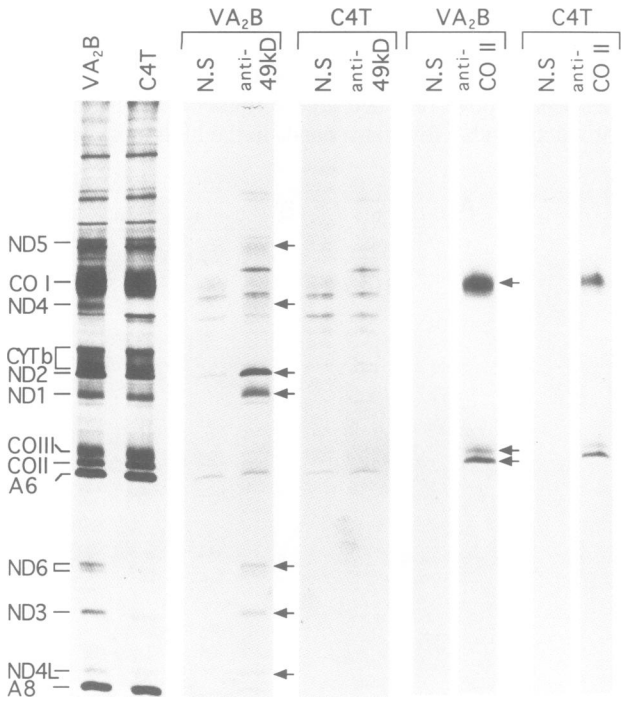

Fig. 5. Antibodies against the nuclear-encoded bovine 49 kDa subunit precipitate the mtDNA-encoded subunits of Complex I from a VA_2B cell Triton X-100 mitochondrial lysate but not from a C4T cell mitochondrial lysate. Shown are fluorograms, after SDS–PAGE, of immunoprecipitates obtained by incubating samples of a 0.5% Triton X-100 mitochondrial lysate from [^{35}S]methionine pulse–chased VA_2B cells or C4T cells with γ-globulins from an antiserum against the bovine 49 kDa subunit, or from an antiserum against the human COII subunit, or from a normal rabbit serum (N.S.). The first two lanes show the patterns of samples of SDS mitochondrial lysates from pulse–chased VA_2B and C4T cells.

representing cytoplasmic proteins labeled during the chase (Chomyn *et al.*, 1986). In the equivalent pattern from C4T cells, the labeled mtDNA-encoded subunits of Complex IV, Complex III and H^+-ATPase were present in the same amounts as in the VA_2B pattern. By contrast, several of the mtDNA-encoded subunits of NADH dehydrogenase were apparently absent (ND4) or strongly reduced (ND2, ND6, ND3 and ND4L), with only ND1 being present in a normal amount. The ND5 subunit could not be clearly recognized due to the presence of closely migrating contaminating bands, but this subunit seemed to be present, though in reduced amount. In view of the previous finding that the rate of labeling of the mtDNA-encoded subunits of NADH dehydrogenase (with the exception of the ND4 subunit) is very similar in VA_2B and C4T cells (Figure 3), the above results clearly pointed to a marked difference in stability among the various Complex I subunits in C4T cells, presumably resulting from defective assembly.

Further evidence in favor of the lack of assembly of all or most of the mtDNA-encoded Complex I subunits came from experiments in which Triton X-100 mitochondrial lysates from VA_2B and C4T cells were incubated with γ-globulins from an antiserum against the purified bovine 49 kDa iron–sulfur protein. As shown in Figure 5, the antibodies precipitated all the mtDNA-encoded subunits of Complex I from the VA_2B control lysate. By contrast, no clear evidence of such subunits, even those that were abundantly represented in the SDS mitochondrial lysate from C4T cells (ND1 and ND2), could be seen in the immunoprecipitate from these cells (Figure 5). This failure of the mtDNA-encoded subunits to be precipitated as a complex is unlikely to be due to instability of this complex in the presence of Triton X-100, because the lack of assembly of these subunits has also been indicated by the results of the above described pulse–chase experiment. In a control experiment, antibodies against the C-terminal undecapeptide of subunit II of human cytochrome *c* oxidase (anti-COII) were able to precipitate the whole enzyme (Mariottini *et al.*, 1986) from the mitochondrial lysates of both VA_2B and C4T (Figure 5). However, the bands corresponding to the three mtDNA-encoded subunits of the precipitated cytochrome *c* oxidase appeared to be less intense in the case of C4T as compared with the VA_2B control. Since the labeling of these subunits was virtually identical in the SDS mitochondrial lysates from pulse–chased VA_2B and C4T cells, it is possible that the lower intensity of the bands in the immunoprecipitate from the C4T lysate is due to a secondary effect of the Complex I defect on the assembly of cytochrome *c* oxidase in the transformant.

Role of the ND4 subunit in Complex I

The conclusion of the experiments described above was that all or most of the mtDNA-encoded subunits of Complex I are not assembled in the C4T transformant. That some of the nuclear-encoded subunits of Complex I are, on the contrary, still assembled in the transformant is suggested by the normal NADH:$Fe(CN)_6$ oxidoreductase activity in the mutant mitochondria. It has been shown in *Neurospora* (Tuschen *et al.*, 1990) that the two arms of the enzyme are assembled independently. If the same situation holds for human cells in culture, it has to be expected that the matrix arm of the enzyme is present in the inner mitochondrial membrane of the mutant.

The results reported here represent a clear-cut case of a drastic mutation in the mitochondrial genome that leads to a non-functional Complex I. Although we cannot absolutely exclude another mutation(s) in mtDNA gene(s) encoding subunits of NADH dehydrogenase, the presence of these subunits in normal amounts and with the expected electrophoretic mobilities in the protein labeling patterns obtained in the short and long pulse experiments makes the lack of the *ND4* gene product the most plausible cause for the failure of the mtDNA-encoded subunits to assemble into Complex I.

There is strong evidence, coming both from biochemical experiments (Earley *et al.*, 1987; Yagi and Hatefi, 1988; Friedrich *et al.*, 1990) and from the study of the effects of mtDNA mutations in human diseases (Wallace, 1992), that at least some NADH dehydrogenase subunits play a role in the function of Complex I. For the *ND4* gene, this evidence has come from studies on a mutation at position 11 778 of the mitochondrial genome, which is associated with Leber's hereditary optic neuropathy (Wallace *et al.*, 1988). Although this mutation leads to decreased Complex I activity, as determined by measurements of the rate of oxidation of NADH-linked substrates in isolated mitochondria from muscle or peripheral lymphocytes of patients carrying the mutation, a direct influence of the mutation on the electron transfer activity of Complex I could not be demonstrated (Larsson *et al.*, 1991; Majander *et al.*, 1991). The suggestion has therefore been made (Majander *et al.*, 1991)

that the ND4 subunit may be involved in forming a domain in Complex I necessary for the association of NADH-linked dehydrogenases with this enzyme complex, which would facilitate the NADH transfer between them. Indeed many of these dehydrogenases have been shown to be associated with Complex I, with possible important kinetic implications (Sumegi and Srere, 1984). On the other hand, the lack of enzyme activity observed in the mutant C4 implies a more essential role of the ND4 subunit than just that of facilitating substrate channeling. In fact, the present observation suggest that the ND4 subunit is important for the assembly of the membrane arm of Complex I or the association between the membrane and the matrix arm of the enzyme; therefore, these results would place some structural constraints on any model involving an additional role for this subunit in promoting substrate transfer to the matrix arm. Whether the ND4 subunit is involved in electron transfer or in proton translocation remains to be investigated.

Materials and methods

Cell lines and media

The human cell line VA_2B (Mitchell *et al.*, 1975) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 3 μg/ml 8-azaguanine. The rotenone-resistant clone C4 was grown in the above medium supplemented with 1 μM rotenone. The ϱ°206 cell line (King and Attardi, 1989), a derivative of 143B.TK$^-$ cells, was grown in DMEM supplemented with 5% fetal bovine serum (FBS), 50 μg uridine per ml and 100 μg 5-bromodeoxyuridine (BrDU) per ml.

Mitochondria-mediated transformation

ϱ° cell transformation by cytoplast fusion was carried out as previously described (King and Attardi, 1989). Transformant C4T was isolated in DMEM supplemented with 5% dialyzed FBS and 100 μg of BrdU per ml.

O_2 consumption by digitonin-permeabilized cells

Cells were grown in rotenone-free medium for 24 h prior to the measurement. About 10^7 cells were harvested by trypsinization, resuspended in 1 ml of buffer (20 mM HEPES, pH 7.1, 10 mM $MgCl_2$, 250 mM sucrose), and then 100–150 μg digitonin (1–1.5 μl of a 10% solution in DMSO) were added while mixing; after addition of 9 ml of buffer, the cells were pelleted and resuspended in ~100 μl of buffer. After introduction of the cells into the chamber of a Gilson 5/6 Oxygraph, this was filled up with 1.8 ml of respiration medium (20 mM HEPES, pH 7.1, 2 mM KP_i, 10 mM $MgCl_2$ and 1.0 mM ADP), and then two small aliquots were removed to determine the exact number of cells in the chamber. Substrates and inhibitors were added with Hamilton syringes from 200 × stock solutions (neutralized with NaOH when necessary). Final concentrations were: malic acid, 5 mM; glutamic acid, 5 mM; succinic acid, 5 mM; Na_2 glycerol-3-phosphate, 5 mM; ascorbic acid, 5 mM; TMPD, 0.2 mM; rotenone, 100 nM; antimycin, 4 nM; KCN, 1 mM.

Enzymatic tests

The mitochondrial fraction was isolated from ~0.5 ml of packed cells as described elsewhere (Storrie and Attardi, 1972), resuspended in 8 ml 20 mM Tris, pH 7.5, at 4°C, and sonicated for 40 s in four 10 s pulses separated by 15 s intervals. Mitochondrial membranes were pelleted by centrifugation at 39 000 r.p.m. in a Beckman Ty 65 fixed angle rotor for 60 min and resuspended in the buffer mentioned above at a protein concentration (determined by the Bio-Rad assay) of ~15 mg/ml. The activities were measured, at a protein concentration of 160 μg/ml for the Q_1 reduction and 30 μg/ml for the $Fe(CN)_6$ reduction, in 20 mM Tris, pH 7.5, 1 mM KCN, 100 μM NADH and 50 μM Q_1 (Esai Co., Japan) or 1 mM $Fe(CN)_6$. The reaction was monitored at 275 nm for the reduction of Q_1 (ϵ = 12 250) and at 410 nm for the reduction of $Fe(CN)_6$ (ϵ = 1.0). The NADH:Q_1 oxidoreductase activity of the VA_2B control was reduced by 97% by 100 nM rotenone.

Mitochondrial protein synthesis analysis

Labeling was performed according to Chomyn *et al.* (1991). Samples of 10^6 VA_2B or C4T cells were plated on 5 cm dishes and grown overnight. After washing the cells with methionine-free DMEM, 5 ml of the same medium containing 100 μg/ml emetine were added to each plate. After 10 min incubation, [^{35}S]methionine was added (0.1 mCi for the 2 h pulse, 0.5 mCi for the 20 and 40 min pulses) and the plates were incubated for the times indicated in the figure. The cells were then trypsinized, washed and lysed in 1% SDS. Samples containing 75 μg protein were electrophoresed through an SDS–polyacrylamide (15–20% exponential) gel.

DNA sequencing

The segment of the C4T and VA_2B mtDNAs between positions 10 420 and 12 161, which contains the *ND4L* and the *ND4* genes, was amplified by PCR with appropriate oligonucleotides in two overlapping portions of 989 and 896 bp in length. The PCR products were electrophoresed on an agarose gel in Tris–acetate–EDTA, eluted from the gel and ethanol-precipitated; the purified double-stranded fragments were directly sequenced by the chain termination method (Sanger *et al.*, 1977) using the CircumVent DNA polymerase (NEB) and [^{35}S]ATP. The products of the sequencing reactions were separated on a 0.4 mm 6% polyacrylamide–urea gel.

Immunoprecipitation experiments

As described by Chomyn *et al.* (1985), samples of 3 × 10^6 cells were plated on 10 cm dishes, grown for 22 h in the presence of 40 μg/ml chloramphenicol (CAP) to accumulate nuclear-encoded subunits of Complex I, washed with methionine-free DMEM to remove CAP and methionine, incubated for 10 min in the same medium with 100 μg/ml cycloheximide (CHX), and then exposed for 2 h to 1 mCi [^{35}S]methionine. Thereafter, the CHX was washed away, and the cells were subjected to a 16 h chase in complete unlabeled medium to allow the incorporation of the labeled mtDNA-encoded subunits into Complex I. The cells were trypsinized and washed, and the mitochondrial fraction was isolated and lysed in 0.5% Triton. Samples containing 120 μg protein were incubated at 4°C with 72 μg of γ-globulins from either an antiserum against the bovine 49 kDa subunit (Chomyn *et al.*, 1986), or an anti-COII antiserum (Mariottini *et al.*, 1986), or a normal rabbit serum. Immunocomplexes were bound to formaldehyde-fixed *Staphylococcus aureus* (Zysorbin, Seemed) (Chomyn *et al.*, 1985), spun down and washed several times. The final pellets were dissolved in 1% SDS and separated on an SDS–polyacrylamide (15–20% exponential) gel.

Acknowledgements

These investigations were supported by NIH grant GM-11726 to G.A. and by a Fellowship from the Deutsche Forschungsgemeinschaft to G.H. We thank Anne Chomyn for help in the immunoprecipitation experiments, and Ms Benneta Keeley and Ms Lisa Tefo for technical assistance.

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Received on February 4, 1993; revised on April 20, 1993