Novel mutations of ND genes in complex I deficiency associated with mitochondrial encephalopathy

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Isolated Complex I (CI) deficiency, the most frequent cause of mitochondrial disease, is a clinically and genetically heterogeneous condition. Complex I is a giant multiheteromeric enzyme composed of seven ND subunits encoded by mitochondrial DNA (mtDNA) genes, and at least 38 subunits encoded by nuclear genes. To establish the contribution to human mitochondrial encephalopathy of ND versus nuclear gene mutations, we have been undertaking a systematic analysis of CI genes in a cohort of 46 adult and paediatric patients with biochemically defined CI defect. Sequence analysis of the entire mtDNA let us identify six patients with mutations in ND genes. The clinical presentations varied, from infantile Leigh syndrome, to childhood MELAS, to adult-onset encephalopathic syndromes of variable severity. Three of the mutations were not previously reported (3481G > A, 14600G > A and 13063G > A, in ND1, ND6 and ND5 genes, respectively) and were further investigated in mutant transmitochondrial cybrids. Tight correlation between mutation load and decrease in CI activity was observed in each of the three mutant cybrid lines, supporting the pathogenic role of the novel mutations. Structural studies on mutant cybrids showed impaired assembly or reduced stability of the holoenzyme complex. In our experience ND gene mutations are relatively common in CI-defective mitochondrial encephalopathy of both children and adults.

Keywords: mitochondrial encephalomyopathy; complex I deficiency; assembly; mtDNA mutation

Abbreviations: BNGE = blue native gel electrophoresis; CI = Complex I; COX = cytochrome c oxidase; RRFs = Ragged Red Fibers


Introduction

Isolated deficiency of respiratory chain Complex I (CI, NADH:ubiquinone oxidoreductase) is a major cause of mitochondrial disease in children and adults (Janssen et al., 2006). The severity of the clinical presentations associated with CI defects varies according to the age of onset, from neonatal lactic acidosis, with or without cardiomyopathy, to infantile Leigh syndrome (OMIM 256000) or leukoencephalopathy, to juvenile/adult LHON (Leber hereditary optic neuropathy, OMIM 535000) and MELAS (mitochondrial encephalopathy with lactic acidosis and stroke-like episodes, OMIM 540000) (Bugiani et al., 2004; Janssen et al., 2006). Mammalian CI is a giant multiheteromeric enzyme located in the inner membrane of mitochondria, consisting of a peripheral arm protruding into the mitochondrial matrix, where the electron transport takes place, and a membrane-embedded arm, where proton translocation takes place. The protein backbone of CI is composed of seven mitochondrial DNA (mtDNA)-encoded NADH-dehydrogenase (ND) subunits, and at least 38 nuclear-encoded subunits (Janssen et al., 2006; Carroll et al., 2006). The identification of mutations associated with CI defects is hampered by the remarkable number of genes involved in its formation and by the incomplete knowledge on the protein machinery controlling its assembly, function and turnover. Although a large
percentage of human CI defects remains genetically undefined, nuclear DNA or mtDNA gene mutations have been documented in a fraction of CI-deficient patients presenting with definite clinical syndromes (Bugiani et al., 2004; Janssen et al., 2006).

Here we report on six CI-deficient patients with mitochondrial encephalomyopathy and mutations in mtDNA, three of which are novel. The patients belong to a larger series of 46 CI-deficient patients (see also Bugiani et al., 2004), 12 of whom, including the 6 presented here, carried pathogenic mutations in ND genes. Although the majority of CI deficiency cases still fails to be defined at the genetic level, and the molecular dissection of all 45 CI subunits has been carried out in only a number of patients, our results show that the impact of ND gene mutations in both infantile and adult CI-defective encephalopathy is considerably higher than previously thought.

**Patients**

The clinical and laboratory findings of our patients are outlined in Table 1.

**Patient 1.** This 8-year-old boy was admitted at our Institute after a 10-month history highly suggestive of MELAS (Fig. 1A). A maternal cousin suffered of recurrent migraine. The psychomotor development was normal until 6 years of age, when he presented with muscle weakness, easy fatigability and a left divergent squint, soon followed by exercise-induced episodic headache. At 7.5 years of age, he had a first acute encephalopathic episode characterized by a generalized seizure followed by persistent headache and drowsiness, recurrent focal motor and visual seizures, and right homonymous hemianopia. Lactate was markedly increased both in blood and CSF. A first conventional MRI study showed a large cortico-subcortical lesion in the left occipital lobe, bordering the inter-hemispheric cortex and corresponding neither to vascular territories nor to watershed areas, and bilateral symmetric signal abnormalities in the thalami. A rod-cone-type retinal dystrophy was also documented, with no optic atrophy. Since then, the boy underwent five additional episodes characterized by recurrent focal seizures, headache with vomiting and appearance of new stroke-like lesions on MRI, with partial regression of the previous lesions (Fig. 2A). The clinical picture has progressively worsened due to the appearance of quadriaparesis, cortical blindness and cognitive deterioration with significant deficits in executive functions.

A skeletal muscle biopsy was obtained when the boy was 8 years old. Histology and histochemistry revealed numerous ragged red fibres (RRFs), which stained strongly for succinate dehydrogenase (SDH) and cytochrome c oxidase (COX).

**Patient 2** was a baby girl, first child of unrelated parents. Three older siblings, two girls and a boy, who were born to different fathers, all died in early infancy due to a progressive encephalopathy with MRI features consistent
with Leigh syndrome (Fig. 1B). A maternal aunt is affected since early adulthood by optic atrophy and mild cerebellar atrophy. The patient was born at term, after an uneventful pregnancy. Since the first months of life, she presented with developmental delay, severe muscle hypotonia and dystonic movements of the limbs, marked irritability and a complex eye movement disorder suggestive of ocular apraxia. A persistent and severe lactic acidosis was documented. A first conventional MRI study, performed at 4 months, showed a moderate cerebral atrophy and symmetric signal changes in basal nuclei. Visual evoked responses were absent, but funduscopy was normal. Auditory evoked responses were consistent with bilateral sensory-neural deafness. At 7 months of age, the patient had an acute episode of metabolic failure, possibly triggered by a trivial infection. Epileptic seizures ensued, as well as respiratory insufficiency with episodes of apnea requiring mechanical support. A second MRI showed progression of cerebral atrophy, and the appearance of additional signal changes in thalami, midbrain tegmentum and deep cerebellar white matter. The girl deceased 1 month later with sepsis.

A skeletal muscle biopsy, taken 3 months before death, revealed only minimal myopathic changes.

Patient 3 is the only child of unrelated parents. At 5 months of age, he presented with developmental delay, soon followed by psychomotor regression, marked hypotonia with brisk deep tendon reflexes, incoordination of eye movements, drooling and mild dysphagia and recurrent focal epileptic fits. A brain MRI at 9 months showed symmetric signal abnormalities in basal ganglia, subthalamic nuclei, thalami and brainstem tegmentum, consistent with a diagnosis of Leigh syndrome (Fig. 2B). Lactate was increased in blood and CSF. The clinical conditions worsened acutely a few months later, due to severe respiratory difficulties, requiring tracheostomy and nasogastric feeding. A dilating cardiomyopathy was documented by ultrasonography. Funduscopy revealed optic disk pallor. The child is now 3 years old, severely disabled.

Skeletal muscle and skin biopsies were performed at 10 months. Muscle morphology was unremarkable.
Patient 4 has already been reported (Antozzi et al., 1995). He was well until the age of 21 years, when he first experienced generalized seizures and focal motor epilepsy partialis continua (EPC). Three months later, he started complaining of poor visual acuity. Bilateral optic atrophy was documented with absent visual evoked responses. Several episodes of EPC occurred thereafter, often in the form of segmental myoclonic jerks involving alternatively either body side. Interictal physical examination was normal for 4 years after the onset, with only mild sensory-motor deficit of the right upper limb. Serial MRI studies showed single or multiple areas of abnormal signal intensity, mainly involving the cortico-subcortical regions, fluctuating in time and localization, not always corresponding to the putative epileptic foci. Lactate was repeatedly detected in the CSF. A first conventional MRI study showed symmetric signal changes in basal nuclei, thalami, basal ganglia (Fig. 2C), substantia nigra and periaqueductal gray matter.

Since the MRI features were suggestive of a mitochondrial disorder, the patient underwent a skeletal muscle biopsy, which was morphologically normal.

Patient 5 was first admitted at the age of 16 years, for the subtle onset of bilateral reduced visual acuity. Funduscopy was normal, but visual evoked responses were markedly delayed. A first MRI study was unremarkable. The patient was readmitted at 20 years for the onset of cerebellar ataxia, dysarthria and hyperreflexia in lower limbs. Optic atrophy was accompanied by delay and disorganization of visual and auditory evoked responses. A second MRI study showed the appearance of bilateral signal abnormalities in basal ganglia (Fig. 2C), substantia nigra and periaqueductal gray matter.

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Patient 6 (Fig. 1C) has partly been reported elsewhere (Pincherle et al., 2006). He was well until 32 years of age, when he complained of a transient visual loss, soon followed by fluctuating gait instability, slurred speech, myoclonic jerks at rest in the lower limbs, and sleep-onset insomnia due to sleep-related involuntary movements. Two years later, the patient was admitted to our Institute. The physical examination showed cerebellar and sensory ataxia, mild dysarthria and a complex eye movement disorder resulting from the combination of nystagmus and a metric alteration of saccades. Cognition was preserved, apart from mild constructional dyspraxia. A polysomnographic study documented an excessive fragmentary hypnic myoclonus mainly expressed during REM sleep. Laboratory tests were unrevealing, except for some oligoclonal bands detected in the CSF. A first conventional MRI study showed symmetric signal changes in basal nuclei, thalami, internal capsule and brainstem tegmentum, suggesting a mitochondrial disorder (Fig. 2D).

A skeletal muscle biopsy revealed scattered RRFs with hyperintense COX.

Material and Methods
Morphological and biochemical analyses
Biochemical assays of the individual respiratory chain complexes were carried out on muscle homogenate, digitonin-treated skin fibroblasts (Tiranti et al., 1995) and cybrids, as described (Bugiani et al., 2004). Enzymatic activity of each complex was normalized to that of citrate synthase, an index of mitochondrial mass.

mtDNA sequence and restriction fragment length polymorphism analysis
According to a standardized protocol (Bugiani et al., 2004), the entire mtDNA was PCR-amplified into eight overlapping fragments using a set of coupled primers. After amplification, the fragments were tested on agarose gel and free oligonucleotides were digested using ExoSAP-IT (USB). Each fragment was then sequenced using four ‘sense’ primers. Restriction fragment length polymorphism (RFLP) analysis was used to confirm and quantify mtDNA mutations.

Fibroblast and cybrid cell cultures
Fibroblast cell lines were cultured continuously in Dulbecco’s modified Eagle’s medium with 10% FCS (Munaro et al., 1997).

Transmitochondrial cybrids were obtained by polyethylene glycol (PEG) fusion, followed by selection in a uridine-free medium, as described (King and Attardi, 1989). Cytoplasts derived from cytochalasin-treated patients’ fibroblasts were fused with a mtDNA-less (ρ−) derivative of the human osteosarcoma 143B cell line. The absence of mtDNA in ρ− cell lines, and its presence in transmitochondrial cybrids, was confirmed by PCR analysis using pairs of primers that amplify the D-loop region, as described (Mariotti et al., 1994).

Blue native electrophoresis and in-gel activity assays
The detection of the assembled respiratory complexes in isolated mitochondria from cybrids and 143B cells was performed using blue native gel electrophoresis (BNGE) as described (Nijtmans et al., 2002), with slight modifications. After first dimensional (1D) or second dimensional (2D) electrophoresis, the proteins were electroblotted onto nitrocellulose filters and sequenced using specific antibodies against CI subunits NDUFa9 (39 kDa) and NDUFb6 (17 kDa), and against SDHB, the 30 kDa subunit of Complex II (CII) (Molecular Probes, Invitrogen). For detection, the ‘ECL western blotting detection system’ from GE Healthcare was used.

In-gel activity assays for CI and cytochrome c oxidase (COX, complex IV, CIV) were performed on 1D-BNGE as described (Zerbetto et al., 1997).

Statistics
Regression analysis was performed for CI/CS values versus heteroplasmy percentages measured in cybrid clones from patients.
The remaining three patients (#1, #2 and #6) harboured novel mtDNA mutations (Fig. 3).

Patient 1 housed a novel heteroplasmatic 3481G > A mutation in ND1. The mutation corresponds to a drastic change from glutamate to lysine (E59K) affecting a highly conserved residue in the first matrix side loop of subunit ND1. Heteroplasmia was 80% in muscle and urinary epithelium, 55% in lymphocytes and 50% in fibroblasts. Patient 2 had a novel 14600G > A mutation in ND6. This mutation, which introduces a proline to leucine change at position 25 of the protein (P25L), was homoplasmic in muscle and fibroblasts of the patient, but heteroplasmic in her mother and maternal aunt (Fig. 1B). Patient 6 harboured a novel 13063G > A mutation, changing a valine to isoleucine at residue 243 (V243I) in a highly conserved region of the ND5 protein. The mutation was 80% heteroplasmic in muscle, 70% heteroplasmic in fibroblasts and 25% heteroplasmic in lymphocytes. RFLP analysis was extended to mtDNA from lymphocytes (Fig. 1) and, whenever possible, fibroblasts and urinary epithelial cells of other maternal relatives of patients 1, 2 and 6 (not shown).

Biochemical studies on cybrids

To establish the pathogenic role of the novel mtDNA mutations, we performed a complementation assay on transmitochondrial cybrids, obtained from fibroblast cell lines of patients 1, 2 and 6, and from fibroblasts from the mother of patient 2. Cybrids were obtained by fusing mitochondria-containing cytoplasts from patients’ fibroblasts with mtDNA-less ρ0 cells derived from 143B human osteosarcoma cell line. We analysed a series of 13 clones from patient 1, and a series of 16 clones.
from patient 6, harbouring different percentages of heteroplasmy. As shown in Fig. 4A and B, the correlation between the CI values, normalized to the activity of a mitochondrial mass index, citrate synthase (CS), against the heteroplasmic mutation loads, was highly significant in both series. Since the mutation was homoplasmic in patient 2, we compared 12 mutant cybrid clones derived from fibroblasts of the patient with 12 homo- plasmic wild-type (wt) cybrid clones obtained from fibroblasts of her mother. As shown in Fig. 4C, the CI/CS mean was 3.0±1.8 in the mutant series versus 14.9±2.3 in the wt series (Student’s t test \( P = 4.3E-12 \)). Taken together, these results clearly indicate that each of these mtDNA mutations is indeed associated with defective CI activity, i.e. pathogenic.

**BNGE analysis of complex I and in-gel activity assays**

To evaluate whether the three novel mutations found in patients 1, 2 and 6 affect the amount and assembly of CI, we carried out western blot (WB) analysis on protein extracts from homoplasmic mutant cybrids versus 143B control cells, separated by 1D and 2D-BNGE. Immunovisualization of CI was performed using antibodies against NDUFB6 and NDUFA9 subunits. NDUFB6 is a 17 kDa polypeptide which is part of the membrane arm of CI; NDUFA9 is a 39 kDa subunit which is part of the peripheral arm but is localized in close proximity to the membrane arm. Both subunits enter the CI assembly pathway at a relatively early stage (Antonicka et al., 2003; Ugalde et al., 2004), thus defining seven distinct sub-complexes as CI assembly intermediates (Janssen et al., 2006). An antibody specific to SDHB, the 30 kDa subunit of succinate dehydrogenase (complex II, CII), was used as a standard.

As shown in Fig. 5A, the intensity of the band corresponding to the 980 kDa bona fide fully assembled CI, obtained by 1D-BNGE WB analysis using either the anti NDUFB6 or the NDUFA9 antibody, was comparable between mutant cybrids from patient 6 and 143B control cells, while it was reduced in mutant cybrids from patient 1 and even more so from those derived from patient 2.
In-gel activity on 1D-BNGE was visualized as a single CI-reactive band in 143B control cells; the corresponding band from homoplasmic mutant cybrids of patient 6 showed clear reduction of CI activity, compared to the control band; more severe reduction of CI activity was detected in homoplasmic mutant cybrids from patient 1. Homoplasmic mutant cybrids from patient 2 showed hardly any CI activity. COX activity was visualized in a single CIV band of comparable intensity in all samples (Fig. 5B).

The assembly state of CI was further investigated by WB analysis on 2D-BNGE. In mutant cybrids from patient 6, NDUFA9 cross-reacting material (CRM) was present at the position corresponding to fully assembled CI. A similar pattern was obtained in mutant mitochondria from patient 1. Homoplasmic mutant cybrids from patient 2 showed hardly any CI activity. COX activity was visualized in a single CIV band of comparable intensity in all samples (Fig. 5B).

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representing partially assembled CI species (Ugalde et al., 2004). The latter were predominant in patient 2 cybrids, which had drastically reduced amounts of fully assembled CI. Low MW CI species were immunovisualized in all three mutant samples using the anti NDUFB6 antibody (Fig. 6B). Again, the signal corresponding to fully assembled CI was prominent in patient 6 and patient 1, but very low in cybrids from patient 2. The smeary appearance of these protein species reflects the hydrophobic nature of both NDUFA9 and NDUFB6 and is a common finding in 2D-BNGE WB analysis of these subunits (Antonicka et al., 2003; Ugalde et al., 2004; Vogel et al., 2007). The drastically reduced amount of patient 2 CI-CRM and its dispersion along the 2D-BNGE blot can explain why low MW species failed to be detected in 1D-BNGE WB analysis (Fig. 5A). No low MW assembly intermediates were immunovisualized by the NDUFA9 antibody in a 143B cell control sample. In the same sample, most of NUDUFb6 CRM was detected in fully assembled CI; a small aliquot was also present in a high molecular weight subcomplex, a frequent finding in 2D-BNGE WB analysis using antibodies specific to hydrophobic subunits of the membrane-bound arm, including NDUFB6 (Ugalde et al., 2004). The NDUFB6-containing subcomplex may reflect partial CI disaggregation upon treatment of cell homogenate with dodecyl-maltoside (Vogel et al., 2007).

As shown in Fig. 6C, assembly levels of CII were similar in patients and control cells, indicating a specific CI abnormality.

**Discussion**

The six patients with mitochondrial encephalopathy reported here belong to a larger series of 46 CI-deficient cases that were systematically screened for mutations in mtDNA. Twelve patients (26%), including the six here reported, harbourcd changes in mtDNA ND genes, while mutations in nuclear-encoded CI subunits were found in only two cases (see also Bugiani et al., 2004). Likewise, in a recent paper reporting the analysis of 12 nuclear-encoded CI subunits performed in 26 patients with severe CI deficiency, only two mutations were identified, in the X-linked NDUFA1 gene in two unrelated hemizygous mutant patients (Fernandez-Moreira et al., 2007). Our results indicate that systematic mtDNA screening is essential in the diagnostic workout of CI defects in both children and adults. Seven of our 12 patients with ND mutations, including two of the six patients reported in this paper, presented with Leigh syndrome, which suggests a prevalent association between Leigh syndrome and mutations in mtDNA ND genes in early-onset CI deficiency (Bugiani et al., 2004).

We found already reported mtDNA mutations in three out of six patients. Patients 3 and 5 carried a 14487T > C missense mutation (M63V) in the ND6 gene. The different mutation loads in the two patients (>95 versus 50%) can explain the different severity of the clinical presentations (Leigh syndrome versus optic atrophy and cerebellar ataxia), including the age of onset (5 months versus 16 years). The M63 residue is located in the most conserved transmembrane region of ND6, in which several mutations have been found in patients with Leigh syndrome, MELAS, LHON and LHON/dystonia syndrome (www.mitomap.org). In particular, the 14487T > C mutation was reported in two children with typical Leigh syndrome (Ugalde et al., 2003; Lebon et al., 2003), in one infant with a combination of Leigh syndrome and cardiomyopathy (Bugiani et al., 2004), and in three other patients with childhood-onset...
progressive generalized dystonia and bilateral striatal necrosis (Solano et al., 2003; Raspall-Chaure et al., 2004). Although the clinical presentation of patient 5, i.e. juvenile-onset cerebellar ataxia with optic atrophy, has never been reported in association with CI deficiency, this patient had MRI signal changes in brain deep gray structures which are typical of Leigh syndrome (Farina et al., 2002). The low mutation load, about 50% in muscle and lymphocytes, could account for the later onset of symptoms in this patient, compared to the nearly homoplasmic mutation load found in all the other cases with the 14487T>C mutation in our patient, compared to the nearly homoplasmic mutation load could account for the later onset of symptoms in this patient.

MRI signal changes in brain deep gray structures which are reported in association with CI deficiency, this patient had atrophy and progressive cognitive decline (Taylor et al., 2001), but also in several children with infantile Leigh syndrome (Lebon et al., 2003; Bugiani et al., 2004; McFarland et al., 2004; Leshinsky-Silver et al., 2004). The low mutation load, about 50% in muscle and lymphocytes, could account for the later onset of symptoms in this patient, compared to the nearly homoplasmic mutation load found in all the other cases with the 14487T>C mutation, including our patient 3, who were affected by full blown, infantile Leigh syndrome. By 2D-BNGE, the M63V change in ND6 was shown to severely affect the stability and assembly of CI (Ugalde et al., 2003). A significant increase in reactive oxygen species production, resulting in lipid and mtDNA oxidative damage, was demonstrated in homoplasmic 14487T>C mutant cybrids (Gonzalo et al., 2005).

Patient 4 harboured a 10191T>C mutation, corresponding to a S45P change in a highly conserved region of the ND3 protein. The S45 residue is relatively conserved throughout metazoans, and is contained within a hydrophilic loop, between two of the three membrane-spanning α-helices of ND3 (Fearnley and Walker, 1992). The 10191T>C transition introduces a hydrophobic P residue in this domain that could affect the folding of the ND3 protein and possibly its interaction with other CI subunits. BNGE studies (McFarland et al., 2004) showed that the 10191T>C change causes a disproportionately greater reduction in enzyme activity than in the amount of fully assembled CI, suggesting that the ND3 subunit may play a predominantly catalytic, rather than structural, role, in electron transport, proton pumping or ubiquinone binding. The 10191T>C mutation was previously reported in an adult patient with a history similar to our patient 4, consisting of epilepsy, stroke-like episodes, bilateral optic atrophy and progressive cognitive decline (Taylor et al., 2001), but also in several children with infantile Leigh syndrome (Lebon et al., 2003; Bugiani et al., 2004; McFarland et al., 2004; Leshinsky-Silver et al., 2005). Considering all the 10191T>C positive patients, earlier onset and more severe phenotype are well correlated to both lower residual activity of CI and higher mutational load.

The three other patients of our series (patients 1, 2 and 6) carried novel mutations in mtDNA ND genes. Different lines of evidence support the pathogenic role of these mutations. First, the mutations were absent in more than 500 mtDNAs from unrelated Italian subjects, taken as controls. Secondly, the biochemical phenotype identified in fibroblast cell lines, consisting in a defect of CI activity, was retained in the cybrid clones generated from the transfer of mutant mitochondria into ρ0 cells. This result indicates that, irrespective of the nuclear background, the OXPHOS defect segregated with mtDNA. Thirdly, at least for the families of patients 1 and 2, the transmission of the clinical disease was compatible with a maternally inherited trait. The entire offspring of the mother of patient 2, generated with three different partners, was clinically affected. The mother of patient 2 was indeed affected by subtle psychiatric symptoms, and her sister by optic nerve and cerebellar atrophy.

We could demonstrate that the novel mutations found in patients 1, 2 and 6 determine the impairment of CI assembly or stability.

The whole matter concerning CI assembly in mammals is highly controversial and subjected to frequent revisions and refinements (see for instance Vogel et al., 2007). With this caveat in mind, in the interpretation of our results we considered two partially alternative models, both deduced by studies in human cells. A step-wise sequential CI assembly pathway has been proposed, based on studies in 143B cells (Ugalde et al., 2004), similar to data previously obtained in Neurospora crassa. According to this model a preassembled peripheral arm, including NDUFA9, associates with a preassembled membrane arm, including the ND1-6 and the NDUFB6 subunits, to give rise to an almost complete CI. A few other subunits are then added to form holo-CI. According to an alternative assembly pathway, deduced from studies on a genetically heterogeneous cohort of CI-deficient patients, subsequent assembly intermediates containing subunits of the peripheral arm, together with a portion of the membrane arm containing ND1, are assembled before the incorporation of the bulk of the membrane arm (Antonicka et al., 2003).

By 1D-BNGE WB analysis on ND1 and ND6 mutant cybrids, we showed selective reduction in the steady-state levels of the CI holoenzyme, while in ND5 mutant cybrids the amount of CI was comparable to that of 143B control cells.

By 2D-BNGE WB analysis on ND1 mutant cybrids, we showed the presence of fully assembled CI, but also the accumulation of low MW species, which contained hardly any NDUFA9, but high amounts of NDUFB6 CRM. The low MW CI species were similar in size and distribution to those attributed to CI intermediate subcomplexes (Antonicka et al., 2003; Ugalde et al., 2004). Since NDUFB6 belongs to the membrane arm, while NDUFA9 is part of the peripheral arm, the results obtained in ND1 mutant cybrids fit better with the first assembly model mentioned earlier, which assigns the ND1 subunit to preassembled membrane arm, rather than with the second assembly model, which attributes ND1 to a subcomplex including the NDUFA9 subunit.

In 2D-BNGE WB on ND6 mutant cybrids, fully assembled CI was present in very low amount, while abundant low MW species were detected by both NDUFA9 and NDUFB6-specific antibodies. These results suggest for ND6 an essential role in the assembly and stability of CI, in line with the previous studies conducted on different organisms (Bai and Attardi, 1998; Cardol et al., 2002).
Finally, 2D-BNGE WB analysis on ND5 mutant cybrids showed the presence of a substantial amount of fully assembled CI, together with some low MW CI species, which were detected by the antibody against the membrane arm NDUF6 subunit, but not by the antibody against the peripheral arm NDUF9 subunit. ND5 is considered non-essential for the assembly of the remaining mtDNA-encoded subunits into the complex, since it is the last ND subunit to be incorporated into the membrane arm (Bourges et al., 2004). Nevertheless, loss of ND5 has been shown to cause instability of the membrane arm (Chomyn, 2001), as also indicated by our own WB results on 2D-BNGE.

The identification of specific CI assembly patterns, obtained by investigation on human mutant cells, can help understand the molecular mechanisms underlying different CI defects, and provide useful insight for the reconstruction of the CI assembly pathway.

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