

## Analysis of the Assembly Profiles for Mitochondrial- and Nuclear-DNA-Encoded Subunits into Complex I<sup>∇</sup>

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Received 15 January 2007/Returned for modification 14 February 2007/Accepted 5 April 2007

**Complex I of the respiratory chain is composed of at least 45 subunits that assemble together at the mitochondrial inner membrane. Defects in human complex I result in energy generation disorders and are also implicated in Parkinson's disease and altered apoptotic signaling. The assembly of this complex is poorly understood and is complicated by its large size and its regulation by two genomes, with seven subunits encoded by mitochondrial DNA (mtDNA) and the remainder encoded by nuclear genes. Here we analyzed the assembly of a number of mtDNA- and nuclear-gene-encoded subunits into complex I. We found that mtDNA-encoded subunits first assemble into intermediate complexes and require significant chase times for their integration into the holoenzyme. In contrast, a set of newly imported nuclear-gene-encoded subunits integrate with preexisting complex I subunits to form intermediates and/or the fully assembly holoenzyme. One of the intermediate complexes represents a subassembly associated with the chaperone B17.2L. By using isolated patient mitochondria, we show that this subassembly is a productive intermediate in complex I assembly since import of the missing subunit restores complex I assembly. Our studies point to a mechanism of complex I biogenesis involving two complementary processes, (i) synthesis of mtDNA-encoded subunits to seed de novo assembly and (ii) exchange of preexisting subunits with newly imported ones to maintain complex I homeostasis. Subunit exchange may also act as an efficient mechanism to prevent the accumulation of oxidatively damaged subunits that would otherwise be detrimental to mitochondrial oxidative phosphorylation and have the potential to cause disease.**

Complex I (NADH-ubiquinone oxidoreductase) is the major entry point of electrons into the electron transport chain and contributes to the establishment of a proton gradient that is required for the bulk of cellular ATP synthesis (30). Complex I is the largest and most complicated structure of the mitochondrial respiratory chain. We have yet to gain a clear understanding of how this enzyme functions in the cell due to the lack of detailed structural information, as well as significant evolutionary divergence between its human and lower cellular forms. In mammals, complex I contains 45 different subunits and forms a complex of ~1 MDa (5). Seven complex I subunits are encoded by mitochondrial DNA (mtDNA), while the remainder are encoded by nuclear genes and then translated in the cytosol before being imported into the organelle via the protein import machineries (11, 40). Following import, the targeting signals are often, but not always, cleaved before the protein is folded and assembled. Assembled complex I is also known to associate with complex III and complex IV into supercomplexes or “respirasomes” (26). The role of these supercomplexes is not clear but may involve substrate channeling, as well as complex stability (1, 17, 27).

Defects in complex I activity are the most common diagnosis in patients with energy generation disorders (32). In most cases, these defects seem to correlate with a reduction in complex I activity and/or defects in its assembly (31). Complex I

defects, and the associated generation of reactive oxygen species, have also been implicated in common neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis, as well as aging and apoptosis (39). A clearer understanding of the complex I assembly pathway, including the roles of additional cofactors and chaperones, will provide insights into how dysfunction of this enzyme results in disease.

While the assembly process is poorly understood, a number of proteins involved in complex I biogenesis have been reported. The human ortholog of *Neurospora crassa* CIA30, termed NDUF1, is required for complex I assembly in a process that has yet to be clearly defined (38). In addition, roles for apoptosis-inducing factor, complex III, and complex IV in complex I biogenesis have been identified (1, 7, 27, 36). More recently, the protein B17.2L was found associated with an ~800-kDa form of complex I present in mitochondria from patients lacking the subunit NDUF54. A patient with a mutation in the *B17.2L* gene also exhibited reduced levels of complex I. Although the exact function of B17.2L is not known, its absence from fully assembled complex I suggests that it acts as a molecular chaperone/assembly factor (19).

Previous studies of mammalian complex I biogenesis have used patient cells containing assembly defects to generate models of complex I assembly (3, 34). Antonicka et al. (3) identified a number of complexes in different patient mitochondria, classifying them as assembly intermediates. These intermediates were then used to construct a unique assembly model for complex I. However, it was argued that these complexes may not be true assembly intermediates but rather mis-

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<sup>∇</sup> Published ahead of print on 16 April 2007.

assembled or partly degraded complexes that are due to the complex I subunit mutations present in the patients studied (34, 37, 42). In a separate study, incorporation of subunits into new complex I assemblies was monitored in cells that had been depleted of complex I holoenzyme by pretreatment with doxycycline (35). Assembly intermediates different from those observed by Antonicka et al. (3) were identified, leading to the proposal that human complex I is assembled in an evolutionarily conserved, modular form (34, 37). In this study, we have addressed the biogenesis of complex I in mitochondria containing the preexisting holoenzyme, as well as in patient mitochondria containing complex I assembly defects. We analyzed the assembly profiles of newly synthesized mtDNA-encoded subunits and performed *in vitro* assays of the mitochondrial import and assembly of individual nuclear-gene-encoded subunits and provide evidence for two processes in complex I biogenesis—*de novo* complex I synthesis and regeneration of existing complexes by subunit exchange.

#### MATERIALS AND METHODS

**Cloning procedures.** The cDNAs encoding human NDUFV3, NDUFS4, NDUFS6, NDUFS7, NDUF9A, NDUF9A10, and NDUFS2 (accession numbers BC021217, BC005270, BC038664, BC111517, BC009311, BC003417, and BC008868, respectively) were obtained from the I.M.A.G.E Consortium (Medical Research Council, Cambridge, United Kingdom). The cDNAs encoding NDUFS1, NDUFV1, NDUFV2, NDUFB6, NDUFB8, and B17.2L (accession numbers NM\_005006, NM\_007103, NM\_021074, NM\_002493, NM\_005004, and NM\_174889) were amplified by PCR from a human placental cDNA library (BD Biosciences, Clontech) and cloned into pGEM-4Z (Promega). For expression of NDUF9A, the cDNA, lacking its initiation codon and mitochondrial targeting sequence, was cloned into pQE-30 (QIAGEN) at BamHI and SmaI restriction sites. All clones were verified by dideoxynucleotide sequencing.

**Cell culture and mitochondrial isolation.** Primary skin fibroblasts grown from patient skin biopsy material were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) containing 10% (vol/vol) fetal calf serum (FCS) at 37°C under an atmosphere of 5% CO<sub>2</sub> and 95% air, supplemented with 50 µg/ml uridine. Mitochondria were isolated by homogenization of cells in 20 mM HEPES (pH 7.6)–220 mM mannitol–70 mM sucrose–1 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride. Cell homogenates were centrifuged at 700 × g at 4°C for 10 min to obtain a postnuclear supernatant. Mitochondria were pelleted by centrifugation at 10,000 × g at 4°C for 10 min.

**mtDNA-encoded subunit labeling and chase assays.** Labeling of mtDNA-encoded subunits was performed essentially as previously described (6, 16). Control lymphoblasts were incubated with or without 50 µg/ml chloramphenicol (CAP) in DMEM containing 5% (vol/vol) FCS for 16 h before washing and addition of 0.1 mg/ml cycloheximide in methionine-free DMEM–5% (vol/vol) dialyzed FCS for 15 min at 37°C. Labeling was performed by addition of 20 µCi of [<sup>35</sup>S]methionine-cysteine (EXPRE<sup>35</sup>S Protein Labeling Mix; Perkin-Elmer Life Sciences) and incubation for 2 h, followed by addition of unlabeled methionine to a final concentration of 1 mM. After 15 min, the solution was removed and replaced with normal DMEM–5% (vol/vol) FCS. Cells were harvested at various chase times, and mitochondria were isolated as described above.

**Mitochondrial import and assembly assays.** Generation of radiolabeled, nuclear-gene-encoded precursor proteins was performed by *in vitro* transcription, followed by translation with rabbit reticulocyte lysates (Promega) in the presence of [<sup>35</sup>S]methionine-cysteine protein labeling mix (Perkin-Elmer) as previously described (23). Translation products were incubated with freshly isolated mitochondria in 20 mM HEPES-KOH (pH 7.4)–250 mM sucrose–80 mM potassium acetate–5 mM magnesium acetate–10 mM sodium succinate–5 mM methionine at 37°C for various times, as indicated in the figure legends. Dissipation of membrane potential ( $\Delta\psi_m$ ) was carried out with 10 µM valinomycin (Sigma). Samples subjected to protease treatment were incubated on ice for 10 min with 100 µg/ml proteinase K (PK; Sigma) before treatment with 1 mM phenylmethylsulfonyl fluoride for 10 min. For Tris-Tricine sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (28), mitochondrial pellets (25 µg) were first precipitated with trichloroacetic acid according to Alconada et al. (2). For blue native PAGE (BN-PAGE), all mitochondrial pellets (50 µg protein) were resuspended in either 50 µl 1% (wt/vol) digitonin (Calbiochem), 1% (wt/

vol) *n*-dodecyl-β-D-maltoside (DDM; Sigma), or 1% (wt/vol) Triton X-100 (Sigma) in 50 mM NaCl–10% (vol/vol) glycerol–20 mM Bis-Tris (pH 7.0) and subjected to 4 to 13% gradient BN-PAGE, with or without SDS-PAGE in the second dimension, according to McKenzie et al. (16). Subunit spots on two-dimensional PAGE were correlated based on migration patterns, assembly into appropriate complexes, and in some cases Western blot analysis. Thyroglobulin (669 kDa), ferritin (440 kDa), and bovine serum albumin (134 and 67 kDa) were used as markers for BN-PAGE.

**Miscellaneous.** NDUF9A polyclonal antibodies were raised in rabbits and used to detect complex I. Antibodies against the 70-kDa subunit and the core I subunit were used to detect complex II and complex III, respectively (Invitrogen). Radiolabeled proteins were detected by PhosphorImager analysis (Molecular Dynamics). Western blotting was performed by a semidry transfer method (9). Horseradish peroxidase-coupled secondary antibodies and ECL chemiluminescent substrate (Amersham) were used to detect immunoreactive proteins in blots. Images were captured with a ChemiGenius chemiluminescence detection system (SynGene).

## RESULTS

**Mitochondrially encoded subunits assemble into complex I via intermediates.** In order to analyze the assembly of mtDNA-encoded complex I subunits, cells were incubated with [<sup>35</sup>S]Met-Cys in the presence of cycloheximide to block cytosolic protein synthesis. This results in the preferential radiolabeling of the seven complex I subunits, as well as the other six subunits of complexes III, IV, and V. The specific labeling of mtDNA-encoded subunits can be seen through SDS-PAGE analysis (Fig. 1A). Cells were directly harvested or incubated for longer times following removal of radiolabel and cycloheximide (chase), before solubilization in 1% Triton X-100 and analysis by BN-PAGE (Fig. 1B). While some complexes (e.g., complex V [CV]) were found to contain radiolabeled subunits immediately after the labeling period (0 h of chase), assembly of subunits into complex I occurred later—between 3 and 24 h of chase (Fig. 1B, lanes 1 to 4). Neither the levels of steady-state complex I holoenzyme nor the labeling profiles of mtDNA-encoded subunits were altered over this chase period (data not shown). The assembly of mtDNA-encoded subunits was enhanced by first treating cells with CAP, which increases the pool of unassembled nuclear-gene-encoded subunits within mitochondria (6, 16). When this was performed (Fig. 1B, lanes 5 to 8), the complexes were more strongly radiolabeled yet assembly of the subunits into complex I still required a chase time of between 3 and 24 h. However, with the CAP pretreatment, additional broadly labeled complexes were visible from 0 to 3 h of chase (Fig. 1B, lanes 5 to 7, labeled a and b) before disappearing after 24 h of chase (Fig. 1B, lane 8).

We next addressed whether these broad complexes contain complex I subunits by performing SDS-PAGE in the second dimension (Fig. 1C). Mitochondria were again solubilized in 1% Triton X-100 for the first-dimension BN-PAGE, but in this case the mitochondrial protein-to-detergent ratio was higher than that used for the experiment shown in Fig. 1B, resulting in the presence of more complex I/complex III<sub>2</sub> supercomplex (SC). In this analysis, spots representing mtDNA-encoded subunits of complexes I (ND1, ND2, ND3, ND4, and ND6), III, IV, and V are seen. At the 0- and 1-h chase times, radiolabeled complex I subunits ND1, ND2, ND3, and ND6 were preferentially found in a number of complexes in the 350- to 500-kDa range (Fig. 1C, labeled b) or in an ~800-kDa complex (Fig. 1C, labeled a). At 3 h of chase, these subunits accumulated more in the ~800-kDa species before being chased to the complex I

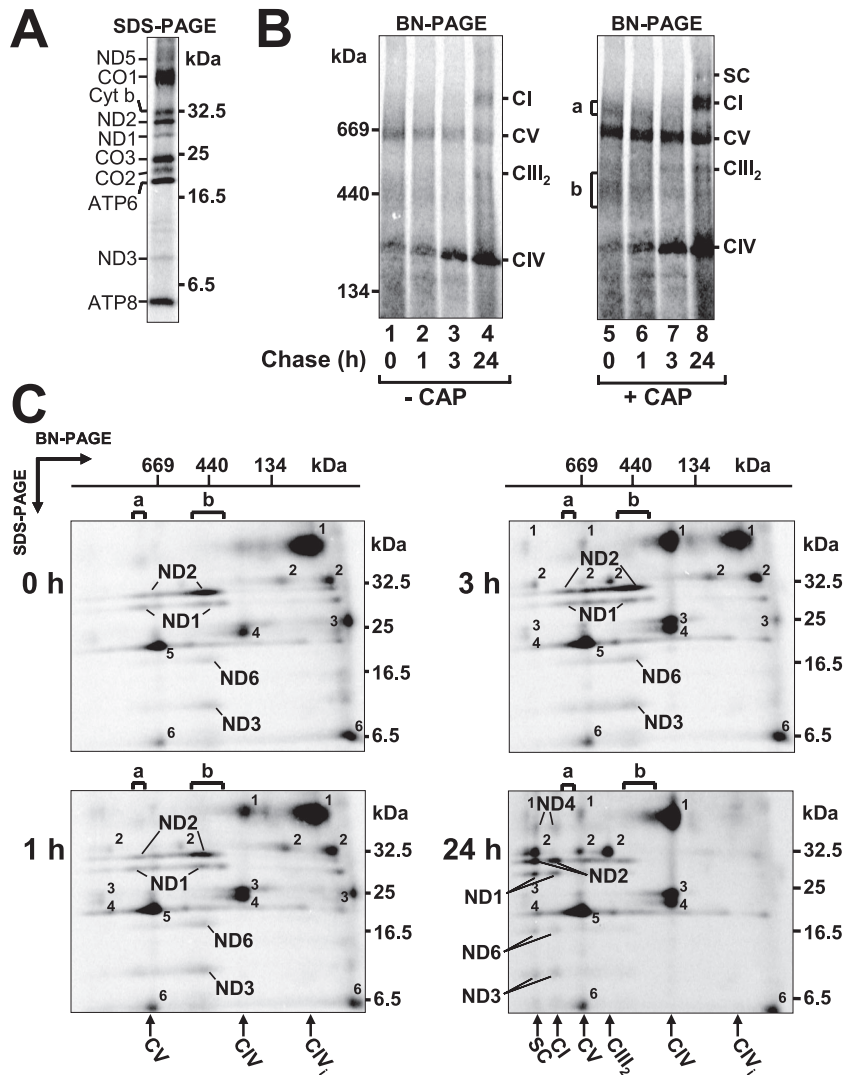


FIG. 1. Mitochondrially encoded subunits assemble into intermediate subcomplexes. mtDNA-encoded subunits were pulse-chase labeled in wild-type lymphoblasts, followed by mitochondrial isolation. (A) Labeling profile of mtDNA-encoded subunits on SDS-PAGE. (B) BN-PAGE analysis of mitochondria solubilized in Triton X-100. mtDNA-encoded subunits were labeled without (left side) or following (right) CAP pretreatment. The letters a and b indicate regions of potential assembly intermediates. CI, complex I; CIII<sub>2</sub>, complex III homodimer; CIV, complex IV; SC, CI/CIII<sub>2</sub> supercomplex. (C) Two-dimensional PAGE analysis of mitochondria isolated from radiolabeled lymphoblasts after 0, 1, 3, and 24 h of chase. For the first-dimension BN-PAGE, mitochondria were solubilized in 1% Triton X-100 as in panel B but at a higher mitochondrial protein/detergent ratio. Regions a and b include complex I intermediate subcomplexes containing subunits ND1, ND2, ND3, and ND6. Subunits CO1 (1), cytochrome *b* (2), CO3 (3), CO2 (4), ATP6 (5), and ATP8 (6) are indicated. CIV<sub>i</sub>, complex IV intermediate.

holoenzyme (CI) and the supercomplex (SC) after 24 h (Fig. 1C). We conclude that mtDNA-encoded subunits assemble into the complex I holoenzyme through a number of intermediates and that significant maturation times are required, even in the presence of a matrix pool of unassembled nuclear-gene-encoded subunits.

**Import and assembly of nuclear-gene-encoded subunits.** The biogenesis of individual nuclear-gene-encoded complex I subunits can be individually characterized by *in vitro* importing radiolabeled subunits into isolated mitochondria and addressing assembly by BN-PAGE. Since no other proteins are made during this time, any assembly represents an interaction between the imported radiolabeled subunit with preexisting subunits or complexes. The subunit NDUFB3 (10 kDa) contains

an N-terminal, cleavable targeting signal and, as a flavoprotein subunit, is predicted to assemble into the tip of the matrix arm of complex I (10, 34). NDUFB3 was *in vitro* translated in rabbit reticulocyte lysate as a <sup>35</sup>S-labeled precursor and incubated for various times with mitochondria that were freshly isolated from cultured human primary fibroblasts. In one sample, the mitochondrial membrane potential ( $\Delta\psi_m$ ) was dissipated with uncouplers prior to addition of <sup>35</sup>S-labeled precursor, thereby blocking the import process. External PK was added to half of each sample to degrade the nonimported radiolabeled precursor. Samples of each were then subjected to SDS-PAGE, and radioactive protein was subsequently detected by phosphorimaging (Fig. 2A). As can be seen, the <sup>35</sup>S-labeled NDUFB3 precursor binds to mitochondria and

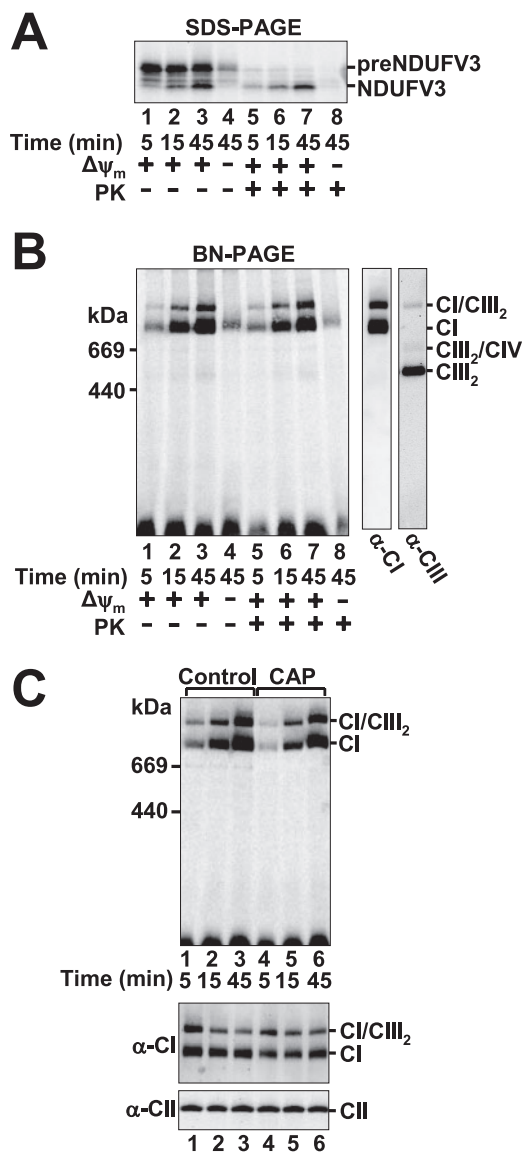


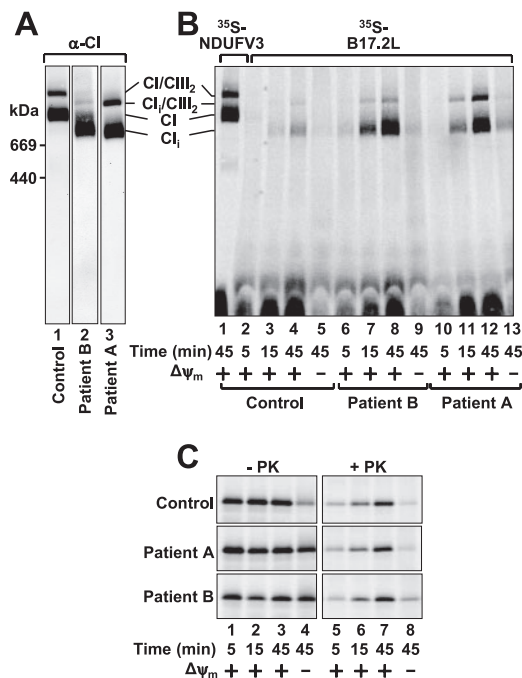
FIG. 2. Import and assembly of NDUFV3 into preexisting complex I. <sup>35</sup>S-labeled precursor to NDUFV3 (pre-NDUFV3) was incubated for different times with mitochondria isolated from fibroblasts in the presence or absence of a membrane potential ( $\Delta\psi_m$ ). Samples were treated with or without PK and subjected to SDS-PAGE (A) or solubilized in DDM-containing buffer and then subjected to BN-PAGE (B). Radiolabeled proteins were detected by PhosphorImager analysis. CI, complex I; CIII<sub>2</sub>, complex III homodimer; CIII<sub>2</sub>/CIV, complex III/complex IV supercomplex; CI/CIII<sub>2</sub>, complex I/complex III<sub>2</sub> supercomplex. As shown at the right side of panel B, the migration of complex I (CI) and its supercomplex with dimeric complex III (CI/CIII<sub>2</sub>) was identified by Western blot analysis with antibodies to complex I subunit NDUFV3 (α-CI) and the complex III core 1 subunit (α-CIII). (C) <sup>35</sup>S-labeled NDUFV3 was incubated for 5 to 45 min with mitochondria isolated from control fibroblasts that had been pre-treated with (lanes 4 to 6) or without (lanes 1 to 3) CAP for 24 h. All samples were treated with externally added PK before being solubilized in DDM-containing buffer and subjected to BN-PAGE, Western transfer, and PhosphorImager analysis (top). After the radiolabeled signals were acquired, complex I (CI) and the CI/CIII<sub>2</sub> supercomplex were identified by Western blot analysis with antibodies to complex I subunit NDUFV3 (α-CI). The anti-70-kDa subunit antibody (α-CII) was used to detect complex II (CII) as a loading control (bottom).

over time an additional faster-migrating species accumulates (Fig. 2A, lanes 1 to 3). Based on (i) its processing, (ii) protection from externally added PK (Fig. 2A, lanes 5 to 7), and (iii) the fact that it does not appear in mitochondria lacking a  $\Delta\psi_m$  (lanes 4 and 8), we conclude that this species represents the imported mature form of NDUFV3.

In addition to SDS-PAGE, each sample was also solubilized in DDM and subjected to BN-PAGE (Fig. 2B). With this detergent, complex I is found in both its monomeric (~980 kDa) and supercomplex (complex I/III<sub>2</sub>) forms (17), as demonstrated by immunoblot analysis with antibodies against complex I subunit NDUFV3 and the complex III core 1 subunit (Fig. 2B, right side). Imported <sup>35</sup>S-labeled NDUFV3 readily assembles into both forms of complex I with the signal accumulating over time, consistent with its import shown in Fig. 2A. When  $\Delta\psi_m$  was dissipated, the assembly of <sup>35</sup>S-labeled NDUFV3 was dramatically reduced (Fig. 2B, compare lanes 4 and 8 versus lanes 3 and 7). These results indicate that <sup>35</sup>S-labeled NDUFV3 assembles into complex I with the other 44 subunits that are already present in the isolated mitochondria. The possibility exists that NDUFV3 is the only complex I subunit in limiting amounts in mitochondria. However, if other individual subunits can also assemble into complex I following their import into isolated mitochondria, it would mean that either (i) newly imported subunits preferentially assemble into new complex I forms at the expense of preexisting subunits or (ii) the imported subunits are exchanged for previously assembled subunits via a dynamic process. We therefore imported <sup>35</sup>S-labeled NDUFV3 into mitochondria isolated from cells that had been preincubated with CAP. In this case, complex I assembly intermediates containing mtDNA-encoded subunits are unlikely to be present while a pool of nuclear-gene-encoded subunits will also accumulate in mitochondria. Radiolabeled NDUFV3 is therefore less likely to assemble into complex I via intermediates and also needs to compete with the increased pool of free NDUFV3. As shown at the top of Fig. 2C, <sup>35</sup>S-labeled NDUFV3 still rapidly assembles into complex I and its supercomplex in mitochondria from control and CAP-treated cells. Western blot analysis of these lanes (Fig. 2C, bottom parts) revealed that the steady-state levels of complex I were reduced in CAP-treated cells, relative to complex II (which lacks mtDNA-encoded subunits), consistent with the prevention of any new complex I assembly by CAP treatment.

We next tested the import and assembly of a variety of different nuclear-gene-encoded complex I subunits. Representative subunits with cleavable (NDUFV1, NDUFV2, NDUFV3, NDUFV8, NDUFV2, NDUFV4, NDUFV6, NDUFV7, NDUFV9, and NDUFV10) and noncleavable (NDUFV1) presequences, predicted transmembrane domains (NDUFV9, NDUFV8, and NDUFV7), and/or cofactor attachment (NDUFV1, NDUFV2, NDUFV1, NDUFV2, and NDUFV7) were chosen. The predicted subunit locations in holocomplex I (based on the models described in references 4, 10, and 25) are shown schematically in Fig. 3A. Radiolabeled precursors were incubated with isolated mitochondria in the presence or absence of  $\Delta\psi_m$ , and all samples were treated with PK before BN-PAGE analysis. As shown in Fig. 3A, in addition to NDUFV3 (lanes 5 to 6), the newly imported subunits NDUFV1, NDUFV2 (lanes 1 to 4), NDUFV4, NDUFV6 (lanes 13 to 16), and NDUFV9 (lanes 19 to 20) clearly assembled into both monomeric complex I (CI)

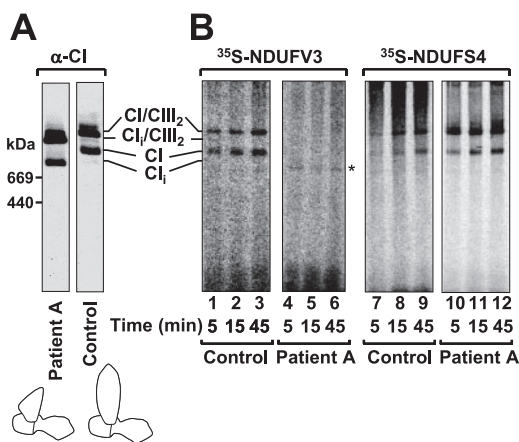




**FIG. 4.** The assembly factor B17.2L is associated with a complex I subassembly in patient and control mitochondria. (A) Mitochondria from control cells (lane 1) and patient cells lacking subunit NDUF6 (patient B, lane 2) or NDUF4 (patient A, lane 3) were solubilized in DDM-containing buffer and subjected to BN-PAGE before Western blot analysis with complex I antibodies (anti-NDUFA9). (B) <sup>35</sup>S-labeled B17.2L was incubated with mitochondria isolated from control or patient cells for increasing times in the presence or absence of a  $\Delta\Psi_m$ . Half of each sample was treated with external PK before being split in two and subjected to BN-PAGE (protease-treated samples only) or (C) SDS-PAGE and phosphorimaging. As shown in lane 1 of panel B, <sup>35</sup>S-labeled NDUFV3 was imported into control mitochondria to illustrate the fully assembled forms of complex I. CI/CIII<sub>2</sub>, complex I/complex III<sub>2</sub> supercomplex; CI<sub>i</sub>/CIII<sub>2</sub>, complex I intermediate/complex III<sub>2</sub> supercomplex; CI<sub>i</sub>, complex I intermediate; CI, complex I.

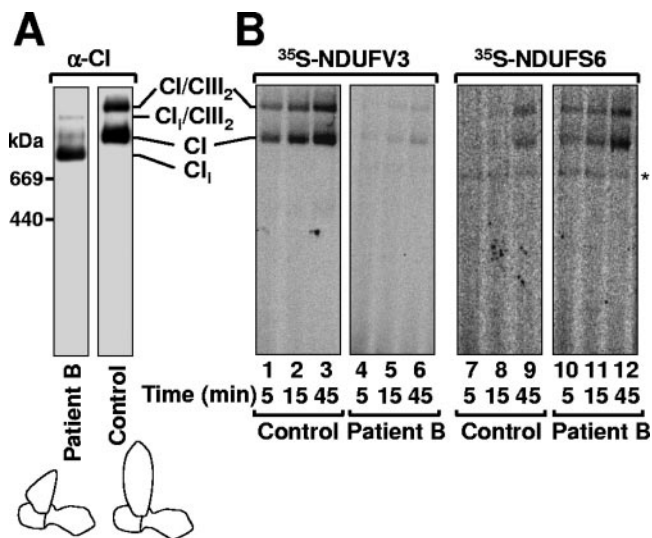
(CI) and supercomplex (CI/CIII<sub>2</sub>) forms of complex I in control mitochondria; however, it did not assemble into patient mitochondria (Fig. 5B, lanes 1 to 3 versus lanes 4 to 6). NDUFV3 is predicted to associate with the tip of the matrix arm of complex I (10), and since no assembly was observed in the patient mitochondria, it follows that this tip is missing. We next imported <sup>35</sup>S-labeled NDUF4 into isolated mitochondria. As previously shown, NDUF4 assembled into complex I in control mitochondria, forming the monomeric (CI) and supercomplex (CI/CIII<sub>2</sub>) forms (Fig. 5B, lanes 7 to 9). In patient mitochondria, imported <sup>35</sup>S-labeled NDUF4 also assembled into both complexes that were identical in size to those in control mitochondria (Fig. 5B, lanes 10 to 12). This indicates that assembly of complex I can be restored upon import of a single subunit. In addition, the assembly of <sup>35</sup>S-labeled NDUF4 into complex I was more efficient in the patient mitochondria than in the control mitochondria. This is consistent with the fact that in control mitochondria, imported <sup>35</sup>S-labeled NDUF4 competes with preexisting NDUF4 for assembly into complex I.

We tested whether restoration of complex I could also be



**FIG. 5.** Import of NDUF4 into patient mitochondria restores complex I assembly. Mitochondria from control or NDUF4-deficient patient A cells were incubated with <sup>35</sup>S-labeled NDUFV3 (as a control) or <sup>35</sup>S-labeled NDUF4 for 5 to 45 min as indicated before BN-PAGE analysis. (A) Western blot analysis of complex I (probed with NDUFA9 antibodies) and (B) PhosphorImager analysis of import and assembly. Schematic models depicting complex I forms are shown. CI/CIII<sub>2</sub>, complex I/complex III<sub>2</sub> supercomplex; CI<sub>i</sub>/CIII<sub>2</sub>, complex I intermediate/complex III<sub>2</sub> supercomplex; CI<sub>i</sub>, complex I intermediate; CI, complex I. The asterisk denotes a nonspecific complex.

seen by importing <sup>35</sup>S-labeled NDUF6 into mitochondria isolated from patient B fibroblasts (lacking endogenous NDUF6). Some <sup>35</sup>S-labeled NDUFV3 appeared to assemble into a complex of the same size as complex I (CI) (Fig. 6B, lanes 4 to 6), suggesting that complex I subunits can form



**FIG. 6.** Import of NDUF6 into patient mitochondria restores complex I assembly. Mitochondria from control or NDUF6-deficient patient B cells were incubated with <sup>35</sup>S-labeled NDUFV3 (as a control) or <sup>35</sup>S-labeled NDUF6 for 5 to 45 min as indicated before BN-PAGE analysis. (A) Western blot analysis of complex I (probed with NDUFA9 antibodies) and (B) PhosphorImager analysis of import and assembly. Schematic models depicting complex I forms are shown. CI/CIII<sub>2</sub>, complex I/complex III<sub>2</sub> supercomplex; CI<sub>i</sub>/CIII<sub>2</sub>, complex I intermediate/complex III<sub>2</sub> supercomplex; CI<sub>i</sub>, complex I intermediate; CI, complex I. The asterisk denotes a nonspecific complex.



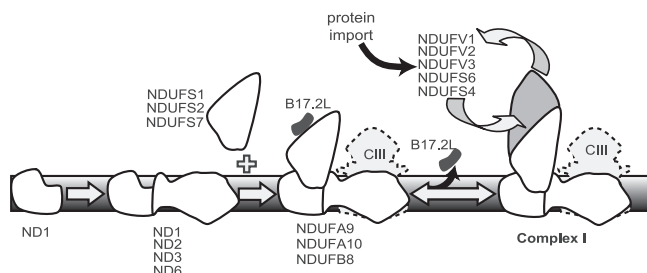


FIG. 9. Model depicting the assembly of complex I. De novo assembly occurs via the seeding of intermediate complexes through the synthesis of mitochondrially encoded (ND) subunits, followed by recruitment of other nuclear-gene-encoded subunits. ND1 is found in a separate, smaller complex before associating with other ND subunits. An intermediate containing the assembly factor B17.2L is present in the absence of NDUFS4 or NDUFS6. Their incorporation into the complex drives complete assembly and releases B17.2L. Complex I homeostasis can also occur by competition of newly imported subunits with preexisting ones for assembly into complex I. Complex I intermediates may be found in supercomplexes that include complex III.

to that of control mitochondria (see Fig. 3B). The survey of assembly in patient B (*NDUFS6* mutation) showed that subunits NDUFS2, NDUFS7, NDUFA9, and NDUFA10 were also able to integrate into the CI<sub>i</sub> subassembly (Fig. 8A, lanes 11 to 12 and 17 to 22). Subunits NDUFV1, FV2, FV3, and FS1 weakly assembled into the residual complex I (CI) that forms in the absence of NDUFS6 (Fig. 8A, lanes 1 to 8). As for patient A, the import of representative subunits into patient B mitochondria appeared to be unaffected (Fig. 8B). In summary, our results suggest that NDUFS4 and NDUFS6 are required for the assembly and stabilization of a portion of complex I that contains a number of subunits including, NDUFV1, NDUFV2, and NDUFV3 (Fig. 9). Furthermore, we conclude that some subunits can assemble with preexisting subunits into complex I while others, such as NDUFS1, NDUFS2, NDUFS7, and the mtDNA-encoded subunits, assemble into complexes that represent earlier intermediates in complex I assembly.

## DISCUSSION

In vitro import and assembly assays, combined with BN-PAGE, have been powerful tools for the analysis of mitochondrial membrane protein complexes, in particular, the protein import translocases (12, 13, 18, 22, 41). Here we have used a similar approach to address the assembly of respiratory complex I—an elaborate membrane protein complex containing subunits encoded by two separate genomes. A greater understanding of complex I assembly will not only provide insights into complex I function and subunit interactions but may also lead to new techniques for the diagnostic screening of patients with mitochondrial disorders.

**Complex I biogenesis.** All proteins encoded by mtDNA are subunits of the respiratory chain complexes. Most are very hydrophobic and appear to be cotranslationally inserted into the inner mitochondrial membrane through the actions of a number of proteins involved in ribosome docking and membrane insertion (8, 20). Labeling and chase assays revealed that complex I subunits encoded by mtDNA required longer chase

times for their assembly into the holoenzyme, compared to mtDNA-encoded subunits of the other respiratory complexes. This was also seen in other cell lines, including primary fibroblasts and lymphoblasts (data not shown). Even in the presence of an increased pool of nuclear-gene-encoded subunits following treatment with CAP, assembly of mtDNA subunits into holoenzyme I was not accelerated, although larger, stable intermediates were observed. The presence of assembly intermediates containing mtDNA-encoded subunits is in concordance with other studies (3, 33, 35) and suggests that even in the presence of fully assembled endogenous complex I, mtDNA-encoded subunits assemble via intermediate modules. The modular model of assembly of mtDNA subunits would be consistent with the highly hydrophobic nature of these proteins and their location in the core of complex I.

In contrast, many nuclear-gene-encoded subunits are not hydrophobic and their assembly profile may differ from that of mtDNA-encoded subunits. Until now, the assembly of newly imported subunits in mitochondria containing endogenous levels of complex I has not been investigated. Initially, NDUFV3 was identified as a subunit that can assemble into both monomeric and supercomplex forms of complex I. Although assembly into the complex was kinetic, an assembly pathway composed of intermediates prior to formation of the holoenzyme was not observed. This indicates that NDUFV3 is incorporated at a late stage in complex I assembly, consistent with its location in the matrix tip of the complex and the fact that it is not present in the ~800-kDa subassembly in patient cell lines. An assembly scan with subunits located in different regions within the complex was performed to identify other subunits that can assemble into complex I in vitro. This analysis also addressed whether there is a correlation between subunit characteristics (such as location, presence of cofactors, etc.) and their competency to assemble into complex I. This scan resulted in the identification of a host of different subunits that could assemble into complex I to various degrees; however, there did not appear to be any correlation between the assembly competency of a subunit and its location in the complex or its posttranslational modifications.

The assembly survey also revealed that a number of subunits, such as NDUFB8, NDUFS1, NDUFS2, NDUFS7, and NDUFA9, integrate into additional complexes. Investigations by Antonicka et al. (3) found an ~480-kDa complex in patients containing at least NDUFA2, NDUFA9, NDUFS2, NDUFS3, and ND1. We also found that subunits NDUFS1 and NDUFS2 assembled into two complexes in the ~480- and 550-kDa ranges. NDUFS7 was also found in a similar-sized complex (Fig. 7). These complexes also resemble those seen for mtDNA-encoded subunit ND1 (Fig. 1C). The bovine orthologs of these subunits (75 kDa [NDUFS1], 49 kDa [NDUFS2], PSST [NDUFS7], and ND1) are thought to integrate into a common assembly intermediate as part of the modular model of complex I assembly (37). This is also consistent with the recent crystal structure of the peripheral arm of complex I from *Thermus thermophilus* where subunits Nqo4, Nqo3, and Nqo6, which are orthologs of NDUFS2, NDUFS1, and NDUFS7, respectively, are found adjacent to one another (24). In addition to these subunits, NDUFV1, NDUFV2, NDUFA9, and NDUFA10 also appeared to partially assemble into these subcomplexes. NDUFV1 and NDUFV2 also form part of the



*T. thermophilus* peripheral arm, consistent with their association in human mitochondrial subassemblies.

Subunits NDUFV3, NDUFS4, and NDUFS6 all assembled efficiently into complex I with an absence of detectable intermediates. Given that no single complex I subunit tested is limiting for assembly, it is unlikely that separate preformed intermediates are present in mitochondria which await the import of a single subunit to drive complete complex I assembly. This therefore indicates that individual, newly imported subunits can compete with their preexisting counterparts to assemble into complex I. In support of this, mitochondria isolated from cells pretreated with CAP did not hinder the assembly of newly imported NDUFV3. This is also consistent with the more efficient assembly of either newly imported NDUFS4 or NDUFS6 in patient mitochondria which lack the subunit compared to control mitochondria which contain preexisting forms.

**B17.2L-associated subassemblies in patient mitochondria.** Ogilvie et al. (19) identified a molecular chaperone, termed B17.2L, that is associated with an ~800-kDa subassembly of complex I in patients with mutations in the genes encoding subunit NDUFV1 or NDUFS4. Our investigations into the import and assembly of B17.2L showed that this subassembly is also present in control mitochondria that already contain fully assembled complex I. The ~800-kDa intermediate is present at very low levels, and this is reflected by our inability to clearly detect the subassembly with antibodies against complex I. However, the signal we observed by using in vitro import and assembly assays is representative of only a small number of molecules imported into each mitochondrion and hence this is why it is possible to detect complexes that are present at such low levels. The presence of this subassembly in control mitochondria provides further evidence that the ~800-kDa complex observed in patient mitochondria is not an artifact of destabilized complex I but in fact represents a stalled assembly intermediate of the holoenzyme.

Although the mutations in patients A and B are in different genes, the subassemblies in both patients comigrate on BN-PAGE. However, patient B also appears to have low levels of a complex I indistinguishable in size from the holoenzyme, which presumably only lacks the 13-kDa NDUFS6 subunit. The ~800-kDa subassemblies appear to be associated with B17.2L, as observed by in vitro import and assembly assays (Fig. 4) and by immunoblot analysis (19). Furthermore, the assembly of B17.2L is much more efficient in patient mitochondria compared to the control, presumably because of the accumulation of the ~800-kDa form in the patients. Interestingly, we did not observe any B17.2L in the small amount of ~970-kDa complex I lacking NDUFS6 in patient B mitochondria, indicating that it must dissociate before complex I is completely assembled.

In addition to its association with the ~800-kDa complex, B17.2L was also found in a high-molecular-weight complex that represents its association with complex III in the supercomplex (CI<sub>I</sub>/CIII<sub>2</sub>). Presumably, the interaction of B17.2L with this higher-molecular-weight complex is still via its association with the complex I intermediate. These results indicate that complex I does not need to be fully assembled before associating into supercomplexes. Indeed, complex III and, perhaps, complex IV may act as scaffolds to facilitate complex I

assembly since loss of either has been found to lead to reduced complex I levels (1, 7).

**Import and assembly of complex I subunits in patient mitochondria.** Reintroduction of either subunit missing from patient mitochondria (NDUFS4 in patient A and NDUFS6 in patient B) by in vitro import facilitated the completion of complex I assembly. This indicates that the patient's mitochondria must still harbor the subunits that are lacking from the unassembled portion of the complex and that these subunits are maintained in a form that allows for their productive assembly. We have been unable to detect whether these unassembled subunits form a separate complex, perhaps in association with novel assembly factors. Of note, NDUFV1 and NDUFV2 assembled into an ~300-kDa complex in mitochondria lacking NDUFS4 (Fig. 7A), which was not observed in control mitochondria. In addition, given that reintroduction of the missing subunit into patient mitochondria could drive complete assembly of complex I, the ~800-kDa subassembly can be viewed as a productive intermediate. This is also consistent with the association of B17.2L with the subassembly in patient mitochondria containing separate gene mutations.

A subunit survey in the patients provided further information as to the predicted locations of certain subunits and also aided in determining those subunits which are found in the ~800-kDa subassembly and those which are not. Subunits NDUFS2, NDUF9, and NDUF10 assembled into the ~800-kDa subassembly in the patients, while NDUFS6, NDUFS4, and NDUFV3 did not, suggesting that the latter are located in the missing tip of the complex.

The ability to follow the assembly of newly imported, nuclear-gene-encoded complex I subunits has opened a new perspective on the biogenesis of complex I. Since complex IV of the respiratory chain is assembled via intermediates that involve a number of chaperones (29), it is suspected that complex I is assembled in a similar fashion. Although it is likely that newly assembled complex I is formed via an assembly pathway, not every subunit that is imported into mitochondria must be incorporated into new assemblies; instead, a subunit may assemble with preexisting subunits that undergo dynamic transitions between intermediate and fully assembled complex I forms. Furthermore, these forms are most likely found in higher-molecular-weight supercomplexes. This is depicted schematically in Fig. 9. Such a mechanism would explain how multiple subunits can individually assemble into complex I in the absence of other subunit synthesis or import.

Complex I transitions between assembled and intermediate forms may enable a mechanism to aid in the turnover of subunits, some of which are prone to oxidative damage. Indeed, it was recently found that a number of complex I subunits had become oxidatively damaged in the brains of patients with Parkinson's disease (14). Given our findings, it is possible that the underlying basis of mitochondrial disease is due to decreased rates in mitochondrial protein import and/or assembly which slows the exchange of preexisting complex I subunits for newly imported ones. Decreased import or assembly may lead to increased oxidative damage and complex I deficiency that results in Bax-dependent apoptosis (21) and subsequent disease.

## ACKNOWLEDGMENTS

We thank Ann Frazier for critical reading of the manuscript.

This work was supported by grants from the Australian National Health and Medical Research Council, the Australian Research Council, the Ramaciotti Foundation, and the Muscular Dystrophy Association (United States). D.T. is supported by an NHMRC Senior Research Fellowship, M.M. is supported by an NHMRC Peter Doherty Fellowship, M.L. is supported by an Australian Postgraduate Award, and A.O. is supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (16591052).

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