ND3 and ND4L Subunits of Mitochondrial Complex I, Both Nucleus Encoded in *Chlamydomonas reinhardtii*, Are Required for Activity and Assembly of the Enzyme

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Made of more than 40 subunits, the rotenone-sensitive NADH:ubiquinone oxidoreductase (complex I) is the most intricate membrane-bound enzyme of the mitochondrial respiratory chain. In vascular plants, fungi, and animals, at least seven complex I subunits (ND1, -2, -3, -4, -4L, -5, and -6; ND is NADH dehydrogenase) are coded by mitochondrial genes. The role of these highly hydrophobic subunits in the enzyme activity and assembly is still poorly understood. In the unicellular green alga *Chlamydomonas reinhardtii*, the ND3 and ND4L subunits are encoded in the nuclear genome, and we show here that the corresponding genes, called *NUO3* and *NUO11*, respectively, display features that facilitate their expression and allow the proper import of the corresponding proteins into mitochondria. In particular, both polypeptides show lower hydrophobicity compared to their mitochondrion-encoded counterparts. The expression of the *NUO3* and *NUO11* genes has been suppressed by RNA interference. We demonstrate that the absence of ND3 or ND4L polypeptides prevents the assembly of the 950-kDa whole complex I and suppresses the enzyme activity. The putative role of hydrophobic ND subunits is discussed in relation to the structure of the complex I enzyme. A model for the assembly pathway of the *Chlamydomonas* enzyme is proposed.

Complex I (NADH:ubiquinone oxidoreductase; EC 1.6.5.3) is the largest and the most complicated enzyme of the mitochondrial respiratory chain. It is a membrane-bound assembly of approximately 1,000 kDa composed of 42 subunits in the land plants *Arabidopsis thaliana* and *Oryza sativa* (36), 42 or 43 subunits in the green alga *Chlamydomonas reinhardtii* (7, 8), 46 subunits in the mammal *Bos taurus* (9, 38), and 39 subunits in the fungus *Neurospora crassa* (52). The complex I enzyme shows a general L shape with a peripheral arm protruding in the matrix and another arm embedded in the inner membrane of the mitochondrion (18, 21, 32). Among the 33 components common to all of these eukaryotes, 14 are homologues of the constituents of the bacterial type I NADH dehydrogenase (ND) and are considered to represent the core of the enzyme (8). Seven subunits of the bacterial enzyme bind the FMN and nine iron-sulfur clusters (37). In contrast, the role of the seven other subunits, which are counterparts of the ND1, -2, -3, -4, -4L, -5, and -6 hydrophobic polypeptides of eukaryotes and are encoded in the mitochondrial DNA (mtDNA) of plants, mammals, and fungi, is still unclear.

In land plants, the ND7 and ND9 polypeptides are also encoded in the organelle genome. In maize, the NCS2 mitochondrial mutation is a deletion of the 3' end of the *nd4* gene, which creates a fused *nd4-nd7* chimeric gene and prevents the assembly of the whole complex (43, 51). NCS2 mutant plants, which are maintained at the heteroplasmic state (a mixture of normal and mutated mitochondrial genomes), display poor growth, pale striping of leaves, and decreased yield of kernels. Cytoplasmic male-sterile mutants I and II of *Nicotiana sylvestris*, with a deletion of the *nd7* mitochondrial gene, show reduced respiration and breakdown of complex I activity and assembly (34, 63). Mutations affecting mitochondrion-encoded subunits are also known to be responsible for many hereditary diseases in humans (12, 74). However, due to the heteroplasmic state of the cells that contain both normal and mutant mitochondria and to the presence of allelic variants, it is often difficult to establish the real impact of these mutations on complex I assembly and activity in patients. In this respect, the unicellular green alga *C. reinhardtii* constitutes an invaluable tool to study the role of the ND subunits since homoplasmic mutations that affect mitochondrial genes encoding respiratory chain components are not lethal in this organism (53, 69). Moreover, genetic transformation allowing the insertion of point mutations into these genes has been very recently achieved (70). Previously, characterization of mutations altering the *nd1*, *nd4*, and *nd6* mitochondrial genes allowed us to determine the impact of the loss of the corresponding subunits on the activity and assembly of the enzyme (6). The mtDNA of *Chlamydomonas reinhardtii* algae has the particularity that it codes for only five complex I subunits (ND1, -2, -4, -5, and -6) and lacks the genes that encode the ND3 and ND4L subunits (4, 57). This observation led to the suggestion that the corresponding genes have been transferred to the nucleus (60). Thanks to the data of the *Chlamydomonas* nuclear genome sequencing (33) (data available at the Joint Genome Institute website, http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), we have identified the *NUO3* and *NUO11* nuclear genes as homologs of the ND3 and ND4L coding sequences, respectively (7, 8).

In this work, we investigate the modifications that have occurred following the transfer of these two genes to the nucleus, which allows their proper expression, and the sorting and import of their polypeptidic products to the mitochondria. More-
over, to analyze the role of ND3 and ND4L subunits, we here describe the suppression of NUO3 and NUO11 gene expression and determine the impact of the loss of the corresponding subunits on the activity and assembly of complex I and other respiratory chain complexes.

**MATERIALS AND METHODS**

**Strains and culture conditions.** The *C. reinhardtii* strain used in this study is the cw15 arg7-8 mt+ mutant. This strain is deficient for the arginine wall and is auxotrophic for arginine because of a mutation in the *ARG7* gene coding for argininosuccinate lyase (16). Cells were grown in liquid or on solid agar medium under continuous illumination (75 µE m⁻² s⁻¹) at 25°C. The routinely used medium was Tris-acetate-phosphate medium (30) in which 7.5 mM NH₄Cl is replaced by 4 mM NaNO₃, and supplemented with 100 µg/ml of t-arginine per ml when necessary.

*Escherichia coli* DH5α was used for cloning gene and cDNA sequences, and *E. coli* transformants were grown in Luria medium in the presence of ampicillin (50 µg/ml) (Fig. 1A).

**Construction of plasmids for dsRNA expression.** The pNB1 plasmid (2,895 bp) was used to express double-stranded RNA (dsRNA) contains a 229-bp chimeric strong promoter comprising 84 bp of the *C. reinhardtii* nitrate reductase (*NIA1* gene) promoter and 98 bp of the *C. reinhardtii* β-tubulin (*TUB1* gene) minimal promoter. *C. reinhardtii* TUB1 promoter is inserted in the XbaI and HindIII sites of the pUC19 vector (50) (Fig. 1A).

**Construction of plasmid pND3-RNAi (3,849 bp).** Two fragments of the ND3 cDNA (GenBank accession numberAY351265) (586 and 404 bp) were amplified by PCR using as forward primer ND3-1F (5’-AAGCTTCCATGGGTGGAAGTACGCCACGCACAG-3’) and ND3-2R primers were used for *NUO9* (GenBank accession numberAY351261); ND7-3F (5’-TGGCAACTGCTATGTTGATCTGCGCAACGAGTACAAGG-5’) and ND7-3R were used for *NUO7* (GenBank accession numberAY374483); ESSS-3F (5’-TCAGGACGGAGCGAGGGAACGCA-5’) and ESSS-6R (5’-TGGCGAACAGTAACAGGAGG-3’) were used for *NUO17* (GenBank accession numberAY358676); NUO11-1F (5’-AGGGTCGCTCCGGGCTCCTGACTG-3’) and NUO11-3R (5’-TCCGCACGTCACATTTACC-3’) were used for NUO11 (GenBank accession numberAY549572).

**Protein synthesis analyses.** The starting material was purified mitochondria (6) or crude membrane fractions (68). The protein content was determined by the Bradford method (2). Activity of complexes I, IV, and NADH:ferricyanide oxidoreductase was measured as previously described (6, 68).

**RESULTS AND DISCUSSION**

**NUO3 and NUO11 genes possess characteristics of *Chlamydomonas* nuclear genes, and their polypeptide products are adapted for sorting and import into mitochondria.** The endosymbiotic event that gave rise to mitochondria was followed by a massive migration of genes to the nucleus (31). Like other highly hydrophobic components of complex I, ND3 and ND4L subunits from vascular plants, animals, and fungi stay encoded in the mitochondrial genome. In contrast, the nd3 and nd4l chemical sequence of the *Chlamydomonas* cw15 arg7-8 mt+ strain was carried out using the glass bead method (46) with 5 µg of plasmid RNAi (linearized with ScaI for pND3- RNAi or SspI for pND4L-RNAi) and 1 µg of pASL, linearized with BamHI. This pASL plasmid bears the *Chlamydomonas* *ARG7* gene that codes for the arginino- succinate lyase (16) and is used as a selectable marker. Prototroph transformants were selected on Tris-acetate-phosphate agar plates. The presence of sequences belonging to the right and to the left part of the RNAi plasmids in the transformants was checked by PCR with primers hybridizing in the *NUO3* or *NUO11* sequences and in the vector (universal primers 5’-GTTAAGACGACCTGGCACG-3’ and 5’-CAGGAAACAGCTTATGAC-3’) on a DNA extract prepared as described elsewhere (58). The stability of the phenotype observed for the transformants mentioned in this study was checked 1 year after their isolation.

**RNA analyses.** Total RNA was extracted from 5 × 10⁶ cells as previously described (49). For RNA blot analyses, RNA (15 µg) was separated on 0.8% agarose-formaldehyde gels and transferred onto Hybond N⁺ membrane (Amersham Pharmacia Biotech). Digoxigenin-labeled PCR products of cDNA fragments were used as gene probes and detected using anti-digoxigenin-AP conjugates and CDP-Star as substrate (Roche). ND4L-1F and ND4L-3R primers were used to synthesize the probe for detection of *NUO4* transcripts; ND3-3F and ND3-2R primers were used for *NUO9*; ND3-3F (5’-CAGGAGCCACGATCATACACACCAG-3’) and ND9-2R (5’-GCCGGTGCTGAAACCTGTCGCA-3’) were used for *NUO7* (GenBank accession numberAY374483); ESSS-3F (5’-TCAGGACGGAGCGAGGGAACGCA-5’) and ESSS-6R (5’-TGGCGAACAGTAACAGGAGG-3’) were used for *NUO17* (GenBank accession numberAY358676); NUO11-1F (5’-AGGGTCGCTCCGGGCTCCTGACTG-3’) and NUO11-3R (5’-TCCGCACGTCACATTTACC-3’) were used for NUO11 (GenBank accession numberAY549572).

**Transformation.** The starting material was purified mitochondria (6) or crude membrane fractions (68). The protein content was determined by the Bradford method (2).

**Activity of complexes I, IV, and NADH:ferricyanide oxidoreductase was measured as previously described (6, 68).** To conduct blue native polyacrylamide gel electrophoresis (BN-PAGE) analyses, protein complexes were first solubilized in the presence of 2.5% (wt/vol) dodecylmaltoside, 375 mM 6-aminohexanoic acid, 250 mM EDTA, and 25 mM Bis-Tris, pH 7.0, and centrifuged for 20 min at 15,000 × g at 4°C to remove insoluble matters. One percent (wt/vol) sodium taurodeoxycholate was then added to the supernatant prior to separation by electrophoresis on a 4 to 12% acrylamide gradient BN gel (73). NADH/nitroblue tetrazolium (NBT) staining and Coomassie blue staining of the gels were performed as previously published (7).

**BN gels were electroblotted** (20) according to standard protocols. The following antiseria raised in rabbits were used: polyclonal antiserum against whole purified complex I from *N. crassa* (provided by H. Weiss and U. Schulte) or monoclonal antiserum against the PSST subunit from *Yarrowia lipolytica* (provided by V. Zickermann and U. Brandt). Detection was performed using a BM Chemiluminescence Western blotting kit (Roche) with anti-rabbit peroxidase-conjugated antibodies.

**In silico analyses.** Frequency of codon utilization was compared with data available for *C. reinhardtii* nuclear genes (http://www.kazusa.or.jp/codon/). Alanine-helix predictions were computed with the Deleage-Roux and Levitt scales with the Protscale program on the Expo server (http://us.expasy.org). The positions of residues along alpha helices (3.6 residues per turn) were determined with the Pepwheel program (http://emboss.sourceforge.net/apps/pepwheel.html).

Hydropathy profiles were determined by using the scale of Kyte and Doolittle with a window of seven residues (48). Local hydropobicity (rH⁵) and average regional hydropobicity (termed mesohydrophobicity) were calculated using scanning windows of 17 and 60 to 80 residues, respectively, with the MITOPROT, version 2.0, program (http://ihg.gsf.de/ihg/mitoprot.html) (11).
genes are missing from the mitochondrial genome of *C. reinhardtii*, *Chlamydomonas eugametos*, and *Polytomella parva*, three members of the “*reinhardtii*” clade (65). In the course of proteomic analysis of the complex I components of *C. reinhardtii*, we have identified the homolog of the ND3 subunit, whereas other small or hydrophobic polypeptides, including the ND4L subunit, escaped detection using our experimental approach (7). We have also identified the NUO3 and NUO11 nuclear genes of *C. reinhardtii* as homologs of the ND3 and ND4L coding sequences, respectively (7, 8). However, no bioinformatic or expression analysis of these two genes has ever been undertaken.

To be efficiently expressed, mitochondrial genes that have been transferred to the nucleus have to acquire several distinct traits typical of nuclear genes and absent in mitochondrial genes (3, 29). Moreover, this transfer of genes from the mitochondrial genome to the nucleus also raises the question of the import of their hydrophobic polypeptidic products. We thus wondered whether these genes and the presence of modifications in their sequences.

(i) **Gene expression.** To analyze NUO3 and NUO11 gene expression, RNA blots from wild-type cells were hybridized with cDNA probes. Strong single signals were detected at 1.5 and 1.6 kb, respectively (Fig. 2, WT lanes in top blots), indicating that both genes are highly expressed. The sizes of the transcripts were in good agreement with the sizes predicted from the GenBank database (1.585 bp for NUO3, accession number AY351265; 1.616 bp for NUO11, accession number AY216718). Several methionine codons were found in the 5′ ends of the NUO3 and NUO11 cDNA sequences. For each sequence, the first ATG was chosen as the putative initiation codon because its neighborhood was the closest to the (A/C)(A/C)(A/C)ATG(G/C)(C/G) consensus sequence defined for initiation codons of *Chlamydomonas* nuclear genes (76). The ND3 and ND4L polypeptides should thus be 279 and 227 amino acids long, respectively (GenBank accession numbers AAQ55461 and AAO61142).

(ii) **Changes in codon usage.** Since the codon usage strongly differs between nuclear and mitochondrial genes in *Chlamydomonas* (76), the pattern of codon utilization in NUO3 and NUO11 genes was determined. Data obtained showed that codon usage in both genes was typical of nuclear genes. For example, we found that among the 25 leucine codons present in the NUO3 coding sequence, 17 are CTG, 5 are CTC, 2 are CTA, and 1 is TTG. The first two codons are preferentially found in nuclear sequences, whereas the latter two are more frequently used in mitochondrial genes. Moreover, the GC content of both genes is about 65% and fits well with that of the nuclear genome including the protein coding sequences (more than 60%) (35). In contrast, the GC content is only 45% for mtDNA (GenBank accession number U03843).

(iii) **Acquisition of polyadenylation signal.** The polyadenylation signal of *C. reinhardtii* nuclear genes (TGTTA) (76) is found at the end of both cDNA sequences.

(iv) **Acquisition of introns.** Intronic sequences with orthodox splicing sites (76) were identified; six were found in the NUO3 gene, and one was found in the NUO11 gene (Joint Genome Institute accession numbers 24195 and 58686).

(v) **Presence of a putative mitochondrial targeting sequence.** The large majority of nucleus-encoded mitochondrial proteins contain an N-terminal targeting sequence (MTS, or mitochondrial targeting sequence) that drives the import to the mitochondria (28). When the deduced full-length ND3 and ND4L sequences were compared to the polypeptidic sequences of other organisms (prokaryotic and eukaryotic), long N-terminal extensions of about 160 and 130 residues, respectively, were found (Fig. 3A). Such unusually long presequences (>100 amino acids) are also present in ATP6, COX IIA, and COX III proteins from *Chlamydomonas* (26, 61, 62) and may improve the efficiency of import of nucleus-encoded, highly hydrophobic proteins across mitochondrial membranes (10, 27). Both ND3 and ND4L N-terminal sequences possess the characteristics of an MTS. (a) Their amino acid composition is similar to the composition described for the MTS of land plants (28); they are rich in Arg (7.8%), Leu (15%), Ala (15%), and Ser (10%) and poor in Cys, His, Ile, Trp, and Asp (about 1% each). (b) The N-terminal residues (1 to 17) have the potential to form an amphipilic α helix with one hydrophilic positively charged face and one apolar face (Fig. 3A). This amphiphilicity seems to be essential for the function of the MTS (23, 28). (c) Putative cleavage sites as defined by Glaser et al. (28) are found at positions that are consistent with the length of their protein homologues encoded by mtDNA (indicated by ↓), i.e., after residue 158 (RTFQ ↓ FT) for ND3 and after residue 123 (RSY ↓ YT) for ND4L. In particular, the deduced molecular mass of mature ND3 subunit (13,547 Da) is in good agreement with its identification by mass spectrometry as a 14-kDa band in a sodium dodecyl sulfate gel (7).

(vi) **Diminished hydrophobicity.** It was proposed that the ND3 and ND4L subunits, which possess three transmembrane domains (24), are too hydrophobic to be imported across the mitochondrial membranes (66). However, the algal polypeptides also showed three hydrophobic segments predicted to be transmembrane alpha helices (Fig. 3A). The likelihood that a
over 17 amino acids (a range that is relevant to determine the hydrophobicity of putative transmembrane segments) (10, 14). Figure 3B shows an <H> versus mesoH plot using the Kyte and Doolittle scale (48) for ND3 and ND4L sequences of various origins. In comparison with mtDNA-encoded polypeptides, the ND3 and ND4L polypeptides from *Chlamydomonas* (Fig. 3B, dots A) exhibit the lowest <H> values while their mesoH values are low or close to the average, respectively. Similar results were obtained with the GES, GVH1, or ECS scales (11) (data not shown). Such reduced <H> and mesoH values compared to their mtDNA-encoded counterparts have also been reported for the nucleus-encoded COX IIA, COX IIB, COX III, and ATP6 proteins of *Chlamydomonas*. Reduced hydrophobicity of the most hydrophobic domains is considered to facilitate the import into mitochondria (29, 60, 61). Daley et al. have demonstrated that structural changes in COX2 polypeptide that diminish hydrophobicity can, indeed, allow import into mitochondria of a nucleus-encoded protein that is normally mtDNA encoded (14).

We thus show here that, following their transfer to the nucleus, both the NUO3 and NUO11 genes have acquired several features allowing their proper expression and the importation of their translation products into mitochondria: acquisition of an efficient promoter, as suggested by the abundance of the transcripts; modification of codon frequencies to the usage of one or several introns that are essential in the expression and subsequent maturation of the mRNA (67); addition of a mitochondrial targeting amino acid presequence; and hydrophobicity diminution of transmembrane stretches that probably promote sorting and import to mitochondria.

**Transformants inactivated for the NUO3 and NUO11 nuclear genes can be isolated by RNAi.** In *Chlamydomonas*, efficient targeted gene disruption by homologous recombination is not yet available for nuclear genes (59). However, interference with the expression of specific gene by dsRNA is a powerful tool to investigate protein function in this organism (25, 75).

To suppress the expression of NUO3 and NUO11 genes, a strain of *Chlamydomonas* lacking cell walls and auxotrophic for arginine was cotransformed with the plasmid pASL (bearing the ARG7 gene) and the plasmid designed for RNAi (pND3-RNAi or pND4L-RNAi) (Fig. 1) (see Materials and Methods). One hundred arg+ colonies of each transformation experiment were selected and tested by PCR for the presence of sequences belonging to the pRNAi plasmids, as described in Materials and Methods. A total of 26 and 28 positive colonies, called coND3 and coND4L clones, respectively, were obtained in transformation experiments with the pND3-RNAi and pND4L-RNAi constructs, respectively.

To investigate whether complex I enzyme activity was suppressed in the cotransformants, high-molecular-mass complexes from crude membrane fractions were separated by BN-PAGE. NADH dehydrogenase activity of the complex I matricial arm was then tested using NBT as an electron acceptor. Wild-type complex I activity was associated with a single band at 950 kDa (6, 7). Among the 28 coND4L clones analyzed, only two (89 and 97) showed no activity or very low activity (observed only after 2 h of incubation). Similarly, only two clones (33 and 43) of the 26 coND3 isolates showed no activity or very low staining activity, whereas the other ones...
exhibited no significant modification of complex I coloration compared to wild type (Fig. 4 and data not shown).

To confirm the alteration of complex I in the four defective clones, enzyme activity was measured in crude membrane fractions compared to wild type and to transformants coND3-32 and coND4L-92 that behaved like wild type in the BN-PAGE experiment (Fig. 4). Cytochrome c oxidase (complex IV) enzyme activity was determined as a control, and no significant difference could be observed between strains (Table 1). In contrast, the NADH: duroquinone oxidoreductase activity sensitive to rotenone (considered to represent the activity of complex I) was found to be null or extremely low in the four mutant cell lines. The NADH:ferricyanide oxidoreductase activity was also measured. In the Chlamydomonas whole-membrane fraction, this reaction is mainly (70 to 85%) catalyzed by the peripheral arm of complex I (6). The four mutant strains exhibited an average activity of 15 to 30% of wild-type activity, again indicating the loss of the peripheral arm activity of complex I. These results fully confirm the complex I defect observed in BN gels by NADH/NBT staining (Fig. 4).

To determine whether the loss of complex I activity detected in coND3-33, coND3-43, coND4L-89, and coND4L-97 strains was correlated to specific degradation of the corresponding transcripts, RNA blotting was performed using NUO3 and NUO11 cDNA sequences as probes (Fig. 2). A specific and drastic reduction of NUO3 transcript level was observed in coND3-33 (more than 95%) and coND3-43 (no transcript detected), while the amounts of NUO11 transcripts were below 2% in coND4L-89 and coND4L-97 clones. The transcription levels of the NUO7, NUO9, NUO17, and NUO1I genes encoding other complex I subunits were also investigated as controls. The three first genes encode homologous subunits of the mammalian 49-kDa, 30-kDa, and ESSS complex I subunits, whereas NUO1I codes for a 10-kDa protein typical of complex I from plants (7, 8). No signal difference was observed between wild-type and mutant strains. The transcript levels from coND3-32 and coND4L-92 strains were also analyzed as controls and were similar to those observed for wild-type cells (data not shown).

Taken together, these results demonstrate that the complex I enzyme activity defect detected in the four mutants is due to th

the absence of or to a drastic decrease in the ND3 and ND4L protein levels triggered by the expression of the NUO3 or NUO11 dsRNA. These results also indicate that no transcription downregulation of nuclear genes coding for other complex I subunits is engaged following inactivation of NUO3 and NUO11 gene expression. As observed in several other experiments designed for RNA interference in Chlamydomonas (75), the rates of clones altered in NUO3 or NUO11 gene expression were less than 10%. Such low frequencies of transformed clones for which suppression of gene expression was effective can be due to rearrangements of the transformant plasmid at the time of the insertion process or to the integration of the construction in transcriptionally inactive areas of the genome, as previously described in Chlamydomonas (46).

Loss of complex I assembly in ND3- and ND4L-deficient strains; function and localization of both proteins within the membrane domain of complex I. In order to investigate the impact of the loss of ND3 or ND4L on complex I assembly, mitochondria were purified from wild-type and mutant cells, and the organelle extracts were subjected to BN-PAGE analyses. By staining the gel with Coomassie blue, four main bands of 250, 500, 950, and 1,700 kDa were observed over 100 kDa for wild type (Fig. 5A). They correspond to respiratory chain complexes IV, III (dimeric state), I, and V (ATP synthase under dimeric state), respectively (7, 78). No signal was observed at 950 kDa in the mutants, while the amount of complexes III, IV, and V was not altered. Moreover, no additional blue band that could correspond to a putative complex I subcomplex was found.

To confirm these results, the protein complexes were investigated by immunological reactions with antisera against whole N. crassa complex I (Fig. 5C) or against the PSST subunit of Y. lipolytica (homologous of C. reinhardtii complex I subunit encoded by the NUO10 gene; GenBank accession number AAQ63698) (Fig. 5D). In wild type, the whole complex I is highlighted (950 kDa) by both antibodies, while no signal was detected in lanes showing results with mutants. Hence, the absence of the ND3 or ND4L subunit prevents the assembly of the whole complex I.

Finally, when the gel obtained from purified mitochondria was submitted to NADH/NBT staining, the 950-kDa complex I activity was observed solely in the wild-type mitochondrion (as

FIG. 4. Screening of clones for complex I defect. Crude membrane extracts (120 µg of protein solubilized with 1.5% [wt/wt] dodecyl-β-D-maltoside) of Chlamydomonas strains were loaded on a BN gel. After electrophoresis, the gel was stained for NADH dehydrogenase activity of complex I using NBT as an electron acceptor. Mitochondrial complex I is detected at 950 kDa. The two lower-molecular-mass bands of complex I using NBT as an electron acceptor. Mitochondrial complex I. These results fully confirm the complex I defect observed in lanes showing results with mutants. Hence, the absence of or to a drastic decrease in the ND3 and ND4L protein levels triggered by the expression of the NUO3 or NUO11 dsRNA. These results also indicate that no transcription downregulation of nuclear genes coding for other complex I subunits is engaged following inactivation of NUO3 and NUO11 gene expression. As observed in several other experiments designed for RNA interference in Chlamydomonas (75), the rates of clones altered in NUO3 or NUO11 gene expression were less than 10%. Such low frequencies of transformed clones for which suppression of gene expression was effective can be due to rearrangements of the transformant plasmid at the time of the insertion process or to the integration of the construction in transcriptionally inactive areas of the genome, as previously described in Chlamydomonas (46).

### Table 1. Activities of respiratory chain complexes in wild-type and transformant strains

| Strain         | Complex I Specific activity (± SD)* | NADH:Fe(CN)₆³⁻ | Complex IV  |
|---------------|-----------------------------------|-----------------|------------|
| Wild type     | 50 ± 22                           | 2,489 ± 379     | 245 ± 29   |
| coND3-32      | 48 ± 9                            | 2,234 ± 175     | 250 ± 12   |
| coND3-33      | 2 ± 1                             | 808 ± 119       | 248 ± 18   |
| coND3-43      | 0                                | 620 ± 74        | 244 ± 5    |
| coND4L-89     | 1 ± 1                             | 739 ± 208       | 245 ± 12   |
| coND4L-92     | 53 ± 19                           | 2,378 ± 89      | 212 ± 28   |
| coND4L-97     | 0                                | 401 ± 219       | 229 ± 41   |

*Values are specific activities (± SD) from three to six experiments. Specific activity was calculated as follows: for complex I, rotenone-sensitive NADH: duroquinone oxidoreductase, as nmol of NADH oxidized min⁻¹ mg of protein⁻¹; for NADH:Fe(CN)₆⁻⁻⁻, NADH:ferricyanide oxidoreductase, as nmol of K₃Fe(CN)₆ reduced min⁻¹ mg of protein⁻¹; for complex IV, cytochrome c oxidase, as nmol of cytochrome c oxidized min⁻¹ mg of protein⁻¹.
previously shown from the crude membrane fraction in Fig. 4), but an additional signal, in the range of 200 kDa, was also observed in both wild-type and mutant strains (Fig. 5B). We previously demonstrated that this 200-kDa signal corresponds to a free soluble enzyme present in *Chlamydomonas* mitochondria and displays NADH:ferricyanide and NADH: duroquinone oxidoreductase activities (6). Immunological studies, moreover, showed (6) that this enzyme comprises the 49-kDa (NUOD) and the 76-kDa (NUOG) polypeptides, two components of the complex I peripheral arm which are thought to be involved in the electron flow from NADH to ubiquinone. Hence, the 200-kDa soluble enzyme can be considered as a fragment originating from the peripheral arm of the complex I enzyme. Interestingly, a similar 200- to 300-kDa NADH dehydrogenase that contains the 76-kDa subunit has also been identified in mitochondria from potato and pea (5). In contrast to the 49- and 76-kDa subunits, the PSST subunit could be absent from the *Chlamydomonas* 200-kDa subcomplex, since no signal was obtained using an antibody directed against the *Y. lipolytica* PSST (Fig. 5D). Finally, it has to be mentioned that the 200-kDa subcomplex is much larger than the type II NADH dehydrogenases (55 to 65 kDa) found in land plant mitochondria (22) and also probably present in *Chlamydomonas*, as suggested from sequence alignment studies (8).

While the presence of ND3 and ND4L in mitochondrial complex I has been demonstrated for more than 20 years (24), very little is known about the role of these two subunits in the activity and assembly of the eukaryotic enzyme. Some data were recently obtained, mainly by studying their prokaryotic counterparts. Concerning the ND3 subunit, it has been shown in the bacterium *Paracoccus denitrificans* that the corresponding polypeptide (Nqo7) directly interacts with subunits Nqo6 (PSST) and Nqo4 (49 kDa) of the peripheral arm (17, 41). Both of these hydrophilic polypeptides are considered to bind the last iron-sulfur cluster (N2) that reduces the quinone at the interface between the two arms and to participate to a quinone binding site (15, 19, 37, 44, 64). In *E. coli*, evidence of the implication of ND3 in the enzyme activity is provided by the double mutant D79N/E81Q, which almost completely lacks the energy-transducing activity of the enzyme (40). Similarly, point mutations affecting the *nd3* gene sequence in human cause specific defects in complex I activity (13, 56, 77). With regard to ND4L, this subunit could be involved in the proton translocation machinery of the enzyme. As a matter of fact, in *E. coli*, NuoK (ND4L) along with NuoL (ND5), NuoM (ND4), and NuoN (ND2) shows primary sequence similarity to antiporters of the prokaryotic Mrp class (54, 55). Moreover, mutations of nearly perfectly conserved Glu-36 in NuoK (ND4L) of *E. coli* lead to almost null activities of coupled electron transfer with a concomitant loss of generation of the electrochemical gradient (42, 45).

Not all hydrophobic subunits of the core enzyme play the same role in the assembly of complex I. As a matter of fact, in the course of the characterization of other *Chlamydomonas* complex I mutants (6), we showed that the loss of ND4 or ND4+ND5 subunits leads to the assembly of a 700-kDa membrane-bound subcomplex; however, as for ND3 and ND4L, the absence of intact ND1 or ND6 subunits totally prevents complex I assembly. This suggests that ND4 and ND5, on one hand, and ND1, ND3, ND4L, and ND6, on the other hand, are located in two different domains of the complex I membrane arm. This hypothesis is in good agreement with the structural models proposed for the localization of these subunits within the bovine complex I (9, 72) and the prokaryotic NDH-1 enzyme (39). Following this hypothesis, we propose a preliminary model for the assembly pathway of *Chlamydomonas* complex I (Fig. 6). In this model, the association of the 200-kDa soluble

![FIG. 5. Complex I assembly in coND3 and coND4L mutants. For each strain, 150 μg of total mitochondrial protein was loaded on a BN gel. After electrophoresis, the gel was submitted to Coomassie blue staining (A) or NADH/NBT staining (B) or blotted and probed with antisera against the *N. crassa* whole complex I (C) or against the *Y. lipolytica* PSST subunit (D). I, III2, IV, and V2 correspond to respiratory chain complexes I, III (dimeric state), IV, and V (dimeric state), respectively.](image-url)
fragment displaying NADH dehydrogenase activity (see above) with other hydrophilic subunits and with hydrophobic proteins (notably ND1, ND3, ND4L, and ND6) would lead to the formation of a membrane-bound 700-kDa subcomplex. Then, the association of another membrane domain located at a distal position and comprising ND4, ND5, and other hydrophobic proteins would give the 950-kDa fully assembled complex I. This simple model is very close to the model that was proposed for the human complex I assembly from the study of various mutant cell lines producing different assembly intermediates (1). A divergent model was proposed for complex I assembly in the fungus N. crassa, based on the existence of a stable peripheral arm composed mainly of hydrophilic proteins and of a membrane arm composed of hydrophobic subunits (79). A priori, the existence of two different complex I assembly pathways is rather surprising. The discrepancy between the two models could be related to the absence or the nondetection of some assembly intermediates in one of the other organisms. Some factors specific to the organisms could also influence the relative stability of the subcomplexes: the rate of proteolysis of unassembled protein sets or the presence of specific chaperones. In this context, only two chaperones involved in complex I assembly have been identified to date: CIA30 and CIA84 (47). While CIA30 is widely found among eukaryotes, homologous sequences of CIA84 are found only in fungi (8). A more detailed analysis of the composition of the various subcomplexes is required to elucidate the assembly pathway of the huge multimeric complex I enzyme.

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