The mtDNA-encoded ND6 subunit of mitochondrial NADH dehydrogenase is essential for the assembly of the membrane arm and the respiratory function of the enzyme

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Seven of the ~40 subunits of the mammalian respiratory NADH dehydrogenase (Complex I) are encoded in mitochondrial DNA (mtDNA). Their function is almost completely unknown. In this work, a novel selection scheme has led to the isolation of a mouse A9 cell derivative defective in NADH dehydrogenase activity. This cell line carries a near-homoplasmic frameshift mutation in the mtDNA gene for the ND6 subunit resulting in an almost complete absence of this polypeptide, while lacking any mutation in the other mtDNA-encoded subunits of the enzyme complex. Both the functional defect and the mutation were transferred with the mutant mitochondria into mtDNA-less (ρ0) mouse LL/2-m21 cells, pointing to the pure mitochondrial genetic origin of the defect. A detailed biosynthetic and functional analysis of the original mutant and of the ρ0 cell transformants revealed that the mutation causes a loss of assembly of the mtDNA-encoded subunits of the enzyme and, correspondingly, a reduction in NADH:Q1 oxidoreductase activity in mitochondrial extracts by ~99%. Furthermore, the ND6- cells, in contrast to the parental cells, completely fail to grow in a medium containing galactose instead of glucose, indicating a serious impairment in oxidative phosphorylation function. These observations provide the first evidence of the essential role of the ND6 subunit in the respiratory function of Complex I and give some insights into the pathogenic mechanism of the known disease-causing ND6 gene mutations.

Keywords: frameshift mutation/mouse cell line/mtDNA-less cells/NADH:Q1 oxidoreductase activity/rotenone resistance

Introduction

In most eukaryotic cells, the first respiratory enzyme, NADH dehydrogenase (Complex I), is a multimeric complex, which consists of ~40 subunits in mammalian cells (Walker, 1992), and of ~30 subunits in Neurospora crassa (Weiss et al., 1991). Seven of the subunits of the enzyme from both mammalian cells and N. crassa are encoded in mitochondrial DNA (mtDNA) (Chomyn et al., 1985, 1986; Weiss et al., 1991). In N. crassa, the enzyme has been shown by electron microscopy to have an overall L-shaped structure, with one arm buried in the mitochondrial inner membrane and the other arm protruding into the mitochondrial matrix (Hofhaus et al., 1991), and it is very likely that the same structure applies to the mammalian enzyme. The membrane arm contains all the mtDNA-encoded subunits, while the peripheral arm contains most of the nuclear-encoded subunits and most of the prosthetic groups involved in the NADH:ubiquinone oxidoreductase reactions. Complex I utilizes the energy produced by these reactions to pump protons out of the matrix, thereby generating an electrochemical gradient across the inner membrane that drives ATP synthesis. The available evidence suggests the involvement of the membrane arm and of the mtDNA-encoded subunits in proton translocation.

Despite the fact that the sequence of the mtDNA-encoded subunits was determined some time ago (Anderson et al., 1981; Chomyn et al., 1985, 1986), nothing is known about the function of these subunits, with the exception of ND1. In particular, concerning the latter subunit, it has been shown that it binds rotenone, a specific inhibitor of Complex I (Earley et al., 1987), and interacts 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. The observation that the much simpler bacterial NADH dehydrogenase contains subunits homologous to the mtDNA-encoded subunits (Weidner et al., 1993; Yagi et al., 1993) strongly suggests that these polypeptides have essential roles in the function of Complex I.

The discovery in the past ten years of a variety of mutations affecting the ND4, ND1 or ND6 subunits of NADH dehydrogenase, with resulting Complex I deficiency, in individuals affected by Leber’s hereditary optic neuropathy (LHON) (Wallace et al., 1988; Johns et al., 1992) or by LHON and dystonia (Howell et al., 1991; Jun et al., 1994) has raised a great interest in the function of the mtDNA-encoded Complex I subunits. Furthermore, these findings have made available to the investigators valuable material for correlating structural and functional changes in these subunits. A further significant advance towards the goal of identifying the functional role of these mitochondrial synthesized polypeptides has been the development in this laboratory of a novel selection scheme, based on resistance to rotenone—a specific inhibitor of Complex I—for isolating from cultured human cells mutants affected in one or another of the ND subunits (Hofhaus and Attardi, 1993, 1995). Thus, mutants lacking completely the ND4 subunit (Hofhaus and Attardi, 1993) or almost completely the ND5 subunit (Hofhaus and Attardi, 1995), due to a frameshift mutation in the corresponding gene, have been isolated from the human cell line VA2B and characterized molecularly and biochemically.

In the present work, the approach described above has
been applied to the mouse fibroblast line A9. The analysis of several rotenone-resistant mutants of this cell line has led to the identification of a variant almost completely lacking the ND6 subunit as a result of a frameshift mutation in the corresponding gene, and without any mutation in the other ND genes. Mitochondrial protein synthesis and immunoprecipitation experiments, analysis of respiration and growth capacity in medium containing galactose instead of glucose, and enzymatic assays have shown that the ND6 subunit is essential for the assembly and function of Complex I.

Results

Isolation of mouse cell mutants defective in Complex I-dependent respiration

The approach previously described for the isolation of human cell mutants defective in one or another of the mtDNA-encoded subunits of the respiratory NADH dehydrogenase, which was based on the cell resistance to high concentrations of rotenone (Hofhaus and Attardi, 1993, 1995), was applied for the selection of Complex I-deficient variants of the mouse fibroblast cell line A9. Preliminary experiments indicated that the growth capacity in mass culture of A9 cells was significantly affected by rotenone concentrations as low as 0.6 μM, with complete inhibition by 1.2 μM. Selection of mutants resistant to 1.2 μM rotenone was achieved by exposing cells to increasing concentrations of the drug. After ~3×10^6 A9 cells were treated with 0.8 μM rotenone for ~3 weeks, 21 independent rotenone-resistant clones were picked up, and 11 were subjected to step-wise increasing concentrations of the drug, and were thus adapted to grow in the presence of 1.2 μM rotenone.

An analysis of the respiratory capacity of the 11 A9 rotenone-resistant cell lines revealed that all exhibited a 43–83% decrease, relative to the A9 level, in malate/glutamate-dependent O_2 consumption rate, which usually reflects the rate-limiting activity of Complex I. By contrast, in the same cell lines, the succinate/glycerol-3-phosphate (G-3-P)-driven respiration, that usually reflects the activity of Complex III, and the N,N',N'-tetramethyl-p-phenylenediamine (TMPD)/ascorbate-driven respiration, which reflects the activity of Complex IV, were not significantly affected (Figure 1B). These results pointed to a specific Complex I defect. Furthermore, seven of the ten cell lines exhibited a 14–64% decrease in overall O_2 consumption (Figure 1A).

A quantification of the mtDNA content of the cell lines, carried out using 32P-labeled mouse mtDNA probes, failed to reveal any pronounced difference (~29%) in mtDNA level from the A9 control value, except in clones 4A and 7A. These clones exhibited 51 and 86% increases in mtDNA level, respectively. These increases presumably reflected a compensatory amplification of the mtDNA, a phenomenon which was observed previously (Yoneda et al., 1994). In fact, among the cell lines analyzed, Clone 4A showed the most severe reduction, relative to the parental A9 cells, in both overall respiration (~64%) (Figure 2A) and malate/glutamate-dependent respiration (~83%) (Figure 2B), while clone 7A had an ~52% reduction in glutamate/malate-dependent respiration. Clone 4A was chosen for further investigation, and its analysis is described below.

The Complex I defect of the 4A clone is due to a mtDNA mutation, while its rotenone resistance is nucleus encoded

Because of the dual genetic control, nuclear and mitochondrial, of the structure and function of the respiratory NADH dehydrogenase, mutations in any of the controlling genes of either genome can potentially cause enzyme defects. Similarly, rotenone resistance could in principle be associated with either nuclear or mtDNA mutations (Endicott and Ling, 1989; Hofhaus and Attardi, 1995). In order to investigate the genetic origin of the rotenone resistance and of the respiratory defect in the isolated A9 variants and the relationship between the two phenomena, advantage was taken of the mtDNA-less (ρ0) cell repopulation approach originally developed for human ρ0 cells (King and Attardi, 1989). The ρ0 LL/2-m21 cell line had been previously isolated in the laboratory from the mouse.
LL/2 cell line (as will be described elsewhere). Mitochondria from A9 parental cells or 4A mutant cells were transferred into the mouse \( \rho^0 \) cells by fusion of the latter with a population of predominantly enucleated cells (cytoplasts) derived from A9 or 4A cells, respectively. The cybrids and hybrids were selected for in DMEM medium not supplemented with uridine [a medium in which \( \rho^0 \) cells cannot survive (King and Attardi, 1989)], and containing HAT medium components (hypoxanthine, aminopterin and thymidine) in order to kill any non-enucleated hypoxanthine/guanine phosphoribosyl transferase-deficient A9 or 4A cells.

One transformant was isolated from a \( \rho^0 \)LL/2\( \times \)enA9 fusion (\( \rho^0 \)LL/2\(-\)A9) and 12 transformants, from a \( \rho^0 \)LL/2\( \times \)en4A fusion (4AT1 to 4AT12). These transformants were expected to contain the A9 and, respectively, the 4A mtDNA, but in the \( \rho^0 \)LL/2 nuclear background. Karyotype analysis of the \( \rho^0 \)LL/2\(-\)A9 transformant and five 4AT transformants (4AT1–4AT5) revealed a range of chromosome numbers (45–48) very similar to those of the LL/2 and \( \rho^0 \) LL/2\(-\)m21 cell lines (i.e. ~42), as expected for cybrids. None of the 12 transformants was able to grow in the presence of 1.2 \( \mu \)M rotenone (data not shown). These results suggested that the resistance to rotenone was due to a mutation in a nuclear gene, as previously observed for the rotenone-resistant human cells (Hofhaus and Attardi, 1993, 1995).

As shown in Figure 2A and B, the rates of overall respiration and, respectively, of malate/glutamate-dependent respiration were significantly lower in the A9 than in the LL/2 parental cell. That this difference was due to some defect(s) in the A9 cell nuclear genes controlling the structure and function of Complex I was strongly suggested by the observation that the A9-derived mitochondrial transformant of \( \rho^0 \)LL/2\(-\)m21 exhibited both overall and malate/glutamate-dependent respiration rates comparable with those of LL/2 cells (Figure 2A and B). Accordingly, it seemed appropriate to compare the \( \text{O}_2 \) consumption rates measured in the \( \rho^0 \)LL/2 transformants derived from the 4A clone with the rates observed in the \( \rho^0 \)LL/2\(-\)A9 transformant. It was found that, in contrast to the rotenone resistance, both the decrease in overall respiratory capacity and the specific Complex I defect of the 4A clone cells were transferred into \( \rho^0 \) cells, together with their mtDNA (Figure 2A and B). In fact, a preliminary screening of the 12 isolated transformants showed a general decrease in overall respiratory capacity to between 35 and 56% of the \( \rho^0 \)LL/2\(-\)A9 level, as well as a specific

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**Fig. 2.** Total respiration rate (A) and activities of the enzymes of the mitochondrial respiratory chain (B, C) in rotenone-resistant clone 4A, parent cell lines A9 and LL/2 and \( \rho^0 \)LL/2\(-\)m21 cell transformants (\( \rho^0 \)LL/2\(-\)A9 and 4AT1 to 4AT5). For details, see legend of Figure 1. (C) shows the glutamate/malate-driven respiration rates normalized to succinate/G-3-P-driven respiration rates.
decrease in malate/glutamate-dependent respiration rate to between 14 and 29% of the \(U^0\)LL/2-A9 transformant rate. The data for five of these transformants (4AT1–4AT5) are shown in Figure 2A and B. An explanation for the much lower decrease in the \(U^0\)LL/2 4A-derived transformants, relative to the wild-type LL/2-A9 transformant, in overall respiratory capacity as compared with the decrease in malate/glutamate-dependent respiration was provided by the interesting observation that the succinate/G-3-P-dependent respiration rate was increased, in all five mutant transformants analyzed, relative to the \(U^0\)LL/2-A9 rate, by between 48 and 115%, while the TMPD/ascorbate-dependent respiration rate was increased by between 67 and 86%. Consistent with this observation is the finding that 52–56% of the overall \(O_2\) consumption in two mutant transformants analyzed was sensitive to rotenone, in contrast to 86–90% in the LL/2 parental line and the wild-type LL/2-A9 transformant.

An analysis of the mtDNA contents of the transformants showed an increase, relative to the LL/2 level of 77, 12, 90, 67 and 98% in 4AT1, 4AT2, 4AT3, 4AT4 and 4AT5, respectively. Therefore, it seems plausible that the increase in succinate/G-3-P- and TMPD/ascorbate-dependent respiration rates reflected a compensatory increase in mtDNA content/cell, as has been observed previously (Yoneda et al., 1994). According to this interpretation, the rate of malate/glutamate-dependent respiration in the five transformant cell lines, normalized to their mtDNA content, would be 8–15% of the \(U^0\)LL/2-A9 level. Similarly, after normalization to the succinate/G-3-P-dependent respiration rate, the malate/glutamate-driven respiration rates in the transformant lines would be 7–19% of the \(U^0\)LL/2-A9 value (Figure 2C).

The transfer of the Complex I defect into \(U^0\) cells with mtDNA excluded the possibility of a deficiency in the transport of the substrates or in the activity of the corresponding dehydrogenases as being responsible for the low Complex I activity, and pointed strongly to a mutation in one of the genes encoding subunits of NADH dehydrogenase. This interpretation was confirmed by an analysis of the activity of Complex I in partially purified mitochondrial membranes, carried out using NADH and a water-soluble ubiniquinone analog \((Q_1)\) to circumvent any transport problems, carried out using NADH and a water-soluble flavoprotein fragment, which contains the mtDNA-encoded subunits of NADH dehydrogenase. It has been shown that, in \(N\).\textit{crassa}, the flavoprotein fragment is assembled independently of the membrane fragment, which contains the mtDNA-encoded subunits (Weiss et al., 1991), and the same is probably true in mammalian cells (Hofhaus and Attardi, 1993, 1995). Therefore, this activity was used to correct for differences in mitochondria content among the crude mitochondrial membrane preparations from different clones. The corrected activities are shown in Table I. It appears that the normalized NADH:Q1 oxidoreductase activity was reduced in 4A to 1.2% of the A9 activity and in 4AT1 to ~0.5% of the LL/2 activity.

### Table I. Measurements of NADH:Q1 and NADH:K3Fe(CN)6 oxidoreductase activities in mitochondrial membranes isolated from A9, 4A, LL/2 and 4T1 cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Average nmol/min/mg *</th>
<th>NADH:Q1 oxidoreductase</th>
<th>NADH:K3Fe(CN)6 oxidoreductase</th>
<th>NADH:Q1/NADH:K3Fe(CN)6 (×10³)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9</td>
<td>10.6 (± 2.2)</td>
<td>1710 (± 57)</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>4A</td>
<td>0.109 (± 0.025)</td>
<td>1425 (± 71)</td>
<td>0.076 (~1.2%)</td>
<td></td>
</tr>
<tr>
<td>LL/2</td>
<td>14.3 (± 1.2)</td>
<td>1185 (± 240)</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>4AT1</td>
<td>0.108 (± 0.017)</td>
<td>1965 (± 14)</td>
<td>0.055 (~0.5%)</td>
<td></td>
</tr>
</tbody>
</table>

| bValues in parentheses represent percentage of normalized NADH:Q1 oxidoreductase activity in 4A relative to A9 and in 4AT1 relative to LL/2. | |

**Enzyme activities are expressed in nmol/min/mg protein.** The values for NADH:Q1 represent the total activity, with >98% being rotenone-sensitive.

**aDeterminations were made in duplicate. Values in parentheses are 2 SE.**

**The 4A cell line and derived \(U^0\) LL/2-m21 transformants harbor a frameshift mutation in the mitochondrial ND6 gene**

In order to obtain some indication of the possible site of the mutation responsible for the Complex I defect, the mitochondrial translation products were labeled with \(^{35}\)S-methionine for 20 min in the presence of emetine, to inhibit cytoplasmic protein synthesis. The individual mitochondrially synthesized polypeptides were identified by comparison of the electrophoretic pattern of the mouse translation products with the pattern of the human \((\text{Chomyn et al., 1991})\) and rat (Loguercio-Polosa and Attardi, 1991) mitochondrial protein synthesis products. As can be seen in Figure 2, in the patterns from both the original mutant 4A (Figure 3A) and the transformant 4AT1 (Figure 3B), the only significant difference from the wild-type patterns of A9 and LL/2 cells was the near-complete absence of the polypeptide identified from its electrophoretic mobility as the ND6 gene product. There were no additional bands in the patterns of the mutant cell lines that could represent truncated or otherwise abnormally migrating polypeptides.

As shown in Table I, the NADH:Q1 oxidoreductase activity of the 4A and 4AT1 mitochondrial membranes was reduced to ~1 and ~0.8% of the A9 and LL/2 activity, respectively. By contrast, the NADH:K3Fe(CN)6 oxidoreductase activity did not appear to be significantly affected in either the 4A or the 4AT1 membrane preparation. It has been shown that, in \(N\).\textit{crassa}, the flavoprotein fragment is assembled independently of the membrane fragment, which contains the mtDNA-encoded subunits (Weiss et al., 1991), and the same is probably true in mammalian cells (Hofhaus and Attardi, 1993, 1995). Accordingly, it would be expected that the NADH:K3Fe(CN)6 oxidoreductase activity would not be affected by a mutation in a mtDNA-encoded Complex I subunit gene, as was indeed shown previously for several mtDNA mutations in rotenone-resistant human clones (Hofhaus and Attardi, 1993, 1995). Therefore, this activity was used to correct for differences in mitochondria content among the crude mitochondrial membrane preparations from different clones. The corrected activities are shown in Table I. It appears that the normalized NADH:Q1 oxidoreductase activity was reduced in 4A to 1.2% of the A9 activity and in 4AT1 to ~0.5% of the LL/2 activity.

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On the basis of these observations, the polymerase chain reaction (PCR)-amplified ND6 gene was sequenced by the chain termination method (Sanger et al., 1977) in both the wild-type cell lines A9 and LL/2 and the mutant cell lines 4A (4AT1 and 4AT2). As shown in Figure 4 for the 4A cell line, an additional C residue, as compared with the A9 sequence, was found in a stretch of six C at positions 13 879–13 884 within the ND6 gene (Bibb et al., 1981). The same insertion was found in the ND6 sequence of the 4AT1 and 4AT2 transformants, when compared with the LL/2 sequence. The mouse ND6 gene encodes a 172 amino acid-long polypeptide. The C insertion introduces a frameshift starting from the 63rd amino acid, and...
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**Fig. 3.** Electrophoretic patterns of newly synthesized mitochondrial translation products of A9 and 4A cells (A) and of LL/2 and 4AT1 cells (B). The cells were exposed to \[^{35}S\]methionine for 20 min in the presence of 100 \(\mu\)g/ml emetine. COI, COII and COIII: subunits I, II and III of cytochrome c oxidase; ND1, ND2, ND3, ND4, ND4L, ND5 and ND6: subunits 1, 2, 3, 4, 4L, 5 and 6 of respiratory chain NADH dehydrogenase; A6 and A8: subunits 6 and 8 of the H^+\textungequal{}ATPase.

**Fig. 4.** Partial sequence of ND6 gene (non-coding strand) from A9 and 4A cells, showing the insertion of a C residue in the six-C stretch at positions 13 879 to 13 884 (Bibb et al., 1981). Note the insertion in the sequence of a relatively small fraction of mtDNA molecules from 4A cells of a second C at position 13 885, which appears as a small C peak under a major T peak.

Creating a stop codon 51–53 base pairs downstream of the C stretch, resulting in a 79 amino acid-long truncated polypeptide. The failure to detect the truncated product at the expected position (close to A8) in the electrophoretic patterns of newly synthesized proteins from the mutant cell lines (Figure 3) suggests that this is an unstable product. In order to exclude the occurrence of any other mtDNA mutation in the 4A cell line, the genes for all the other six mtDNA-encoded Complex I subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) were completely sequenced and no mutation was found.

In the protein synthesis patterns from the 4A (Figure 3A) and 4AT1 cell lines (Figure 3B), a very light band can be seen at approximately the position of the ND6 product. However, no evidence of wild-type ND6 gene was observed in the DNA sequence patterns from the mutant cell lines (Figure 4 and data not shown). A more sensitive experiment, involving allele-specific termination of primer extension (Hofhaus and Attardi, 1995), was carried out to detect the presence of wild-type mtDNA in the mutant cell lines. As can be seen in Figure 5, in A9 wild-type cells, there is no detectable mutant mtDNA. In the five 4A transformants tested (4AT1–4AT5), the mutation appears to be homoplasmic or nearly homoplasmic (Figure 5 and data not shown). Interestingly, the primer extension pattern from the original 4A mutant cells revealed, besides a C insertion present in ~80% of the mtDNA molecules, a second C insertion in ~20% of the molecules. This second C insertion is also recognizable in the 4A DNA sequence pattern of Figure 4 as a small C peak under the T peak at position 13 885.

**Fig. 5.** Quantification of C insertions in the ND6 gene of 4A, 4AT1 and 4AT2 cells. Allele-specific termination of primer extension was carried out, and the products were separated on a 20% polyacrylamide–7 M urea sequencing gel. PR, unextended primer; WT, wild-type product; MT, mutant product with one C insertion; MT*, mutant product with two C insertions.

The mtDNA-encoded subunits of the 4A and \(\rho^0\) transformant cell lines are not assembled into Complex I

In order to investigate whether, in the cell lines carrying a frameshift mutation in ND6, the assembly of the other mtDNA-encoded subunits is affected, immunoprecipitation experiments were carried out using antibodies against the cytoplasmically synthesized 49 kDa iron–sulfur subunit, located in the peripheral arm of the bovine Complex I, which cross-react extensively with the human homologous subunit (Cleeter and Ragan, 1985), and antibodies against the C-terminal synthetic heptapeptide of the mtDNA-encoded subunit ND4L of human Complex I, which is located in the membrane arm (Mariottini et al., 1986). Both types of antibodies had previously been shown able
Mouse cell mutant deficient in ND6 subunit of Complex I

Fig. 6. Electrophoretic patterns of SDS mitochondrial lysates from [35S]methionine pulse-labeled LL/2 cells (C) or pulse–chased A9, 4A, LL/2 and 4AT1 cells (A and B), and of immunoprecipitates obtained by incubating 0.5% Triton X-100 mitochondrial lysates from pulse–chased cells with γ-globulins from an antiserum against the bovine 49 kDa subunit (lanes 49) or an antiserum against the human ND4L subunit (lanes ND4L), or from rabbit normal serum (lanes NS) (B and C). For details, see Materials and methods.

to precipitate the whole human Complex I from a 0.5% Triton X-100 mitochondrial lysate (Chomyn et al., 1985, 1986). Because of the conservation of the 49 kDa subunit among mammalian species (Cleeter and Ragan, 1985) and the 100% homology between the C-terminal heptapeptides of the human and the mouse ND4L subunits (Anderson et al., 1981; Bibb et al., 1981), it was expected that both types of antibodies would precipitate the mouse Complex I, and thus reveal whether the mtDNA-encoded subunits of the mutant cell lines are assembled into Complex I.

For the immunoprecipitation experiments with the anti-49 kDa and anti-ND4L antibodies, 4A and 4AT1 cells, which had been grown for 22 h in the presence of 40 μg/ml of the mitochondrial protein synthesis inhibitor chloramphenicol, were labeled for 2 h with [35S]methionine in the presence of 100 μg/ml of the reversible inhibitor of cytoplasmic protein synthesis, cycloheximide. Cells were then chased for 19 h in complete unlabeled medium in the absence of the inhibitor, in order to allow the incorporation of the labeled mtDNA-encoded subunits into the complexes (see Materials and methods for the rationale behind the above protocol).

Figure 6A and C (lanes 6 and 7) show the electrophoretic patterns of samples of SDS mitochondrial lysates from pulse–chased A9 and 4A cells and, respectively, LL/2 and 4AT1 cells. One can recognize the general mitochondrial protein synthesis pattern obtained from the short-pulse-labeled cells (compare the pulse–chase patterns with the pulse patterns of Figure 3 and Figure 6C, lane 1), with some extra bands, especially in the high molecular weight region, which were more abundant in the A9 and 4A cell patterns. These extra bands represent cytoplasmic proteins labeled during the chase (Chomyn et al., 1986). In the 4A (Figure 6A) and 4AT1 patterns (Figure 6C), the labeled mtDNA-encoded subunits of Complex IV (COI, COII and COIII), Complex III (CYTb) and H⁺-ATPase (A6 and A8) were present in approximately the same amounts as in the A9 pattern (Figure 6A) and LL/2 pattern (Figure 6C), whereas the recognizable mtDNA-encoded subunits of NADH dehydrogenase (ND1, ND2 and ND3) were strongly decreased. In view of the evidence presented above that the rate of labeling of the mtDNA-encoded subunits of Complex I is very similar in A9 and 4A cells and in LL/2 and 4AT1 cells (Figure 3), the above results pointed to a considerably lower stability of these subunits in mutant cells, resulting possibly from defective assembly.

More direct evidence indicating the lack of assembly of all or most of the mtDNA-encoded subunits of Complex I came from the immunoprecipitation experiments. As shown in Figure 6B, when a 0.5% Triton X-100 mitochondrial lysate from A9 cells was incubated with γ-globulins from an antiserum against the bovine 49 kDa subunit, the antibodies precipitated several mtDNA-encoded subunits, in particular, clearly recognizable, ND1, ND2 and ND3 (ND4 appears as a broad diffuse band overlapping the sharp non-specific band). By contrast, no clear evidence of these subunits could be seen in the corresponding immunoprecipitate obtained with normal serum γ-globulins. Furthermore, no mtDNA-encoded subunits were immunoprecipitated from a 0.5% Triton X-100 mitochondrial lysate from 4A cells by γ-globulins from the anti-49 kDa subunit antiserum nor by normal serum γ-globulins.
Fig. 7. Growth curves of A9, 4A, LL/2, 4AT1 and 4AT2 cells in glucose-containing DMEM and in galactose-containing DMEM. Both floating and attached cells were counted. After 3–4 days, the mutant cells in galactose medium were all floating.

It has been shown that cells deficient in respiratory function are severely impaired in their growth capacity in medium containing galactose instead of glucose (Hayashi et al., 1991; Robinson et al., 1992; Guan et al., 1996). Figure 7 compares the growth curves in glucose and galactose media of the mutant 4A and of its parental line A9, and the growth curves in the same media of transformants 4AT1 and 4AT2 and of the LL/2 parent of their recipient p0 cell line (LL/2-m21). It appears that the 4A cells grew well in glucose-containing medium [initial doubling time (DT) = 24.5 h], although at a slower rate than the parental A9 cells (initial DT = 15.6 h). Similarly, the 4AT1 and 4AT2 cell lines grew well in glucose medium (initial DT = 15.4 h and 14.2 h, respectively), although they tended to slow down before reaching a concentration of $10^7$ cells/ml. It appeared that the ND6 frameshift mutation-carrying cells had already become adapted to a predominantly glycolytic ATP production during the selection process, so as to be independent of the oxidative phosphorylation function to a considerable extent (Donnelly and Scheffler, 1976). By contrast, in medium in which glucose was substituted by galactose, that is not utilized efficiently by mammalian cells as a glycolytic substrate and in which these cells are forced to rely almost exclusively on oxidative phosphorylation for ATP production (Chu et al., 1972), the mutant cell lines failed to grow (Figure 7) and died (after 3–4 days, they were all floating). In contrast, the A9 and LL/2 cells showed in this medium a growth rate only slightly reduced, as compared with that in glucose medium. Interestingly, the LL/2 cells stopped growing in the galactose medium when their density reached approximately $10^7$/ml. This behavior, which was observed in repeated experiments (data not shown), is suggestive of an apoptotic phenomenon.

Discussion

Genetic basis of mutant isolation

In the present work, the application to a cultured mouse cell line of the method previously developed for the isolation of human cell mutants affected in any of the mtDNA-encoded subunits of the respiratory NADH dehydrogenase (Hofhaus and Attardi, 1993, 1995) has led
to the isolation of the first natural mutant cell line affected in the ND6 subunit. The results reported here have fully confirmed the power of this approach, which is based on the selection of variants resistant to high concentration of rotenone, a specific inhibitor of Complex I. Of 11 randomly chosen clones that had been adapted to grow in the presence of 1.2 μM rotenone, all showed a specific decrease in malate/glutamate-dependent O₂ consumption. As previously shown for the rotenone-resistant human cell mutants, the rotenone resistance of the mouse mutants was not transferred with mitochondria into mouse p0 cells, while their respiratory defect was, clearly indicating the nuclear origin of the mutation responsible for the drug resistance and the mtDNA origin of the mutation underlying the putative Complex I deficiency. In particular, as suggested for the selection mechanism operating in the isolation of the human cell mutants defective in Complex I, it seems very likely that, also in the mouse system investigated here, the rotenone resistance was due to a nuclear gene mutation pre-existing in A9 cells, and possibly involving hyperepression or amplification of the genes encoding the P-glycoproteins, the cell membrane-associated energy-dependent drug efflux pumps responsible for the multi-drug resistance phenotype (Endicott and Ling, 1989). Similarly, the Complex I deficiency of the selected rotenone-resistant mutants could reflect the occurrence in these of pre-existing mtDNA mutations affecting one or the other of the genes coding for subunits of the enzyme. As suggested for the human cell mutants, it is possible that a combination of a replicative advantage of the mutant mtDNA molecules, such as previously described for mtDNA carrying the mutation associated with the MELAS encephalomyopathy (Yoneda et al., 1992), and of the progressive adaptation to a glycolytic metabolism accompanying the mutation amplification may have led to the selection of cells which the mutation had made Complex I-independent and, therefore, insensitive to the relatively small amounts of rotenone leaking into the cells.

In view of the fact that most of the mtDNA mutations in protein-coding genes which are associated with diseases in man occur in genes encoding subunits of Complex I, the successful application to mouse cell lines of the rotenone resistance selection scheme for isolation of mtDNA mutations affecting one or the other of these subunits introduces a valuable approach towards the goal of constructing mouse models of such mutations.

An interesting observation in the present work has been that the mutant mtDNA of the A9 rotenone-resistant cell line most severely affected in its malate/glutamate-dependent respiration (clone 4A) consisted of a mixture of ~80% molecules carrying an insertion of a C residue in a row of six C residues and ~20% molecules carrying two insertions of a C residue in the same stretch. Both insertions created stop codons downstream and caused the formation of unstable, prematurely terminated translation products. In another rotenone-resistant A9 cell derivative, an insertion of an A residue in a stretch of eight A residues at positions 9818–9825 in the sequence encoding the variable loop of the tRNAArg in ~60% of the mtDNA molecules has recently been detected (unpublished observations). These findings suggest that in mouse mtDNA, as previously found in human mtDNA (Hofhaus and Attardi, 1993, 1995), homopolymeric stretches may be hot spots for nucleotide insertions because of stuttering of the mitochondrial DNA polymerase. In mouse mtDNA there are 25 homopolymeric stretches of six or more identical residues (Bibb et al., 1981), and one could predict that a significant proportion of the Complex I-deficient mutants isolated among the rotenone-resistant mouse clones will turn out to carry frameshift mutations. In fact, in the previous analysis of the rotenone-resistant human cell variants, four of the six Complex I-deficient mutants investigated exhibited frameshift mutations in their mtDNA, which produced prematurely terminated polypeptides (Hofhaus and Attardi, 1993, 1995). It is intriguing, in this connection, that no frameshift mutation has so far been detected among the mtDNA mutations associated with diseases in man. A plausible interpretation of this difference between the in vitro systems investigated previously and in the present work and the in vivo situation is that frameshift mutations within protein-coding genes are likely to be lethal. It should be mentioned that, in spite of the preferential occurrence of frameshift mutations in mtDNA of the cell lines subjected to rotenone selection, variants carrying base substitutions have also been detected among the rotenone-resistant human cell mutants (Hofhaus and Attardi, 1995) and mouse cell mutants (Y.Bai and G.Attardi, unpublished results).

**Physiological effects of the ND6 frameshift mutation**

The absence of any mutation, other than the C insertion, in the mtDNA genes of the 4AT1 transformant which encode subunits of NADH dehydrogenase and the lack in the original clone 4A or in the transformants of any change in the rate or pattern of mitochondrial protein synthesis, apart from the strong decrease in the intensity of the ND6 subunit band, have clearly indicated that the physiological changes observed in these cell lines are only due to the ND6 gene frameshift mutation. The trace amounts of a band migrating as ND6, which were visible in some of the protein synthesis patterns, possibly reflected a residual synthesis of this subunit. However, no evidence of any residues of wild-type gene was detected by DNA sequencing or by allele-specific termination of primer extension in the mtDNA of clone 4A and of the five analyzed transformants.

Polarographic analysis of the five 4A p0 cell transformants has revealed a drastic decrease in malate/glutamate-dependent respiration rate to 8–15% of that measured in the control p0 cell transformant (p0LL/2-A9), after normalization for differences in mtDNA content. More dramatically, enzymatic assays of NADH:Q1 oxidoreductase activity in sonicated mitochondrial membranes have detected in 4A and 4AT1 cells only 0.5–1.2% of the activity measured in the parental lines. It is very likely that the observed difference between the decrease in enzyme activity and the decrease in O₂ consumption rate reflects a difference in sensitivity of the methods used. In any case it is clear that very little, if any, Complex I activity is present in cells carrying the ND6 gene frameshifting mutation.

As to the mechanism underlying the loss of enzyme activity caused by this mutation, the Complex I immunoprecipitation tests utilizing antibodies against a Complex
I subunit (49 kDa) located in the peripheral arm or against a mitochondrially synthesized subunit of the membrane arm (ND4L) have clearly indicated a defective assembly of the mtDNA-encoded subunits of the enzyme complex. The metabolic instability of these subunits, as revealed by the pulse–chase experiments, presumably results from their failure to assemble in the absence of ND6. Previously, the essential role for the assembly of the membrane arm of another subunit of the enzyme, the ND4 subunit, has been demonstrated in human cells (Hofhaus and Attardi, 1993). In contrast, the absence of the ND5 subunit has been shown not to affect the assembly of the membrane arm in human cells (Hofhaus and Attardi, 1995) and in mouse cells (Y. Bai and G. Attardi, unpublished results).

As previously observed in human cells which lacked the ND4 subunit due to a frameshifting mutation in the corresponding gene, the NADH:K3Fe(CN)_6 oxidoreductase activity of the mitochondrial membranes was unaffected in the five transformants analyzed which carried the ND6 gene frameshift mutation. This result fully confirms the evidence from _N. crassa_ (Weiss et al., 1991) and human cells (Hofhaus and Attardi, 1993, 1995), which indicated that the assembly of the flavoprotein fragment is independent of that of the membrane fragment. Therefore, it seems possible that the entire peripheral arm of the enzyme is assembled in mutant cells.

**Relevance for pathogenic ND6 mutations**

Still very little is known about the role of the ND6 subunit in the electron-transfer and proton-pumping activities of Complex I. Useful information concerning the role of this subunit in the respiratory function of the enzyme is coming from an analysis of the effects of mutations of the ND6 gene which are associated with human diseases. Indeed, among the mtDNA-encoded subunits of Complex I, the ND6 is one of the most frequently affected by primary mutations associated with LHON. In particular, a mutation at position 14 484 has been found to be associated with pure LHON (Johns et al., 1992), or, in conjunction with a 4160 ND1 mutation, with LHON and dystonia (Howell et al., 1991). A mutation at position 14 459 has likewise been found in families exhibiting LHON and/or dystonia (Jun et al., 1994). Both the 14 484 and the 14 459 mutations, which have arisen independently on multiple occasions, have been shown to cause Complex I deficiencies (Oostra et al., 1995; Jun et al., 1996), with a resulting decrease in ATP synthesis (Oostra et al., 1995). A kinetic analysis of the 14 459 mutation-carrying Complex I has revealed a reduction in the _V_\text{max} of the enzyme without any change in the _K_\text{m}. Furthermore, evidence has been reported suggesting that the 14 459 mutation may alter the coenzyme Q-binding site of Complex I (Jun et al., 1996). Other mutations at positions 14 482, 14 498, 14 568 and 14 596 in the ND6 gene have also been found to be associated with LHON (De Vries et al., 1996; Wissinger et al., 1997; Howell et al., 1998). It is remarkable that all the mutations listed above occur in a stretch of <50 amino acids near the N-terminus of the protein, which is the most conserved portion of the ND6 polypeptide, and appear to reside in a single membrane-spanning domain (Fearnley and Walker, 1992). An advantage of the selection approach applied in this work to mouse cultured cells, as compared with the analysis of available disease-causing mutations, is that it allows the isolation of knock-out mutants which may reveal in a more dramatic form the functional role of a given subunit of the enzyme (Hofhaus and Attardi, 1993). In this connection, an interesting possibility suggested by the results presented in this work is that the Complex I deficiencies caused by the pathogenic ND6 mutations result from defective assembly of the membrane arm, and that the membrane-spanning domain mentioned above plays a crucial role in this assembly.

**Materials and methods**

**Cell lines and media**

All the cell lines used in the present work were grown in monolayer culture. The cell line A9 (ATCC CCL-1.4) is a derivative of the L mouse fibroblast cell line deficient in pyroxanthine/guanine phosphoribosyl transferase and is thus resistant to 8-azaguanine and 6-thioguanine (Littlefield, 1963) and incapable of growing in HAT medium (Freshney, 1994). This cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 3 μg/ml 8-azaguanine. The rotenone-resistant clone 4A was grown in the above medium supplemented with 1.2 μM rotenone. The mouse cell line LL/2 (Bertram and Janik, 1980; ATCC CRL-1642) was grown in DMEM supplemented with 10% FCS. The mtDNA-less p° LL/2-m21 cell line, a derivative of LL/2 cells (see below), was grown in DMEM supplemented with 10% FCS and 50 μg of uridine per ml.

**Mitochondria-mediated transformation**

The mtDNA-less p° LL/2-m21 cell line was isolated by a modification of a method described earlier (Desjardins et al., 1985; King and Attardi, 1989), which involved treatment of LL/2 cells with high concentrations of ethidium bromide, as will be reported in detail elsewhere. p° cell transformation by cytoplast fusion was carried out as described (King and Attardi, 1989), by fusing A9 or 4A mutant cells, which had been enucleated by centrifugation in the presence of cytochalasin B, with p° LL/2-m21 cells in the presence of 40% polyethylene glycol 1500 (BDH). Mitochondrial transformants were isolated in DMEM supplemented with HAT medium components (hypoxanthine, aminopterin and thymidine) (Freshney, 1994) and 10% FCS. The transformant clones were subsequently cultured in DMEM medium with 10% FCS.

**Chromosome analysis**

To distinguish the cybrids from hybrids among the p° LL/2-m21 transformants, cells were arrested in metaphase by treatment with 0.05 μg/ml colchicine for 3 h. Karyotype analysis was carried out as described previously (Mitchell and Attardi, 1978).

**O_2 consumption measurements**

The medium of the cell lines to be analyzed was changed with fresh medium (rotenone-free in the case of the original mutant cell lines) 24 h before the measurements. _O_2 consumption determination in intact cells was carried out as described previously (King and Attardi, 1989). For measurements of _O_2 consumption in digitonin-permeabilized cells (Hofhaus et al., 1996), ~5×10^6 cells were resuspended in 1 ml of buffer (20 mM HEPES pH 7.1, 10 mM MgCl₂, 250 mM sucrose), and then 100 μg of digitonin (1 μl of a 10% dimethylsulfoxide solution) in 1 ml buffer was added. After incubation for 1 min at room temperature, the cell suspension was diluted with 8 ml buffer. The cells were rapidly pelleted, and resuspended in respiration buffer (20 mM HEPES pH 7.1, 250 mM sucrose, 2 mM K-phosphate, 10 mM MgCl₂, 1.0 mM ADP). The measurements were carried out in chambers of a 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: 5 mM malate; 5 mM glutamate; 5 mM succinate; 5 mM glycerol-3-phosphate; 10 mM ascorbate; 0.2 mM TMPD; 100 nM rotenone; 20 nM antimycin; and 1 mM KCN.

**Enzymatic tests**

The mitochondrial fraction of the desired cell type was isolated from ~0.5 ml packed cells as described previously (Storrie and Attardi, 1972), resuspended in 8 ml of 50 mM Tris pH 7.5 (4°C), and sonicated with a Branson sonifier for 40 s (four 10-s pulses separated by 15-s intervals) on ice. Mitochondrial membranes were pelleted by centrifugation at 39 000 r.p.m. in a Beckman Ty65 fixed angle rotor for 60 min, and...
resuspended in 500 μl of the above buffer. The oxidoreductase activities were measured, at a protein concentration of 85 μg/ml for Qr reduction and 20 μg/ml for K2Fe(CN)6 reduction, in medium containing 20 mM Tris pH 7.5 (25°C), 1 mM KCN, 100 μM NADH and either 50 μM Qr (Eisai Co., Japan) or 1 mM K2Fe(CN)6. The reaction was monitored by absorbance measurements at 275 nm for the reduction of Qr (ε = 12 250 M−1cm−1) and at 410 nm for the reduction of K2Fe(CN)6 (ε = 10 000 M−1cm−1). The NADH:Qr oxidoreductase activity of the A9 and LL/2 control samples was >98% sensitive to 100 nM rotenone.

**Growth measurements**

Multiple identical samples of 2×105–2×106 cells (in different experiments) were grown for 7 days on 10 cm Petri dishes in the appropriate medium [DMEM, which contains 4.5 mg/ml glucose and 0.11 mg/ml pyruvate, or DMEM lacking glucose and containing 0.9 mg/ml galactose and 0.5 mg/ml pyruvate (Hayashi et al., 1991), both supplemented with 10% dialyzed FCS], and counted on a daily basis. Both floating and attached cells were counted.

**Mitochondrial protein synthesis analysis**

To measure the rate of mitochondrial protein synthesis, pulse-labeling experiments with [35S]methionine were performed according to Chomyn (1996). Samples of 2×106 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 cytoplasmic translational inhibitor emetine. Thereafter, [35S]methionine [0.2 μCi (1175 Ci/mmol)] was added, and the cells were incubated for 20–30 min. To test the stability of the mitochondrial translational products, pulse-chase labeling experiments were performed (Chomyn, 1996). Samples of 2×105 cells were plated on 10 cm Petri dishes, and grown for 22 h in the presence of 40 μg/ml of the mitochondrial translational inhibitor chloramphenicol (CAP), in order to allow accumulation of the nuclear-encoded subunits of the ox-phos complexes and, therefore, facilitate the incorporation into these complexes of the mtDNA-encoded subunits synthesized after the removal of CAP. Labeling was carried out as described above, except that emetine was replaced with cycloheximide, and incubation time with [35S]methionine was extended to 2 h; thereafter, the cells were washed and subjected to a 19 h chase in complete unlabeled medium in the absence of cycloheximide to allow incorporation of the labeled mtDNA-encoded subunits into the complexes. The labeled cells were trypsinized, washed and lysed in 1% sodium dodecyl sulfate (SDS). Samples containing 30–50 μg of protein were electrophoresed through an SDS–polyacrylamide gel (15–20% exponential gradient).

**Immunoprecipitation experiments**

Approximately 5×106 pulse–chase labeled cells were pelleted, and mixed with 2×106 unlabeled cells of the same type to yield ~0.5 ml of packed cells. The mitochondrial fraction was isolated from these cells by homogenization and differential centrifugation, and lysed with 0.5% Triton X-100 (Mariotti and Chomyn, 1996). Samples of 120 μg protein were incubated at 4°C with 72 μg t-globulins from an antiserum against the bovine 49 kDa subunit (Chomyn et al., 1986), or from an antiserum against the C-terminal heptapeptide of the human mtDNA-encoded subunit ND4L (Mariottini et al., 1986), both supplemented with 10% diazyed FCS, and counted on a daily basis. Both floating and attached cells were counted.

**DNA analysis**

For mtDNA sequencing, total DNA samples were isolated from cells with an Applied Biosystems 340A DNA extractor, and then subjected to PCR amplification of the ND6 gene, using the primers ND6-5'-2 and ND6-3'-2 (see below). DNA sequencing of the purified PCR products was carried out by the ABI PRISM™ Dye Terminator Cycle Sequencing Core (Perkin-Elmer), using the primers ND6-5'-2 and ND6-3'-2 (see below).

The mtDNA content of the various cell lines was determined, at the time of O2 consumption measurements, by DNA transfer hybridization of total cell DNA carried out with a slot–blot apparatus (Yoneda et al., 1994). For this purpose, samples of 2×106 cells were lysed in PCR buffer containing 1% NP-40 and 100 μg proteinase K/ml, incubated for 1 h at 55°C and then for 10 min at 95°C, blotted in triplicate and hybridized with a mixture of three mouse mtDNA-specific probes [plasmid MumX1.9, containing the mouse mtDNA sequence from position 8984 to 10 907, plasmid MumX5.1, containing the mtDNA sequence from position 10 907 to 15 973, and plasmid MumX7.6, containing the mtDNA sequence from position 953 to 8529 (Bibb et al., 1981)]. In view of the very limited variability in chromosome number per cell which has been found in the rho0L2-derived transformant cell lines analyzed (45–48 in rho0L2-A9 and five rho0L2–4A transformants), the normalization of the hybridization data to an equal number of cells, according to the protocol described above, is substantially equivalent to a normalization to an equal amount of nuclear DNA.

Quantification of the mtDNA frameshifting mutation (a C insertion in a row of six C residues at positions 13 879–13 884) was carried out by allele-specific termination of primer extension (Hofhaus and Attardi, 1995). For this purpose, the PCR-amplified fragments were used as templates, and the 5'-end 32P-labeled ND6PE oligodeoxynucleotide (see below) was used as a primer in a 1:1 molar ratio. Nucleoside triphosphate concentrations were 100 μM for dCTP and 300 μM for ddTTP. The mixtures were heated to 95°C for 3 min, and then cooled to 45°C for 5 min, to 37°C for 10 min, and finally chilled on ice. After addition of 1 μl of 1:3 diluted Sequenase (Amersham), the mixtures were incubated at 45°C for 5 min. The reaction products were denatured and separated on a 50 cm-long 20% polyacrylamide–6 M urea gel. Quantification of the intensity of the bands was done by using a PhosphorImager (Molecular Dynamics) and the IMAGE-QUANT program. The sequences of the primers used were:

**Acknowledgements**

We are very grateful to Anne Chomyn for advice in the immunoprecipitation experiments and for providing mouse mtDNA clones, and Götz Hofhaus for advice in the enzymatic assays. We also thank C. Lin, R. Shakeley, R. Zedan, A. Drew and B. Keeley for expert technical assistance. These investigations were supported by Eisai Co., Ltd, Japan and by National Institutes of Health Grant GM-11726 (to G.A.).

**References**


