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Identification and Characterization of a Common Set of Complex I Assembly Intermediates in Mitochondria from Patients with Complex I Deficiency*

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Deficiencies in the activity of complex I (NADH: ubiquinone oxidoreductase) are an important cause of human mitochondrial disease. Complex I is composed of at least 46 structural subunits that are encoded in both nuclear and mitochondrial DNA. Enzyme deficiency can result from either impaired catalytic efficiency or an inability to assemble the holoenzyme complex; however, the assembly process remains poorly understood. We have used two-dimensional Blue-Native/SDS gel electrophoresis and a panel of 11 antibodies directed against structural subunits of the enzyme to investigate complex I assembly in the muscle mitochondria from four patients with complex I deficiency caused by either mitochondrial or nuclear gene defects. Immunoblot analyses of second dimension denaturing gels identified seven distinct complex I subcomplexes in the patients studied, five of which could also be detected in nondenaturing gels in the first dimension. Although the abundance of these intermediates varied among the different patients, a common constellation of subcomplexes was observed in all cases. A similar profile of subcomplexes was present in a human/mouse hybrid fibroblast cell line with a severe complex I deficiency due to an almost complete lack of assembly of the holoenzyme complex. The finding that diverse causes of complex I deficiency produce a similar pattern of complex I subcomplexes suggests that these are intermediates in the assembly of the holoenzyme complex. We propose a possible assembly pathway for the complex, which differs significantly from that proposed for *Neurospora*, the current model for complex I assembly.

NADH:ubiquinone oxidoreductase (complex I; EC 1.6.5.3) is the largest and the least understood of all the respiratory chain complexes. Mammalian complex I is composed of at least 46 subunits, which are encoded by both nuclear (39 subunits) and mitochondrial DNA (7 subunits) (1–3). The complex, which has an estimated molecular mass of almost 1 MDa, catalyzes the transfer of two electrons from NADH to ubiquinone coupled to translocation of four protons across the inner mitochondrial membrane.

Low resolution structures based on the electron microscopy of the bovine (4), *Escherichia coli* (5, 6), and *Neurospora crassa* (7) complex I show that the complex has an L-shaped form with one arm in the membrane and a peripheral arm protruding into the mitochondrial matrix. A second, horseshoe-shaped conformation of the *E. coli* complex I has also recently been proposed (8). The complex can be dissociated by treatment with detergent into three subcomplexes: Iα, corresponding to the peripheral arm and composed of ~21 mostly hydrophilic subunits, and Iβ and Iγ, both of which make up the membrane arm (9). Subcomplex Iα contains the NADH binding site and most of the redox centers. All of the mtDNA-encoded[1] subunits and at least 16 of the nuclear-encoded subunits are found in the Iβ and Iγ fractions of the membrane arm.

Deficiencies in complex I are among the most common respiratory chain defects (10–15). Mutations in the mtDNA-encoded subunits of complex I were the first to be associated with a respiratory chain disorder, Lebers hereditary optic neuropathy (10, 16). Lebers hereditary optic neuropathy presents with the comparatively mild phenotype of adult-onset blindness due to optic nerve degeneration, and most cases are caused by mutations in one of four complex I subunit genes (*MTND1*, *MTND4*, *MTND5*, and *MTND6*) (17, 18). In contrast, the majority of early onset complex I deficiencies are severe, and often fatal, autosomal recessive disorders. DNA sequence analysis in more than 20 complex I patients has revealed mutations in seven structural genes: *NDUFS2* (11); *NDUFS4* (12, 19, 20); *NDUFS7* (13); *NDUFS8* (14); *NDUFV1* (15, 21); *NDUFS1* (21); and *NDUFV2* (22). Structural gene mutations were not found in about 50% of patients in these studies, suggesting that genes coding for assembly factors may be important causes of complex I deficiency. An assembly defect in complex I was described in a patient with a mutation in *NDUFS4* (19) and in a patient with a mutation in the mitochondrially encoded subunit ND4 (23). *Saccharomyces cerevisiae* is widely used as a

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[1] The abbreviations used are: mtDNA, mitochondrial DNA; BN-PAGE, Blue-Native PAGE; COX, cytochrome *c* oxidase; Alu, restriction endonuclease; Alu-FISH, Alu-PCR repeats fluorescence *in situ* hybridization; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

model organism for mitochondrial respiratory chain function but does not contain a complex I (24, 25). The current model of assembly of complex I is based on pulse-chase labeling of assembly intermediates in *N. crassa* and on the characterization of assembly subcomplexes in *N. crassa* mutants (26–30); however, our knowledge of mammalian complex I assembly is very limited.

Here, we report the identification of complex I subcomplexes in patients with complex I deficiency due to mutations in either nuclear or mitochondrial DNA and in a human/mouse hybrid cell line with a severe complex I deficiency due to an almost complete failure to assemble the holoenzyme complex. Using Blue-Native PAGE and a panel of antibodies specific for structural subunits of complex I, we identified a common set of complex I subcomplexes, and we propose a possible pathway for mammalian complex I assembly which differs significantly from that proposed for *Neurospora*.

EXPERIMENTAL PROCEDURES

Characterization of Patients—At the time of biopsy, patient S was a 28-year-old male (described previously in Ref. 31) who suffered from lifelong exercise intolerance. Cardiac and pulmonary functions were normal, and he was otherwise well. Both parents and a 23-year-old sister were healthy. The isolated myopathy was associated with severe lactic acidosis, and a muscle biopsy revealed the presence of ragged-red fibers. Biochemical analysis of the patient's muscle revealed a selective complex I deficiency (<10% of control), whereas complexes II–IV had normal activity. The analysis of mtDNA showed a heteroplasmic 2-bp deletion in the *MTND2* gene (90% of mtDNA in the patient muscle carries the mutation) causing a premature stop codon, which predicts a truncated protein.

At the time of biopsy, patient G was a 20-year-old male with a lifelong history of severe aerobic exercise intolerance associated with exertional dyspnea and tachycardia. Biochemical studies of patient muscle biopsy revealed a selective decrease in complex I activity (<20% of control), whereas other respiratory chain activities were normal. Histochemical analysis revealed normal mitochondrial staining, with no ragged-red fibers. The patient's sister (23 years old) also suffers from a pure myopathy and severe exercise intolerance. Myoblasts from both siblings showed slightly decreased complex I activity (75% of control); fibroblasts from both siblings showed no abnormality. Fusion of myoblasts from both siblings with 143B.TK$^-$ rho^0 cells rescued the complex I deficiency, indicating that the defect in patient G and his sister is of nuclear origin.

Patients B1 and B2 are brothers, 39 and 38 years old, respectively, at the time of biopsy. They both suffer from mitochondrial encephalomyopathy and pigmentary retinopathy. Patient B1 had developmental problems, clumsiness, and borderline mental retardation since birth. He developed seizures at 19 years. Patient B2 has a lifelong history of learning disabilities. He had adult onset epilepsy and has a Wolff-Parkinson-White conduction defect. Muscle histochemistry showed ragged-red fibers in both patients, with increased SDH staining and no COX negative fibers. Complex I activity in muscle mitochondria from patient B1 was 11% and from patient B2 was 10% of the normal mean. All other respiratory chain enzyme activities were normal. Southern blot analysis of mtDNA from patient B1 muscle showed no evidence of a deletion, and sequencing of all the tRNA genes and all the mtDNA-encoded complex I genes showed no mutation. Low complex I activity was also found in fibroblasts from patient B1, and all other respiratory chain enzyme activities were normal. Fusion of patients fibroblasts with 143B.TK$^-$ rho^0 cells rescued the complex I deficiency, indicating that the defect in patients B1 and B2 is of nuclear origin. Fusion of fibroblasts from patient B1 with fibroblasts from patient B2 showed low complex I in-gel activity, proving that they belong to the same genetic complementation group, as expected. Informed consent was obtained from all patients, and protocols were approved by the relevant Institutional Review Boards

Cell Lines—Primary skin fibroblasts were established from biopsy material and immortalized by transduction with retroviral vectors expressing the type 16 human papilloma virus E7 gene and the catalytic component of human telomerase (32). The fibroblasts were grown in high glucose Dulbecco's modified Eagle's medium containing 110 mg/ml pyruvate and 10% fetal bovine serum. The LM.TK$^-$ rho^0 mouse cell line (a kind gift of Dr. Eric Schon) was grown in high glucose Dulbecco's modified Eagle's medium containing 110 mg/ml pyruvate, 10% fetal bovine serum, and 50 μg/ml uridine.

TABLE I
Complex I subunits described in this study

Name[a]	Gene name	Localization
49 kDa	*NDUFS2*	Peripheral arm, Iα subcomplex
39 kDa	*NDUFA9*	Peripheral and membrane arm boundary, Iα and Iγ subcomplexes
30 kDa	*NDUFS3*	Peripheral arm, Iα subcomplex
24 kDa	*NDUFV2*	Peripheral arm, Iα subcomplex
20 kDa	*NDUFS7*	Peripheral arm, Iα subcomplex
18 kDa	*NDUFS4*	Peripheral arm, Iα subcomplex
17 kDa	*NDUFB6*	Membrane arm, Iβ subcomplex
15 kDa	*NDUFS5*	Peripheral and membrane arm boundary, Iα and Iγ subcomplexes
14 kDa	*NDUFA6*	Peripheral arm, Iα subcomplex
8 kDa	*NDUFA2*	Peripheral arm, Iα subcomplex
ND1	*MTND1*	Membrane arm, Iγ subcomplex

[a] The names of the nuclear-encoded subunits used in this study are based on the apparent molecular size.

Cell Fusion—LM.TK$^-$ rho^0 cells were infected with a retroviral construct conferring resistance to puromycin (33). Control fibroblasts were plated together with LM.TK$^-$ rho^0 cells and fused 16 h later using polyethylene glycol-1500:Me_2SO (40%:10%). The fused cells were selected in medium containing 1 μg/ml puromycin and 400 μg/ml G-418 for 3 weeks.

Muscle Tissue, Preparation of Mitochondria—Muscle biopsies from patients S, G, B1, and B2 and controls were obtained and stored frozen at −80 °C. Muscle homogenates (5%) prepared in 150 mM KCl, 20 mM Tris-HCl, 2 mM EDTA (pH 7.5) were centrifuged twice for 10 min at 600 × *g* to obtain postnuclear supernatant. Mitochondria were pelleted by centrifugation for 20 min at 10,000 × *g*. Protein concentration was measured by the Bradford method (34).

Electrophoretic Methods—Mitoplasts were prepared by treatment with 0.8 mg (fibroblasts) or 1.2 mg (myoblasts) of digitonin/mg of protein, as described previously (35). Mitoplasts or mitochondria were solubilized with 1% lauryl maltoside, and 10–20 μg of solubilized protein was used for electrophoresis. BN-PAGE (36) was used for separation of samples in the first dimension on 6–15% or 5–12% polyacrylamide gradients. In-gel qualitative assays for complex I and cytochrome *c* oxidase (COX) activity were performed as described (37). For the two-dimensional analysis, strips of the first dimension gel were incubated for 45 min in 1% SDS and 1% β-mercaptoethanol, and then 10% polyacrylamide SDS-PAGE was used to separate the proteins in the second dimension (38). Respiratory complexes I–IV were detected by Western blot analysis using monoclonal and polyclonal antibodies. Anti-COX IV, anti-complex II/70 kDa, anti-complex III/Core1, and complex I antibodies against subunits 30, 20, and 14 (Table I) were from Molecular Probes, Eugene, OR. Other antibodies against complex I subunits were kindly provided by Dr. R. Capaldi (39, 15, and 8 kDa); Dr. B. Robinson (49 and 17 kDa), Dr. J. Walker (24 kDa), Dr. A. Lombes (ND1), and Dr. V. Petruzzella (18 kDa).

RESULTS

Identification of Complex I Subcomplexes—BN-PAGE analysis of mitochondria from muscle biopsies of patients S, G, B1, and B2 showed decreased in-gel activity of complex I, whereas the activity of COX was normal (Fig. 1*A*). Western blot analysis showed normal levels of immunodetectable COX, complex II (not shown), and complex III; however, fully assembled complex I was markedly decreased in all four patients (Fig. 1*B*). Interestingly, antibodies against both nuclear (39 and 49 kDa) and mitochondrial (ND1) subunits of complex I identified several common subcomplexes with apparent molecular masses of 250, 310, 380, 480, and 650 kDa (Fig. 1*B*, Table II). An additional subcomplex of about 830 kDa was seen only in patients B1 and B2. Although the pattern of the subcomplexes was similar in all four patients, the intensities of immunodetectable signals varied among the patients. The ratio between the unassembled (found in subcomplexes) and the fully assembled subunits in patients B1 and B2 was lower than in patients S and G, in whom the amount of ND1 and 49-kDa subunits in the subcomplexes was similar or higher than in the fully assembled complex.

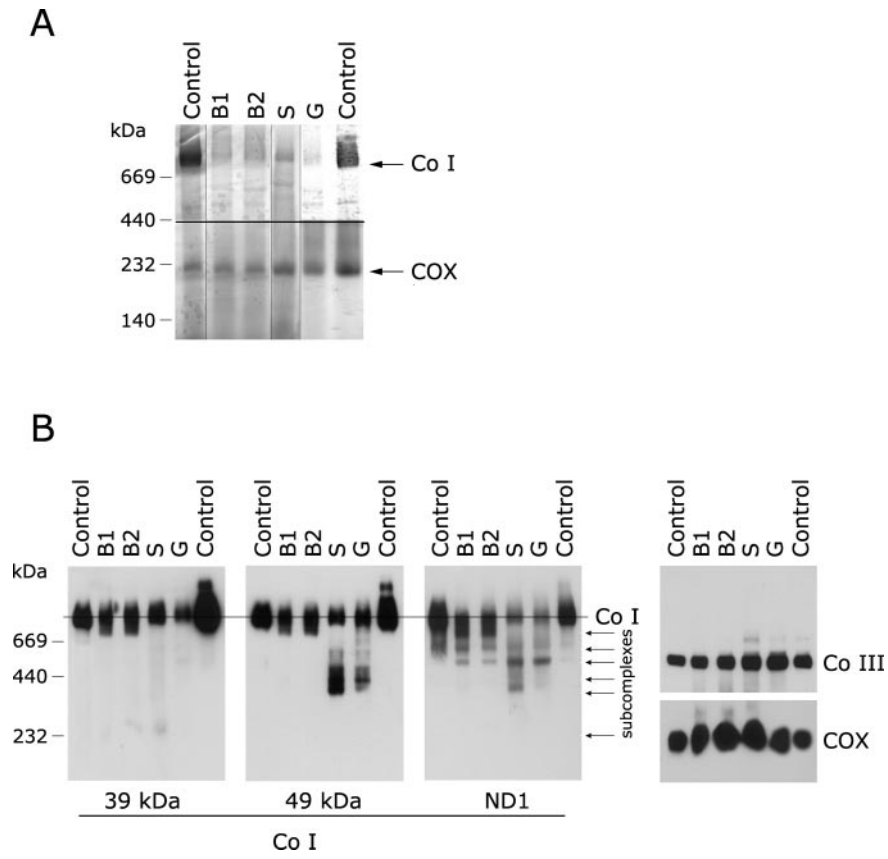

FIG. 1. **Defect of complex I in patient muscle mitochondria analyzed by Blue-Native PAGE.** Muscle mitochondria (10 μg of protein) from two control subjects and patients S, G, B1, and B2 were analyzed by BN-PAGE. *A,* In-gel activity staining of complex I (*Co I*) and COX. *B,* Western blot analysis using complex III (*Co III*) and complex IV (*COX*) antibodies as loading controls and the following antibodies against complex I: subunits 39 kDa, 49 kDa, and ND1. Patient mitochondria show decreased amounts of fully assembled complex I. Complex I subcomplexes are marked by *arrows*. The migration of molecular mass standards is indicated on the *left*.

TABLE II
Subunit composition of complex I subcomplexes
The presence of individual complex I subunits in identified subcomplexes is marked by X.

Subcomplex	MS[a]	Subunit										
		49	39	ND1	15	30	20	14	18	24	17[b]	8
	kDa											
I	200	X	X			X						
II	230						X		X	X		
III	250	X	X			X						
IV	310	X	X	X		X						
V	380	X	X	X		X						X
VI	480	X	X	X		X						X
VII	650	X	X	X	X	X	X	X	X	X		X
Fully assembled	950	X	X	X	X	X	X	X	X	X	X	X

[a] MS, molecular size.
[b] Antibody-detected pattern of the 17-kDa subunit presented as a streak and could not be assigned to any subcomplex.

Subunit Composition of Complex I Subcomplexes—To determine the subunit composition of the complex I subcomplexes, we used a set of 11 antibodies directed against structural subunits of complex I (described under "Experimental Procedures") in combination with two-dimensional electrophoretic analysis. Muscle mitochondria (20 μg of protein) from patient S were analyzed using BN-PAGE in the first dimension and Tricine/SDS-PAGE in the second dimension. Fig. 2 shows markedly decreased amounts of individual subunits in the fully assembled complex I from the patient when compared with control. Seven distinguishable subcomplexes were present in patient S mitochondria (marked I–VII). In addition to the five subcomplexes described in the one-dimensional gels above, subcomplexes of molecular mass 230 and 200 kDa were identified (Table II). A largely similar pattern was seen in muscle mitochondria from patients G, B1 (Fig. 3), and B2 (data not shown), although the relative intensities varied. The specific 830-kDa subcomplex of patient B1 mitochondria contained all analyzed subunits (49, 39, 15, 30, 20, 14, and 18 kDa and ND1). In all patients, antibodies against subunits 49 and 30 kDa detected these subunits in all the subcomplexes except for subcomplex II. A similar pattern was found for subunit 39 kDa; however, it appeared more as a streak than strong individual subcomplexes. A second pattern was seen using antibodies to subunits 24, 20, and 18 kDa, which were present in subcomplexes II and VII. The 8-kDa subunit and ND1 also shared a similar pattern and were not present in the lower molecular mass subcomplexes. Although the 24-kDa antibody does detect some subcomplexes in the control, the ratio between the fully assembled enzyme and the subcomplexes is substantially higher than seen in the patient. The 14- and 15-kDa subunits were only detected in the 650-kDa subcomplex (VII) (and the 830-kDa subcomplex unique to patients B1 and B2), and the 17-kDa subunit formed a very weak streak that could not be linked to any of the subcomplexes. Subcomplexes of complex I were also detected in fibroblasts from patient B1 (data not shown); however, the intensities were markedly decreased in comparison with those seen in muscle mitochondria. In control

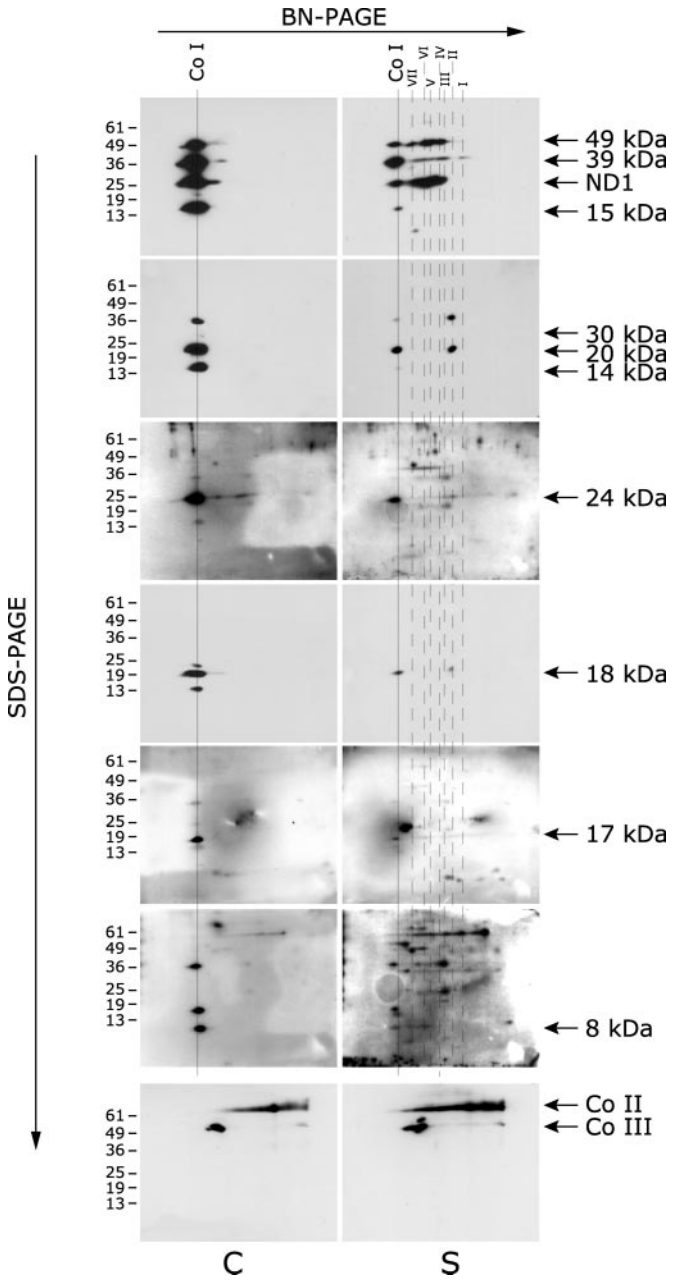

FIG. 2. **Identification and subunit composition of complex I subcomplexes in patient S mitochondria.** Control and patient S muscle mitochondria (20 μg of protein) were separated in the first dimension using BN-PAGE and in the second dimension using SDS-PAGE, and the presence of individual complex I subunits was determined by immunoblotting. The *filled line* marks the position of fully assembled complex I, and the *dashed lines* indicate subcomplexes I–VII. Complex II (*Co II*) and complex III (*Co III*) antibodies were used to assess the loading of the gels. The migration of molecular mass standards (in kDa) is indicated on the *left*.

mitochondria, a weak 650-kDa subcomplex could be detected with antibodies against the ND1 and 18-kDa subunits (Fig. 2); however, the other subcomplexes seen in the patients were not observed even after long exposure of the control immunoblot.

Presence of Complex I Subcomplexes in a Human/Mouse Hybrid Cell Line—A standard method to determine whether a respiratory chain defect maps to nuclear or mitochondrial DNA is to fuse patient cells with rho^0 cells to test for complementation. Complementation of the biochemical phenotype in this experiment indicates a recessive nuclear gene defect. To test whether mouse nuclear genes could rescue human nuclear respiratory chain gene deficiencies, we previously carried out this experiment in several patient cell lines, but never observed complementation. In fact, fusion of a control human fibroblast line with mouse LM.TK$^-$ rho^0 cells resulted in severely decreased COX and complex I activities. BN-PAGE analysis showed a strong dominant-negative effect of the mouse nucleus on complex I assembly, whereas complex II was normal (Fig. 4*A*). This phenotype does not arise from a loss of human chromosomes as Alu-FISH analysis shows a normal diploid chromosome number on metaphase spreads (data not shown). Rather, it appears to result from the dominant negative effects of specific mouse genes present in the human nuclear and mitochondrial background. Two-dimensional analysis of the fusion cells showed the presence of complex I subcomplexes (Fig. 4*B*) with apparent molecular masses of 650, 480, 380, 310, and 250 kDa, corresponding to subcomplexes III–VII. Almost no fully assembled complex could be detected in these cells, and the subunit pattern in the complex I subcomplexes was similar to that found in complex I patients.

DISCUSSION

Blue-Native PAGE is a powerful technique for the analysis of respiratory chain complexes and for detecting and diagnosing respiratory chain disorders (39). Pulse-chase labeling of mitochondrial proteins with [^{35}S]methionine, together with BN-PAGE, allowed the identification of three intermediates in the assembly of COX in mammalian cells (40). Patients with Leigh syndrome caused by mutations in *SURF1* show a characteristic early assembly defect (41) in which COX assembly is arrested before the incorporation of subunit II into the catalytic core, resulting in the accumulation of two early assembly intermediates whose presence discriminates between COX-deficient *SURF1* and non-*SURF1* patients (42). BN-PAGE has also been used to characterize the assembly of Tom7, a component of the preprotein translocase complex in the outer mitochondrial membrane (43). Here, we have used BN-PAGE and a panel of structural subunit antibodies to identify complex I subcomplexes in patients with either nuclear or mtDNA complex I defects. The finding that diverse causes of complex I deficiency produce a similar pattern of complex I subcomplexes, albeit with very different relative abundances, suggests that the subcomplexes represent stalled intermediates in the assembly of the holoenzyme complex.

Antibodies against the 39-kDa, 49-kDa, and ND1 subunits of complex I identified five distinct subcomplexes (250, 310, 380, 480, and 650 kDa) in the one-dimensional BN-PAGE in muscle mitochondria from the four patients studied, and two-dimensional PAGE revealed two additional lower molecular mass subcomplexes (200 and 230 kDa). As the respiratory chain complexes maintain their native conformation on the one-dimensional PAGE, some of the subunits will not be accessible to the antibodies, and the inability to detect these two smaller subcomplexes on the one-dimensional PAGE likely reflects this limitation.

Although the relative intensities of the subcomplexes varied considerably among the patients, a consistent pattern was observed. In general, complex I subunits could be divided into four groups. Subunits 49, 39, and 30 kDa, all part of the Iα structural subcomplex described by Sazanov *et al.* (9), were present in all subcomplexes, except subcomplex II. The 39-kDa subunit is also found in the Iγ subcomplex, indicating that it is probably found on the periphery of the complex at the boundary between the peripheral and the membrane arms (Table I). The streaky nature of the antibody detection pattern of the 39-kDa subunit in the second dimension gel suggests a weak attachment to the other subunits in the subcomplexes. Subunits 24, 20, and 18 kDa, which form part of the peripheral arm Iα subcomplex, were present in subcomplexes II and VII. Subcom-

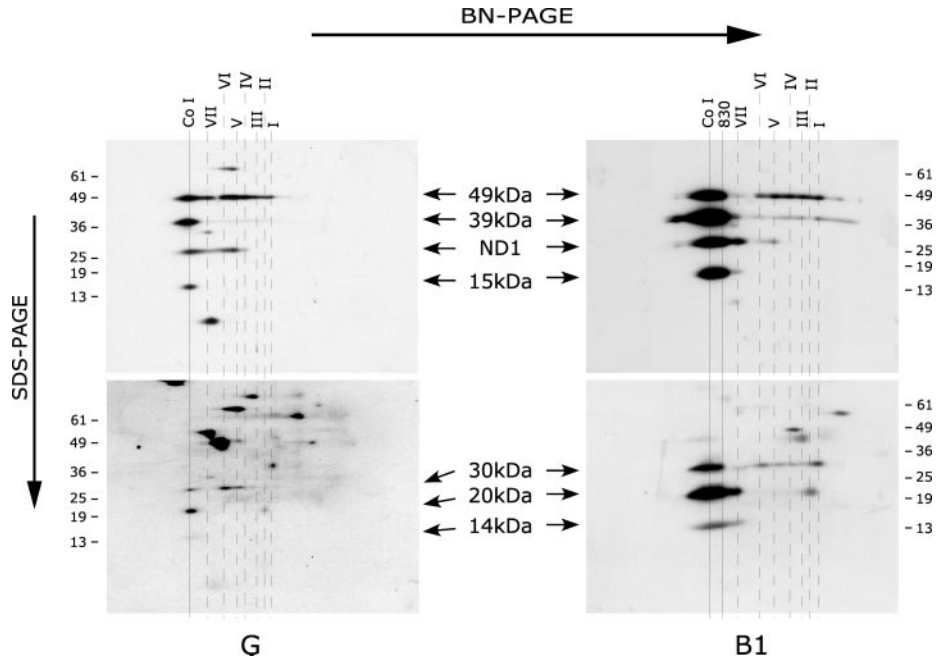

FIG. 3. **Identification and subunit composition of complex I subcomplexes in patients G and B1.** Muscle mitochondria from patient G and patient B1 (20 μg of protein) were analyzed by two-dimensional PAGE, and the presence of individual complex I subunits in the subcomplexes was determined by immunoblotting. The *filled line* marks the position of fully assembled complex I, and the *dashed lines* indicate subcomplexes I–VII. The patient B1 specific subcomplex of 830 kDa is marked with a *filled line*. The migration of molecular mass standards (in kDa) is indicated.

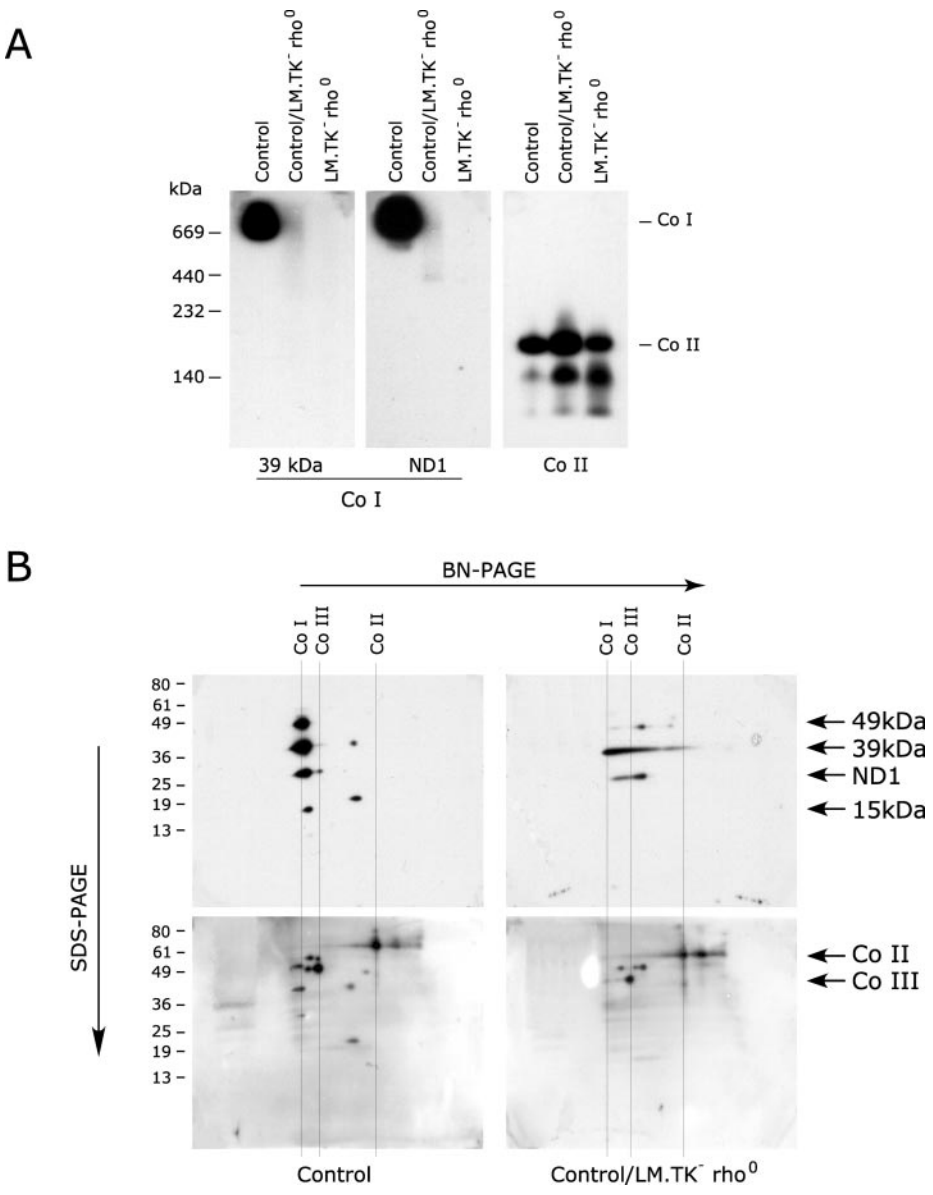

FIG. 4. **Identification of complex I subcomplexes in human/mouse hybrid cell line.** Mitoplasts (20 μg of protein) isolated from control fibroblasts, the human/mouse hybrid line (*control/LM.TK$^-$ rho^0*), and mouse rho^0 cell line (*LM.TK$^-$ rho^0*) were analyzed by BN-PAGE (*A*) and two-dimensional PAGE (*B*). As shown in *A*, a markedly decreased amount of fully assembled complex I was determined by immunoblotting with antibodies against complex I subunits 39 kDa and ND1. As shown in *B*, complex I subcomplexes were identified using antibodies against the complex I 49-kDa, 39-kDa, 15-kDa, and ND1 subunits. The *filled lines* indicate the positions of complex I (*Co I*), complex II (*Co II*), and complex III (*Co III*). The migration of molecular mass standards (in kDa) is indicated on the *left*.

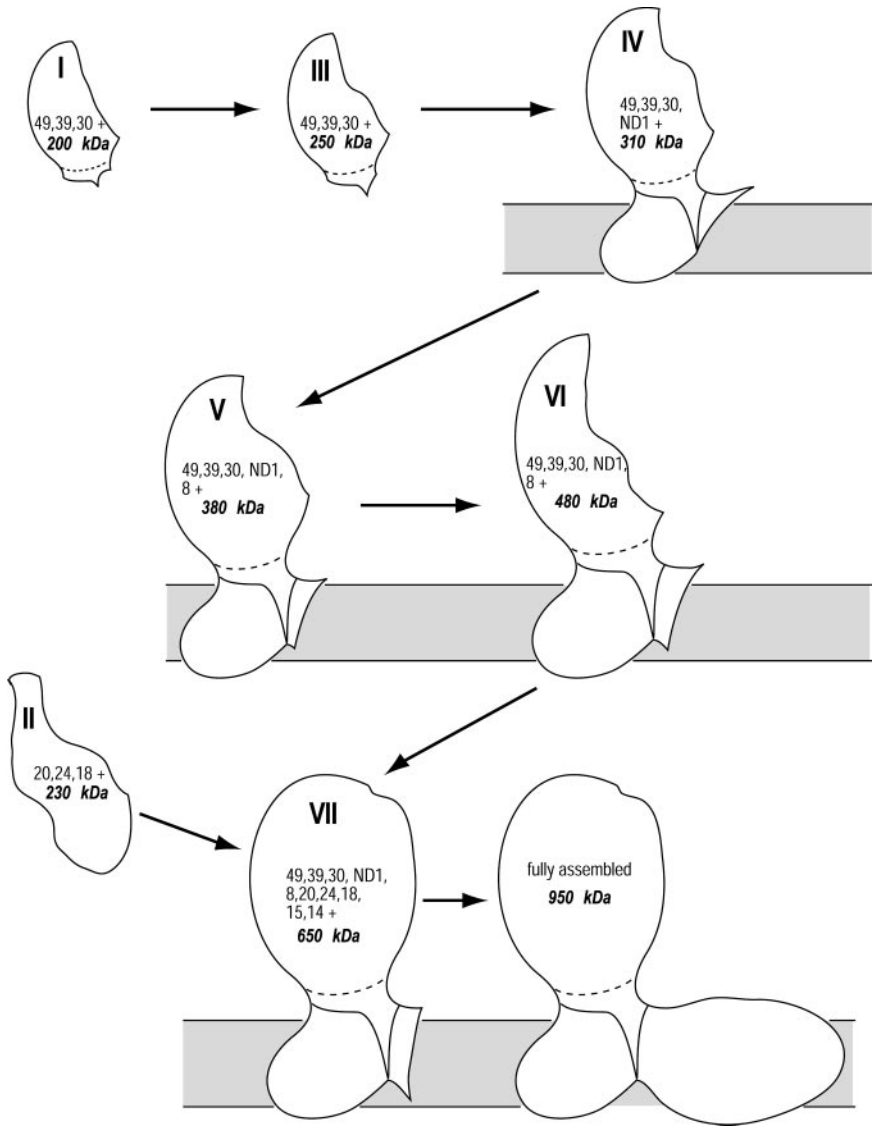

FIG. 5. **Complex I assembly model.** A proposed model for the assembly of complex I based on the studies presented in this report and the subcomplexes identified by dissociation with detergent in Ref. 9 is shown. The molecular mass of the subcomplexes is based on the migration on BN-PAGE and is indicated in *bold*. The different subcomplexes are identified with *Roman numerals* as in Table II. + indicates that other (not studied) subunits are present in the subcomplex.

plex II, with a molecular mass of ~230 kDa, did not contain any of the other subunits studied here. Subunits ND1 and 8 kDa showed a similar distribution among the subcomplexes, although they belong to different parts of the holoenzyme complex, the membrane and peripheral arms, respectively. They were both detected in subcomplexes V–VII, and the ND1 subunit was also found in subcomplex IV. The last group contains the 15- and 14-kDa subunits, which are part of subcomplex VII. The 17-kDa subunit, described as belonging to the membrane Iβ subcomplex (9), appeared as a very faint streak on the immunoblot and was the only subunit that we were not able to assign to any subcomplex. The additional subcomplex of about 830 kDa observed in mitochondria from patients B1 and B2 was not found in fibroblasts or muscle from 10 other complex I patients (data not shown). Two-dimensional PAGE showed the presence of ND1, 49-, 39-, 30-, 20-, 18-, 15-, and 14-kDa subunits in the 830-kDa subcomplex with intensities similar to those found in the fully assembled complex, suggesting a late assembly defect in patients B1 and B2.

Triepels *et al.* (44) studied complex I subcomplexes in several patients with complex I deficiency using a standard immunoblot analysis to measure the steady-state levels of individual structural subunits (which would include free subunits and those in partially or fully assembled complexes) and sucrose gradient centrifugation to investigate assembly patterns. They divided the subunits into three classes depending on their expression levels and complex I activity in the studied patients: expression levels of 39- and 30-kDa subunits most closely paralleled the loss of activity; levels of 20- and 18-kDa subunits were higher and levels of 15- and 8-kDa subunits were lower than predicted from the activity measurement. In this study, we also assigned 39- and 30-kDa subunits and 20- and 18-kDa subunits into two separate groups based on their presence in complex I subcomplexes; however, the 15- and 8-kDa subunits belong to different groups in our study.

Several studies have focused on determining the role of mtDNA-encoded subunits of complex I on enzyme assembly. The deletion of human ND4 subunit caused a failure of other mtDNA-encoded subunits to assemble; however, the nuclear subunits involved in the redox reactions appeared to form an intermediate with normal NADH:$Fe(CN)_6$ oxidoreductase activity (23). A similar result has been reported in an ND4 mutant in plants, where a subcomplex with NADH dehydrogenase activity was characterized (45). MtDNA subunits ND2 and ND3 are required for the membrane arm assembly in *N. crassa* (46), and the ND6 protein is necessary for the membrane arm assembly in mouse mitochondria (47). In contrast, a lack of the ND5 subunit does not influence complex I assembly in humans (48). In the unicellular green alga *Chlamydomonas reinhardtii*, the absence of the ND1 or ND6 subunit prevented the assembly

of complex I, whereas the loss of the ND4 or ND4/ND5 led to the formation of a 650-kDa subcomplex with NADH dehydrogenase activity (49). The loss of the ND2 protein in patient S compromised the assembly of complex I and led to the formation of complex I subcomplexes; however, NADH dehydrogenase activity could not be detected in these subcomplexes by an in-gel activity stain. This indicates a significant role for the ND2 subunit in the assembly and/or stability of complex I. The location of the ND2 subunit in the center of Iγ subcomplex in the vicinity of the ND1 protein (9) suggests that the absence of this subunit causes the disruption of the complex in patient mitochondria.

Much less is known about the role of nuclear-encoded subunits of mammalian complex I in the assembly of the complex. A patient with a mutation in the *NDUFS4* gene encoding the 18-kDa subunit was unable to assemble complex I (19), but no subcomplexes were described in that case. Interestingly, when *nuo21*, the *NDUFS4* orthologue in *N. crassa*, was disrupted, the mutant was able to assemble an almost intact enzyme. Subcomplexes of ~500 and 200 kDa, containing the 20- and 39-kDa subunits, respectively (44), were described in a patient suggested to have a mutation in a complex I assembly factor.

The current model of complex I biogenesis and assembly is based on studies in *N. crassa*, which contains about 35 subunits, at least three of which are not found in mammalian complex I and whose function remains unknown (50, 51). The identification and characterization of complex I subcomplexes in this study allow us to propose a model for complex I assembly in humans (Fig. 5) that differs significantly from that proposed for *N. crassa*. Four assembly intermediates have been identified in the *N. crassa* enzyme: the peripheral arm, a large and a small subcomplex of the membrane arm, and the entire membrane arm itself (52). The mature complex is thought to assemble by stepwise association of these modules. Disruption of specific complex I structural subunits disturbs holoenzyme assembly, resulting in the accumulation of one or more of these enzyme subcomplexes (53); however, mutations in subunits belonging to one arm do not interfere with assembly of the other arm. On the contrary, they lead to the accumulation of the other subcomplexes of the enzyme. Subcomplexes of the peripheral arm have not been observed, nor have subcomplexes involving the peripheral arm and part of the membrane arm. The results of our study on the human enzyme stand in sharp contrast to this picture: many more assembly intermediates are observed, including subcomplexes of the peripheral arm and subcomplexes containing parts of both arms, suggesting that the peripheral and membrane arms are not assembled in separate, independent pathways. In our model, the peripheral arm of mammalian complex I is assembled as two independent units (subcomplexes I and II). Subcomplex I associates with other subunits, forming subcomplex III, which is in turn attached to subunit(s) that are part of the membrane arm, forming subcomplex IV. Since ND1 subunit is a hydrophobic integral membrane protein, we can speculate that this subunit is inserted into the membrane prior to its association with the subcomplex III, hence subcomplex IV, which contains part of the peripheral arm and part of the membrane arm, is membrane-bound. Additional subunits are added to this subcomplex, resulting in subcomplexes V and VI. The next step of assembly requires coupling of subcomplex II (which forms part of the peripheral arm) and subcomplex VI and addition of other subunits, resulting in the formation of subcomplex VII. The remaining subunits are further associated with subcomplex VII and produce a fully assembled complex.

Two chaperones, CIA30 and CIA84, play an essential role in the assembly of the membrane arm of the *N. crassa* complex I, interacting with the large subcomplex of the membrane arm and promoting its association with the smaller component to form the entire membrane arm (28). A ubiquitously expressed human homologue of only one of these, CIA30, has been described (54), but its function is not yet known, and DNA sequence analysis of 13 candidate complex I patients (with putative assembly defects) has not revealed any mutations in this gene (54).

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