

Unusual Location of a Mitochondrial Gene

SUBUNIT III OF CYTOCHROME *c* OXIDASE IS ENCODED IN THE NUCLEUS OF CHLAMYDOMONAD ALGAE*

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Xochitl Pérez-Martínez‡, Miriam Vázquez-Acevedo‡, Elena Tolkunova§, Soledad Funes‡,
Manuel G. Claros¶, Edgar Davidson§, Michael P. King§, and Diego González-Halphen‡¶

From the ‡Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, 04510, Distrito Federal México, the §Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, and the ¶Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, E-29071 Málaga, Spain

The algae of the family Chlamydomonadaceae lack the gene *cox3* that encodes subunit III of cytochrome *c* oxidase in their mitochondrial genomes. This observation has raised the question of whether this subunit is present in cytochrome *c* oxidase or whether the corresponding gene is located in the nucleus. Cytochrome *c* oxidase was isolated from the colorless chlamydomonad *Polytomella* spp., and the existence of subunit III was established by immunoblotting analysis with an antibody directed against *Saccharomyces cerevisiae* subunit III. Based partly upon the N-terminal sequence of this subunit, oligodeoxynucleotides were designed and used for polymerase chain reaction amplification, and the resulting product was used to screen a cDNA library of *Chlamydomonas reinhardtii*. The complete sequences of the *cox3* cDNAs from *Polytomella* spp. and *C. reinhardtii* are reported. Evidence is provided that the genes for *cox3* are encoded by nuclear DNA, and the predicted polypeptides exhibit diminished physical constraints for import as compared with mitochondrial-DNA encoded homologs. This indicates that transfer of this gene to the nucleus occurred before *Polytomella* diverged from the photosynthetic *Chlamydomonas* lineage and that this transfer may have occurred in all chlamydomonad algae.

Mitochondrial cytochrome *c* oxidase (EC 1.9.3.1), the terminal component of the respiratory chain, is an oligomeric membrane protein complex of 10–13 subunits that contains four redox components: a binuclear center Cu_A, heme *a*, heme *a*₃, and Cu_B. The transfer of electrons from reduced cytochrome *c* to molecular oxygen is coupled to proton translocation from the matrix to the intermembrane space. In most eukaryotic cells, the three largest subunits of cytochrome *c* oxidase (COX I, COX II, and COX III) are encoded by the mitochondrial DNA

(mtDNA)¹ and are synthesized inside the organelle (1). These subunits are homologous to the three major polypeptides of bacterial cytochrome *c* oxidases. There are strong similarities in the primary, secondary, and tertiary structures of these subunits, as evidenced by the x-ray crystallographic models of cytochrome *c* oxidases from *Paracoccus denitrificans* and bovine mitochondria (2, 3). In addition, a variable set of nuclear-encoded subunits that exhibit either no transmembrane stretch or a single transmembrane stretch are synthesized in the cytoplasm and imported into mitochondria (4).

A striking example of simplicity, in size and composition, is the 15.8-kilobase linear, double-stranded mtDNA from the green alga *Chlamydomonas reinhardtii*. This compact and highly diverged mitochondrial genome has been entirely sequenced (5). Several genes that encode essential components of oxidative phosphorylation that are usually found in mitochondrial genomes are absent in this mtDNA: *nad3* and *nad4L* (encoding subunits 3 and 4L of NADH-ubiquinol oxidoreductase), *cox2* and *cox3* (encoding COX II and COX III), and *atp6* and *atp8* (encoding subunits *a* and *A6L* of the F₀ portion of ATP synthase). These genes are also absent in the mtDNAs of the closely related algae *Chlamydomonas smithii* (6), *Chlamydomonas eugametos* (7), *Chlamydomonas moewussii* (8), and *Chlorogonium elongatum* (9). The absence of this set of genes seems to be a common feature of the algae from the family Chlamydomonadaceae and suggests that the complexes that participate in oxidative phosphorylation lack some of their classical polypeptide constituents or that the corresponding genes were transferred to the nucleus.

The algae of the colorless genus *Polytomella* are members of the family Chlamydomonadaceae (10) that diverged from the *Chlamydomonas* lineage by losing both the cell wall and the photosynthetic apparatus (11). The close relationship between the genera *Polytomella* and *Chlamydomonas* is supported by numerous morphological (10, 12), molecular genetic (13–16), and biochemical (17) studies. This colorless alga has been used to characterize the mitochondrial complexes of the chlamydomonad algae, because there is no interference by thylakoid components or by the cell wall during purification (17, 18). In the experiments described below, we report the isolation of an active, cyanide-sensitive cytochrome *c* oxidase from *Polytomella* spp. that contains COX III. The corresponding cDNAs of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF233514 and AF233515.

¶ To whom correspondence should be addressed: Dept. de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, 04510, D.F. Mexico. Tel.: 525-622-5620; Fax: 525-622-5611; E-mail: dhalphen@ifisiol.unam.mx.

¹ The abbreviations used are: mtDNA, mitochondrial DNA; Cr-COX III, cytochrome *c* oxidase subunit III protein from *C. reinhardtii*; *mesoH*, mesohydrophobicity; MTS, mitochondrial targeting sequence; PCR, polymerase chain reaction; Ps-COX III, cytochrome *c* oxidase subunit III protein from *Polytomella* spp.; RACE, rapid amplification of cDNA ends; <H>, mean hydrophobicity of a sequence segment; μ H, hydrophobic moment.

cox3 were cloned and sequenced from both *Polytomella* spp. and *C. reinhardtii*. Evidence is provided that in these algae, this gene is not localized in the mitochondrial genome but in the nuclear genome. This contrasts with the location in the majority of eukaryotes. To our knowledge, this is the first example of a *cox3* gene that is found in the nuclear genome. Therefore, our data indicated that the transfer of the *cox3* gene occurred before the genus *Polytomella* diverged from the *Chlamydomonas* lineage and that such transfer is a common feature of the Chlamydomonadaceae family. The results also show that the nuclear-localized *cox3* gene encodes a polypeptide that exhibits diminished values for <H> and *mesoH* (19), when compared with their mitochondrial counterparts in other organisms.

EXPERIMENTAL PROCEDURES

Strain and Culture Conditions—*Polytomella* spp. (198.80, E.G. Pringsheim) from the Sammlung von Algenkulturen (Gottingen, Germany), was grown as described previously (18).

Isolation and Solubilization of Mitochondria—Mitochondria from *Polytomella* spp. were obtained and solubilized in the presence of lauryl maltoside as described previously (18).

Isolation of Cytochrome *c* Oxidase from *Polytomella* spp.—Solubilized mitochondria were dialyzed against 50 mM Tris-HCl (pH 8.0), 1 mM MgSO₄, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml of *N*^α-p-tosyl-L-lysine chloromethyl ketone (TM buffer) containing 100 mM NaCl. The mixture was taken to 40% saturation of ammonium sulfate and 1.6% sodium cholate and then centrifuged at 10,000 × *g* for 15 min. The resultant green pellet was resuspended in TM buffer containing 100 mM NaCl and lauryl maltoside (1.2%). The mixture was centrifuged at 80,000 × *g*, and the supernatant was dialyzed against 10 volumes of TM buffer. The sample was applied to a DEAE-Biogel A column equilibrated with TM buffer containing 0.1% lauryl maltoside. The column was washed with three bed volumes of the same buffer and a linear gradient from 0 to 100 mM NaCl was applied. A cytochrome *c* oxidase-enriched fraction identified by spectroscopy was eluted with the same equilibration buffer that contained 200 mM NaCl. The samples were concentrated by ultrafiltration on an Amicon YM100 filter and stored at -70 °C until use.

Purification of Cytochrome *c* Oxidase from Bovine Heart Mitochondria—Bovine heart cytochrome *c* oxidase was purified according to the method of Capaldi and Hayashi (20) and stored in small aliquots at -70 °C until use.

Spectroscopic and Activity Measurements—Cytochrome *c* oxidase activity was measured spectrophotometrically in a final volume of 3 ml that had 50 mM Tris-HCl (pH 8.0), 1 mM MgSO₄, 0.1 mg/ml dodecyl maltoside, 20 μM antimycin, and 30 μM reduced cytochrome *c*. The reaction was started by addition of the enzyme, and the absorbance change at 550 nm was followed. Cytochrome *a* concentration was calculated using the extinction coefficient 16.5 mM⁻¹ cm⁻¹ (21) and cytochrome *c* determination was done as described previously (22). Visible spectra were recorded at room temperature with a DW-2a UV/Vis SLM-Aminco spectrophotometer modified with the OLIS DW2 Conversion and OLIS software (On-line Instrument System Inc.).

Polyacrylamide Gel Electrophoresis, Immunoblots, and Protein Determination—Polyacrylamide gel electrophoresis was performed as described by Schägger *et al.* (23), using 1.2-mm-thick slab gels (16% acrylamide). Gels were fixed and stained as described in the same work. Apparent molecular masses were calculated based on the reported molecular masses of bovine cytochrome *c* oxidase (24). Immunoblotting was carried out as in González-Halphen *et al.* (25). Antibodies against yeast COX III were obtained from Molecular Probes (Eugene, Oregon). Protein concentrations were determined according to Markwell *et al.* (26).

Sequencing of Subunit III by Edman Degradation—The isolation of polypeptides for N-terminal sequencing was carried out as described previously (18). N-terminal sequencing was carried out by Dr. J. D'Alayer on an Applied Biosystems Sequencer at the Laboratoire de Microséquence des Protéines (Institut Pasteur, Paris, France).

Nucleic Acid Preparation—Two-liter cultures of *Polytomella* spp. grown for 72 h were collected and resuspended in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 2% Triton X-100, and 1% SDS. Total DNA was extracted from broken cells two times with phenol/chloroform 1:1 and once with chloroform. The aqueous phase was precipitated with 3 M sodium acetate (pH 5.3) in the presence of ethanol, and the pellet was resuspended in water free of nucleases. The mixture was incubated in the presence of 2.5 μg of RNase DNase-free (Roche Molecular Bio-

chemicals) for 3 h, and DNA was extracted and precipitated as above. *C. reinhardtii* cells were collected and washed with TE buffer and resuspended in 100 mM sodium citrate (pH 7.0). The cells were frozen in liquid nitrogen and incubated at 60 °C for 15 min in the presence of 1 volume of 2% SDS. Total DNA was extracted and precipitated from broken cells as above. Total RNA from *Polytomella* spp. was obtained using the kit RNeasy Mini Kit (Qiagen).

Cloning and Sequencing of the Gene *cox3* from *Polytomella* spp.—A genomic *Polytomella* spp. *cox3* fragment was amplified by PCR using two degenerate oligodeoxynucleotides. The first one was based on the N-terminal sequence of the protein SDAGHHLSP: 5'-TC(C/T) GA(C/T) GC(C/T) GG(C/T) CA(C/T) CA(C/T) CT(C/T) TC(C/T) CC-3'. The second one was based on an internal highly conserved sequence of the protein WH(F/M)VDVWVWL: 5'-AG CCA (G/A)AC (G/A)AC (G/A)AC (C/G)A(T/A) (G/A)TG CC-3'. For PCR amplification using Vent polymerase (New England Biolabs), samples were denatured for 5 min at 94 °C and subjected to 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 40–55 °C, and 2 min extension at 72 °C. A 780-nucleotide product containing a fragment of the *cox3* gene was obtained and cloned with the pGEM-T Easy Vector System from Promega.

Cloning and Sequencing of cDNA of *cox3* from *Polytomella* spp.—cDNA sequence from *cox3* cDNA from *Polytomella* spp. was obtained with 5'- or 3'-RACE-PCR (27) using primers based on the genomic sequence obtained above. First strand cDNA templates were prepared from 1–2 μg of total RNA with Moloney murine leukemia virus reverse transcriptase from Promega or Display Thermo RT from Display Systems Biotech and using oligo dT/adaptor as first strand cDNA primer (oligo dT/adaptor, 5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TT-3'). For 3' end cDNA cloning, oligo dT/adaptor and B1 primer 5'-GCA TTA CCT CGT CCA CAC TGC-3' were used. A 1.1-kilobase product was amplified. Nested PCR was done with primers B2 (5'-GAT GGG CAT GCA TAC CGA TG-3') and B3 (5'-CAT GAA GAA GGT GGT ACC GTA GG-3') to confirm *cox3* identity. For 5' end cloning, a poly(A) tail was attached to 5' end with terminal transferase from Roche Molecular Biochemicals. For PCR amplification primers B4 (5'-CAT CGG TAT GCA TGC CCA TC-3') with oligo dT/adaptor and B5 (5'-CAA CGG ATC CGA ACA ACA AGG-3') with adaptor for nested PCR were used. A 600-nucleotide PCR product was obtained. Both RACE products were cloned with the pGEM-T easy vector system from Promega. The cDNA sequence was confirmed using primers B6 (5'-GAG GTC TCA GCT TCT TAA GGC TC-3') and B7 (5'-CGC ATA ACG CGA AGT CAC TA-3'). For PCR amplification, samples were denatured for 5 min at 94 °C and subjected to 30 cycles of 45 s denaturation at 94 °C, 1 min annealing at 48 °C, and 2.5 min extension at 72 °C. Primers B8 (5'-ATG AGG TCT CAG CTT CTT AAG GCT C-3') and B9 (5'-CGG ATA ACG CGA AGT CAC TAC-3') were used to amplify the complete *cox3* gene.

Cloning and Sequencing of cDNA of *cox3* from *C. reinhardtii*—A *C. reinhardtii* cDNA library in λgt10 (28) was screened using the *Polytomella* spp. 780-base pair PCR product corresponding to a portion of the genomic *cox3* gene. Eight positive clones were obtained from 5 × 10⁴ plaque-forming units screened. Two deoxyoligonucleotides based on λgt10 sequences were used to identify the longest positive clones (forward, 5'-AGC AAG TTC AGC CTG GTT AAG T-3', and reverse, 5'-CTT ATG AGT ATT TCT TCC AGG GTA-3'). Phage DNA from the clone containing the largest cDNA was isolated with the Qiagen Lambda Mini Kit. The *cox3* gene was subcloned into pBluescript. The 5' end of cDNA was completed by RACE PCR (the primers used were: forward, oligo dT/adaptor, and reverse, 5'-TGC TCC ATG TAG AAC TCC TTG G-3'). The sequences for nested PCR were: forward, oligo adaptor, and reverse, 5'-GTT GGG GAC CTG AGG CTG C-3').

Sequence Analysis *in Silico*—Sequences were compared using the GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, Wisconsin) (29). Alignments and construction of the cladogram were carried out with the Clustal X program (30) using sequences in the Swissprot data bank. Mitochondrial targeting sequences were analyzed and predicted using MitoProt II (31), including calculations of hydrophobic moment (μH), high local hydrophobicity (<H>), and *mesoH*. Protein transmembrane stretches were predicted using the program TodPred II (32). Three dimensional structure modelling was carried out using SWISS-MODEL (33).

Data Base Accession Numbers—The nucleotide sequences discussed in this paper will appear in the DDBJ/EMBL/GenBank™ nucleotide sequence data base under the accession numbers AF233514 (*cox3* cDNA sequence from *Polytomella* spp.) and AF233515 (*cox3* cDNA sequence from *C. reinhardtii*).

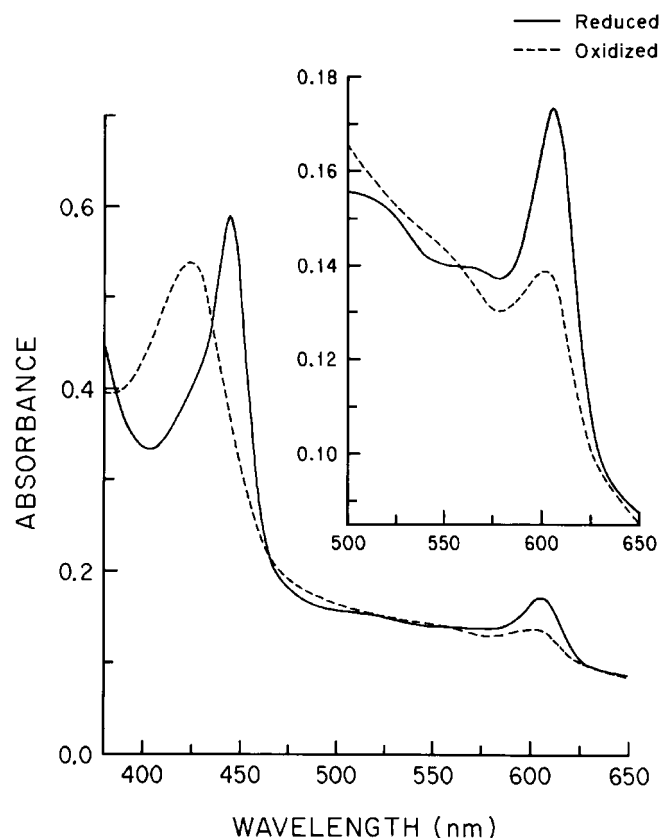


FIG. 1. Visible spectra of cytochrome *c* oxidase from *Polytomella* spp. The cytochrome *c* oxidase was diluted in 50 mM Tris-HCl (pH 8.0) containing 1 mM MgSO₄ and 0.1 mg/ml of lauryl maltoside. Broken lines, oxidized sample, as obtained. Continuous line, cytochrome *c* oxidase fully reduced in the presence of a small amount of dithionite. Inset, enlargement of the α absorption bands of the oxidized and reduced samples.

RESULTS

Isolation and Characterization of the Cytochrome *c* Oxidase Complex from *Polytomella* spp.—Cytochrome *c* oxidase was purified from the colorless alga *Polytomella* spp. The complex catalyzed electron transfer from horse heart cytochrome *c* to oxygen with a specific activity of 2.8 μ mol O₂/mg of protein/min, an activity that was completely abolished by cyanide or azide (data not shown). Absorption spectra of the cytochromes of the complex are shown in Fig. 1. The oxidized complex displayed a major absorbance peak in the Soret region at 425 nm; after reduction with dithionite, there was an increase in intensity and a shift of its maximal absorbance to 445 nm. The α -absorption peak exhibited a maximum at 605 nm in its reduced form, shifted 4–5 nm toward the red when compared with the absorption spectrum of cytochrome *c* oxidase type *aa₃* from other species. A red-shifted α -absorption peak at 606 nm was also described for reduced cytochrome *c* oxidase of *C. reinhardtii* (34). From the difference spectra (reduced with dithionite minus air-oxidized), a heme content of 3.03 nmol of heme *a*/mg of protein for the cytochrome *c* oxidase of *Polytomella* spp. was calculated.

The cytochrome *c* oxidase of *Polytomella* spp. exhibited seven polypeptides with molecular masses of 54.6, 29.6, 18.6, 14.5, 13.4, 10.8, and 9.6 kDa (Fig. 2A). The 29.6-kDa band was identified as subunit III of cytochrome *c* oxidase (see below). Two additional bands were present in this preparation, with apparent molecular masses of 80.0 and 41.8 kDa. These bands were considered contaminants and were not further explored.

In immunoblots, the 29.6-kDa polypeptide of *Polytomella*

spp. cytochrome *c* oxidase exhibited cross-reactivity with an antibody raised against COX III of *Saccharomyces cerevisiae* (Fig. 2B, lane 3). This band had a molecular mass similar to that of the corresponding subunit III of cytochrome *c* oxidase from yeast (Fig. 2B, lane 1). The 29.6-kDa polypeptide of *Polytomella* spp. was excised from the gel and extracted. The purified polypeptide still showed cross-reactivity with the anti-yeast antibody (Fig. 2B, lane 2). Accordingly, it was subjected to N-terminal sequencing. The N-terminal sequence obtained (SSDAGHHLSPRERYLV) showed no similarity with any other COX III in the NCBI sequence data banks.

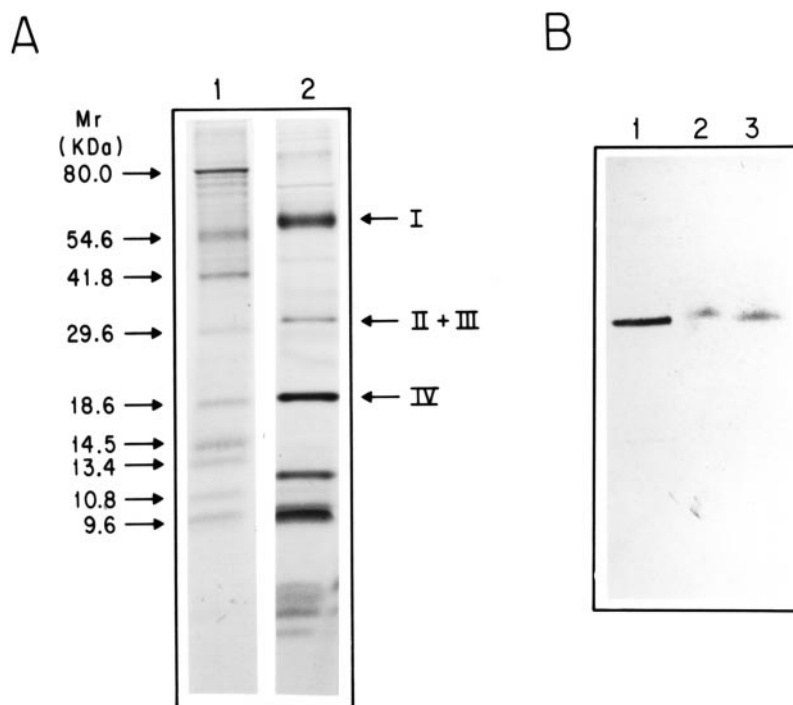
Characterization of the *cox3* Gene from *Polytomella* spp. and the *cox3* cDNA from *C. reinhardtii*—Two degenerate deoxyoligonucleotides were designed based on the N-terminal sequence of COX III from *Polytomella* spp. (Ps-COX III) and on a highly conserved internal sequence of COX III present in different organisms. With these oligonucleotide primers, a PCR amplification product of 780 nucleotides was obtained using total DNA from *Polytomella* spp. as a template. The DNA sequence from the amplified product was predicted to encode a COX III protein. This sequence was used to design primers for use in 5'- and 3'-RACE using cDNA made from *Polytomella* spp. total RNA (27). The overlapping cDNA clones thus obtained were sequenced and a full-length cDNA sequence (DDBJ/EMBL/GenBank™ accession number AF233514) was obtained. The amplified genomic fragment was also used as a probe to screen a λ gt10 cDNA library from *C. reinhardtii*, and eight positive plaques were identified, isolated, and sequenced. The clone containing the longest cDNA was identified by PCR and sequenced, confirming the cDNA as encoding COX III. The overlapping regions of the genomic and cDNA sequences were identical. The sequence of the *cox3* cDNA from *C. reinhardtii* is not shown but is available in DDBJ/EMBL/GenBank™ with accession number AF233515.

Translation of the cDNA sequences predicts a mature protein of 272 residues with a molecular mass of 29,978 Da for Ps-COX III, and a mature polypeptide of 272 residues (29,967 Da) for its close relative *C. reinhardtii* (Cr-COX III). Comparison of Ps-COX III with Cr-COX III indicated that the first 17 residues of the mature COX III sequences are highly conserved between *Polytomella* spp. and *C. reinhardtii* but are not present in the COX III sequence of the chlorophyte alga *Prototheca wickerhamii*. The alignment of the overall amino acid sequences of Ps-COX III and Cr-COX III (Fig. 3A) revealed an identity of 66.5% and a similarity of 73.9%. The similarity between the two subunits III of the cytochrome *c* oxidase is very high and extends over the complete protein sequences.

The *Polytomella* spp. *cox3* cDNA contains an open reading frame of 1113 base pairs, our identification of the N terminus of the mature protein as amino acid 99, allows us to predict a 98-amino acid mitochondrial targeting sequence (MTS). In *C. reinhardtii* the *cox3* cDNA contains an open reading frame of 1146 base pairs. Assuming that the N-terminal sequence of the mature protein corresponds to that of *Polytomella* spp., three different ATG codons could correspond to the initiation of the MTS. The upstream methionine predicts a presequence of 109 amino acids, the second methionine predicts a 51 residues MTS, and the downstream one predicts a presequence of 40 amino acids. It is known that the sequence surrounding start codon affects the efficiency of the translation; in *C. reinhardtii* there is a consensus of (A/C)A(A/C)(A/C)ATG(G/C)C(C/G) for the start codon (35). According to these data, the upstream methionine that predicts a MTS of 110 amino acids is appropriate for translation initiation site.

The alignment of the presequences of Ps-COX III and Cr-COX III revealed 45.4% identity and a 50.5% similarity. This

FIG. 2. Subunit composition and immunoblot analysis of the cytochrome *c* oxidase complex from *Polytomella* spp. A, the cytochrome *c* oxidase preparation was analyzed on a 16% acrylamide gel stained with Coomassie Brilliant Blue (23) and compared with the bovine enzyme. Lane 1, cytochrome *c* oxidase from *Polytomella* spp. (20 μ g of protein). Lane 2, cytochrome *c* oxidase from beef-heart mitochondria (30 μ g of protein); its four major subunits are indicated. The apparent molecular masses are shown in kDa. B, blot immunostained with antibodies raised against COX III from *S. cerevisiae*. Lane 1, yeast cytochrome *c* oxidase (20 μ g of protein per lane). Lane 2, purified COX III from *Polytomella* spp. Lane 3, isolated cytochrome *c* oxidase from *Polytomella* spp. (20 μ g of protein/lane).



values are much higher when the first 17 residues MRSQLL(K/R)ALTRAPAGFS are compared or when the 8-residues region ALAALPPR just before the mature protein is compared. These sequences must play an important role in the processing of the MTS or in the import of COX III in these algae. Immediately upstream of the N terminus of the mature protein (as determined by protein sequencing of *Polytomella* spp. COX III) in both algae there is a methionine that could have been retained from the ancestral mitochondrial copy.

A cladogram was generated with COX III sequences from different organisms (Fig. 3B). The result obtained showed that Ps-COX III clearly affiliates with Cr-COX III, but surprisingly, these chlamydomonad COX III sequences appear close to yeast COX III sequences and relatively far away from the mitochondrial COX III sequences from other algae and from plants.

The pattern of codon utilization for the *cox3* gene of *Polytomella* spp. was compared with the pattern of codon usage of known nuclear and mitochondrial genes of this alga (Table I). As in other nuclear-localized genes, there is a significant bias in each codon family; this is because triplets that end in A are rare in the nuclear genome of this alga (14). The codon usage of the *cox3* gene of *Polytomella* spp. is typically nuclear and different from mitochondrial codon usage. A similar analysis was carried out for the *cox3* cDNA from *C. reinhardtii*. The codon usage pattern was similar to nuclear codon usage and differed from codon usage in the mitochondrial genome. In addition, the polyadenylation signals TGTA (35) were found at the end of the cDNA sequences.

DNA blot analysis was carried out to ascertain that the *cox3* genes were encoded by the nuclear genome. Total DNA isolated from *Polytomella* spp. was electrophoresed through agarose. The mtDNA separated as a discrete band running below the major band representing nuclear DNA. The DNA from these gels was transferred to nylon membranes and subjected to hybridization analysis with a battery of probes from mitochondrial and nuclear origins. The smaller band hybridized with three different mitochondrial probes (Fig. 4A) *cob1*, encoding cytochrome *b* from *Polytomella* spp. (36); *nad4*, encoding subunit 4 of NADH-ubiquinone oxidoreductase from *Polytomella*

spp.²; and *cox1*, encoding subunit I of cytochrome *c* oxidase from *Polytomella* spp. (15). In contrast, nuclear DNA hybridized with the following nuclear probes: *Cytc1*, a partial sequence of the gene encoding cytochrome *c*₁ (18), and *TubB1*, the gene encoding β -tubulin from *Polytomella agilis* (now renamed *Polytomella parva*) (14). The *cox3* gene hybridized with the major DNA fraction and not with the mtDNA band, confirming its nuclear localization. A similar analysis was carried out with total DNA from *C. reinhardtii* (Fig. 4B). The smaller band hybridized with three different mitochondrial probes from *C. reinhardtii*, *cob1*, *nad2*, and *cox1* (5). In contrast, nuclear DNA hybridized with the following nuclear probes from *C. reinhardtii*: the gene *Cyc* encoding cytochrome *c* (37), the gene *Fes1* encoding the Rieske iron-sulfur protein (38), the gene *AtpB* encoding the β subunit of the ATP synthase (28), and the *cox3* gene obtained in this study.

Hydrophobicity and Importability of the Nuclear-encoded Subunit III of Cytochrome *c* Oxidase from *Chlamydomonas Algae*—Import studies suggest that the highest average hydrophobicity over 60–80 amino acids of a polypeptide chain (termed mesohydrophobicity), along with the maximum hydrophobicity of likely transmembrane segments, are useful indicators of the likelihood that a protein could be imported into the mitochondrion (19). The predicted Ps-COX III and Cr-COX III subunits were tested for their physical characteristics *in silico*. The computational analyses suggested that both proteins contain a bipartite MTS. The first 25 amino acids are predicted to be a MTS, whereas the segment up to residue 50 is predicted to be a mitochondrial inner membrane signal that should direct the peptide to its final location (Fig. 5). Both COX III polypeptides were compared with those encoded by other complete *cox3* genes in the data base; all are located at mitochondrial genomes. Fig. 6A shows a *mesoH* versus maximal local hydrophobicity (<H>) plot for different COX III sequences. In comparison with all their mitochondrial counterparts, Ps-COX III and Cr-COX III display both decreased local hydrophobicity and

² S. Funes, A. Antaramian, and D. González-Halphen, unpublished results.

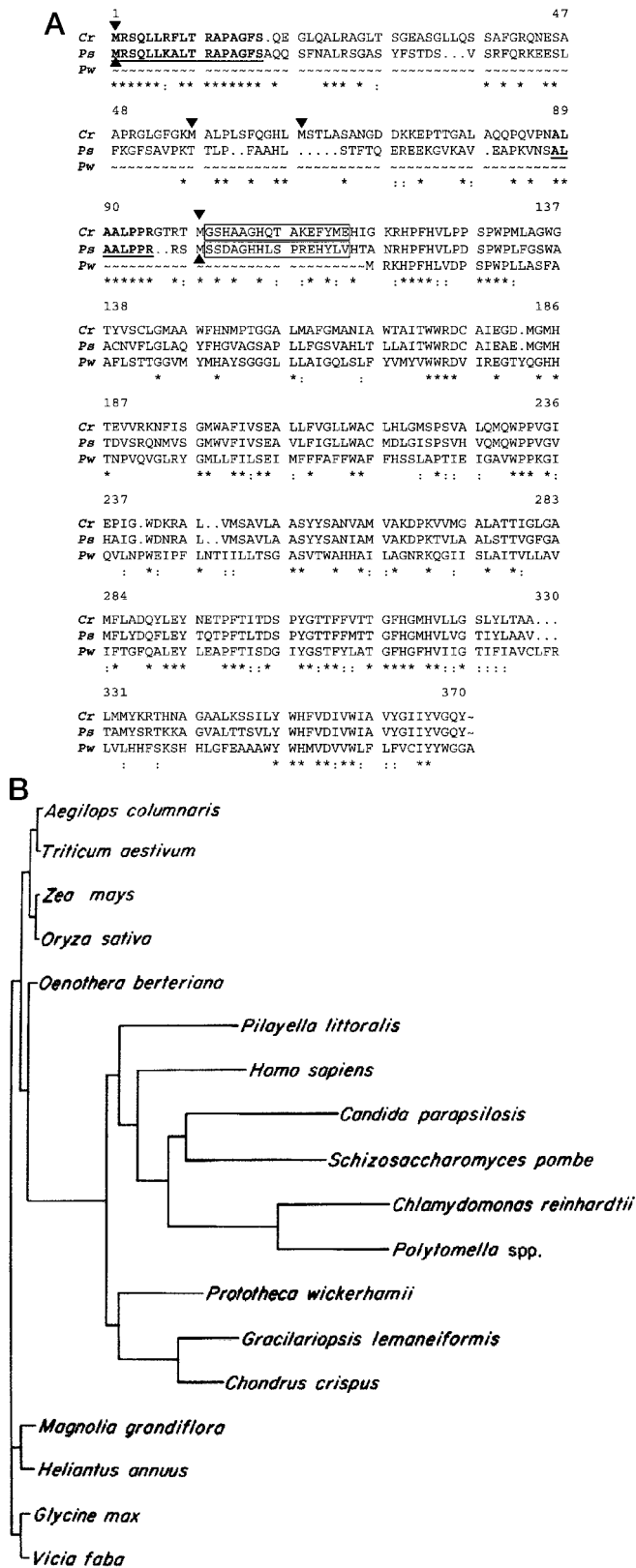


FIG. 3. Sequence alignment and phylogenetic analysis of cytochrome *c* oxidase subunit III sequences. A, sequence alignment of COX III from *Polytomella* spp. (Ps), *C. reinhardtii* (Cr), and *P. wickerhamii* (Pw) (62). Numbering is referred to the *Polytomella* spp. sequence. Black triangles indicate methionine residues present in the putative MTS sequences. The boxed regions indicate the N-terminal sequence of COX III from *Polytomella* spp. determined by Edman degradation and its homologous region in *C. reinhardtii*. The underlined sequences shown in bold are highly conserved amino acids present before the N-terminal sequence. Asterisks denote identical residues.

TABLE I

Codon usage of nuclear and mitochondrial genes of *Polytomella* spp.

Values are shown as percentages. Conspicuous differences in the codon usage are indicated in bold characters and gray boxes. Nuclear gene sequences used to construct this table were the TubB1 gene encoding β -tubulin from *P. agilis* (now *P. parva*) (14), partial sequence of the gene *Cytc1* encoding cytochrome c_1 (18), and partial sequence of *AtpA*, encoding subunit α of ATP synthase from *Polytomella* spp. (Xiao, Antaramian, and González-Halphen, unpublished results). Mitochondrial gene sequences from *Polytomella* spp. were *cox1* (15), *cob1* (36), and *nad4* (Funes, Antaramian, and González-Halphen, unpublished results).

Res	Codon	Mito	Nuc	<i>cox3</i>	Res	Codon	Mito	Nuc	<i>cox3</i>
Gly	GGG	8	0	0	Trp	TGG	100	100	100
Gly	GGA	34	2	5	End	TGA	0	0	0
Gly	GGT	46	75	65	Cys	TGT	43	0	0
Gly	GGC	12	23	30	Cys	TGC	57	100	100
Glu	GAG	47	95	100	End	TAG	33	0	0
Glu	GAA	53	5	0	End	TAA	67	100	100
Asp	GAT	64	64	30	Tyr	TAT	45	17	0
Asp	GAC	36	36	70	Tyr	TAC	55	83	100
Val	GTG	19	4	9	Leu	TTG	24	7	12
Val	GTA	34	0	0	Leu	TTA	28	1	0
Val	GTT	35	37	35	Phe	TTT	50	8	10
Val	GTC	12	59	56	Phe	TTC	50	92	90
Ala	GCG	5	0	0	Ser	TCG	4	0	0
Ala	GCA	14	1	2	Ser	TCA	6	2	4
Ala	GCT	66	36	51	Ser	TCT	21	32	43
Ala	GCC	15	63	47	Ser	TCC	8	49	39
Arg	AGG	4	9	18	Arg	CGG	4	0	0
Arg	AGA	3	0	0	Arg	CGA	21	0	0
Ser	AGT	29	1	0	Arg	CGT	43	62	46
Ser	AGC	32	15	14	Arg	CGC	25	29	36
Lys	AAG	32	98	90	Gln	CAG	41	96	100
Lys	AAA	68	2	10	Gln	CAA	59	4	0
Asn	AAT	57	11	0	His	CAT	64	24	27
Asn	AAC	43	89	100	His	CAC	36	76	73
Met	ATG	100	100	100	Leu	CTG	6	1	6
Ile	ATA	25	2	0	Leu	CTA	12	0	0
Ile	ATT	58	37	50	Leu	CTT	25	44	44
Ile	ATC	17	61	50	Leu	CTC	5	47	38
Thr	ACG	3	0	0	Pro	CCG	2	0	0
Thr	ACA	33	0	0	Pro	CCA	59	0	6
Thr	ACT	50	36	44	Pro	CCT	17	38	33
Thr	ACC	14	64	56	Pro	CCC	22	62	61

mesohydrophobicity. This strengthens the observation that mitochondrial imported proteins have diminished physical constraints (<H> and *meso*H) when compared with polypeptides encoded by mitochondrial genes. The figure presents the results using the scale PRIFT (32), but similar results were obtained with the scale GES and with other scales based on physicochemical amino acid properties OMH or KD (39) (results not shown). It is noteworthy that all COX III proteins that are encoded in the mitochondrial genome have higher hydrophobicity values and are grouped in the upper right corner of the graph.

Hydropathy profile analysis, carried out with different scales (32), predicted seven transmembrane stretches for COX III polypeptides from both algae (Fig. 6B). This suggests a structure of these polypeptides similar to the ones determined by

Two dots indicate similar residues. Sequence comparisons made without considering the putative presequences showed 66.5% identity and 73.9% similarity between *Polytomella* spp. and *C. reinhardtii*; 33.6% identity and 42.7% similarity between *Polytomella* spp. and *P. wickerhamii*; and 35.6% identity and 46.6% similarity between *C. reinhardtii* and *P. wickerhamii*. B, phylogenetic analysis of cytochrome *c* oxidase subunit III sequence. To construct the cladogram, the amino acid sequences of cytochrome *c* oxidase subunit III were compared among different organisms and the sequences obtained in this study, corresponding only to the mature proteins.

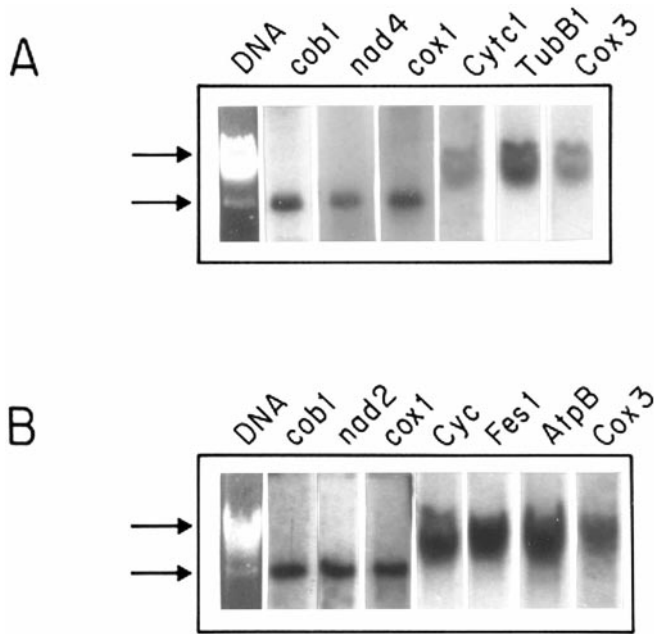


FIG. 4. The gene *cox3* is nuclear-localized in *Polytomella* spp. and in *C. reinhardtii*. A, 30 μ g of total DNA from *Polytomella* spp. was run in a 0.7% agarose gel. The gel transferred to a nylon membrane and hybridized with different nuclear and mitochondrial probes described in the text. Arrows indicate the positions of nuclear DNA and mtDNA. B, 30 μ g of total DNA from *C. reinhardtii* were run in a 0.7% agarose gel. Hybridization analysis was carried out with different nuclear and mitochondrial probes as indicated (see text).

Ps :	1	MRSQLLKALTRAPAGFSAQCSFNALRSGASYFSTDS---VSRFORKEESLFKGFSAVPEKT	57
Cr:	1	MRSQLLRFLTRAPAGFS-QEGLQALRACLTSGEASGLLQSSAFGRQNESAAPRGLGFQKGM	59
Ps :	58	TLP--FAAHL-----STFTQEREKGVKAV-EAPKVNLSALALPPR--RSMSSDAGHLS	107
Cr:	60	ALPLSFQGHMLSTLASANGDDKKEPTTGALAQQPQVFNALALPFRGTRTWSSRAAGHOT	119
Cr α ATP	1	MRSQALSARAGLLQLSSQTGASLEGGFALSKRAEQALIRASRAFASDAKAL	52
CrFeS	1	MALRRVASFLPKLAGAAETLPAASHASSFSQLICTPLDVVERPEQPSARSRFSASDNEVEF	60

FIG. 5. Comparison of presequences in nuclear encoded mitochondrial proteins in *Polytomella* spp. and *C. reinhardtii*. Identical and similar residues are denoted with straight vertical lines and double dots, respectively. Only the sequences from *Polytomella* spp. (Ps) and *C. reinhardtii* (Cr) are compared. These sequences exhibited 45.4% identity and 50.5% similarity. The arrow indicates the site where presequences are cleaved by the mitochondrial processing protease. The N-terminal sequences of the mature proteins are shown in bold characters. The data shown for the α subunit of ATP synthase (Cr α ATP) and the Rieske iron sulfur protein (CrFeS) of *C. reinhardtii* were taken from Nurani and Franzén (63) and from Atteia and Franzén (38), respectively. The box indicates conserved residues before and after the cleavage site according to the consensus sequence R(A/S/T)(M/F)↓(A/S/G)S(D/H)A (45).

x-ray crystallography for the bacterial and mammalian COX III subunits (2, 3). As an initial approach to gain insights on its topological arrangement, Ps-COX III was modelled over the three-dimensional structure of the bovine COX III (3). The predicted structure of Ps-COX III shows an overall topology similar to the bovine COX III but exhibiting shorter or incomplete transmembrane stretches as compared with the bovine counterpart (results not shown). Altogether, these observations suggest that the cytoplasmic-synthesized COX III polypeptides from chlamydomonad algae are imported into mitochondria and assembled in the inner mitochondrial membrane, with a topology similar but not identical to that of its mitochondrial-synthesized counterparts in other organisms.

DISCUSSION

Subunit III Is a Bona Fide Constituent of Cytochrome c Oxidase from Polytomella spp.—Cytochrome *c* oxidase from *C.*

reinhardtii has been purified and partially characterized (34, 40). In those works, the presence of Cr-COX III was not ascertained. In our hands, Ps-COX III was present in the intact cytochrome *c* oxidase of *Polytomella* spp. and was shown to be a *bona fide* constituent of this complex by immunochemical analysis. Therefore, we suggest that this subunit must exist in the mitochondrial complexes of algae of the family Chlamydomonadaceae. Moreover, because the corresponding gene is absent in the mitochondrial genomes of these algae (Refs. 5–9 and this work), it is likely that it was transferred to the nucleus early in evolution but previous to speciation.

COX III Is Nuclear-encoded in the Algae of the Family Chlamydomonadaceae—This work also describes the cloning and complete sequencing of two new members of the *cox3* gene family from two chlamydomonad algae. Up until now the genes that encode COX III have been found only in mitochondrial genomes. The gene *cox3* is found even in the most reduced mitochondrial genome known to date, that of *Plasmodium falciparum* (41). The existence of a nuclear-encoded *cox3* gene was proposed for the lycopod *Selaginella*, because it was not present in the mitochondrial genome (42). However there is no evidence for its presence in the nuclear genome. Here we show that the *cox3* gene is nuclear-localized in the algae of the family Chlamydomonadaceae, as shown by Southern blot hybridization (Fig. 4), the presence of a biased codon usage typical of nuclear-localized genes in chlamydomonad algae (Table I), the presence of a polyadenylation signal TGTA usually found in the nuclear-localized genes of these algae, the existence of a sequence encoding a putative bipartite MTS (Fig. 5), a diminished <H> and mesoH of the predicted protein product (Fig. 6A), and the presence of introns in the corresponding *cox3* genomic sequences.³ The *cox3* gene is expressed as demonstrated by Northern blot hybridization (data not shown). In addition, the corresponding subunit is present in the mature and isolated cytochrome *c* oxidase complex from *Polytomella* spp., as shown by N-terminal sequencing and immunochemical analysis. To our knowledge, this is the first report of a nuclear-localized and active *cox3* gene. Some portions of the *cox3* gene of *C. reinhardtii* described in this work are similar to three cDNA sequences (AV386752, AV391757, and AV393074) recently deposited in the expressed sequence tags data base (43).

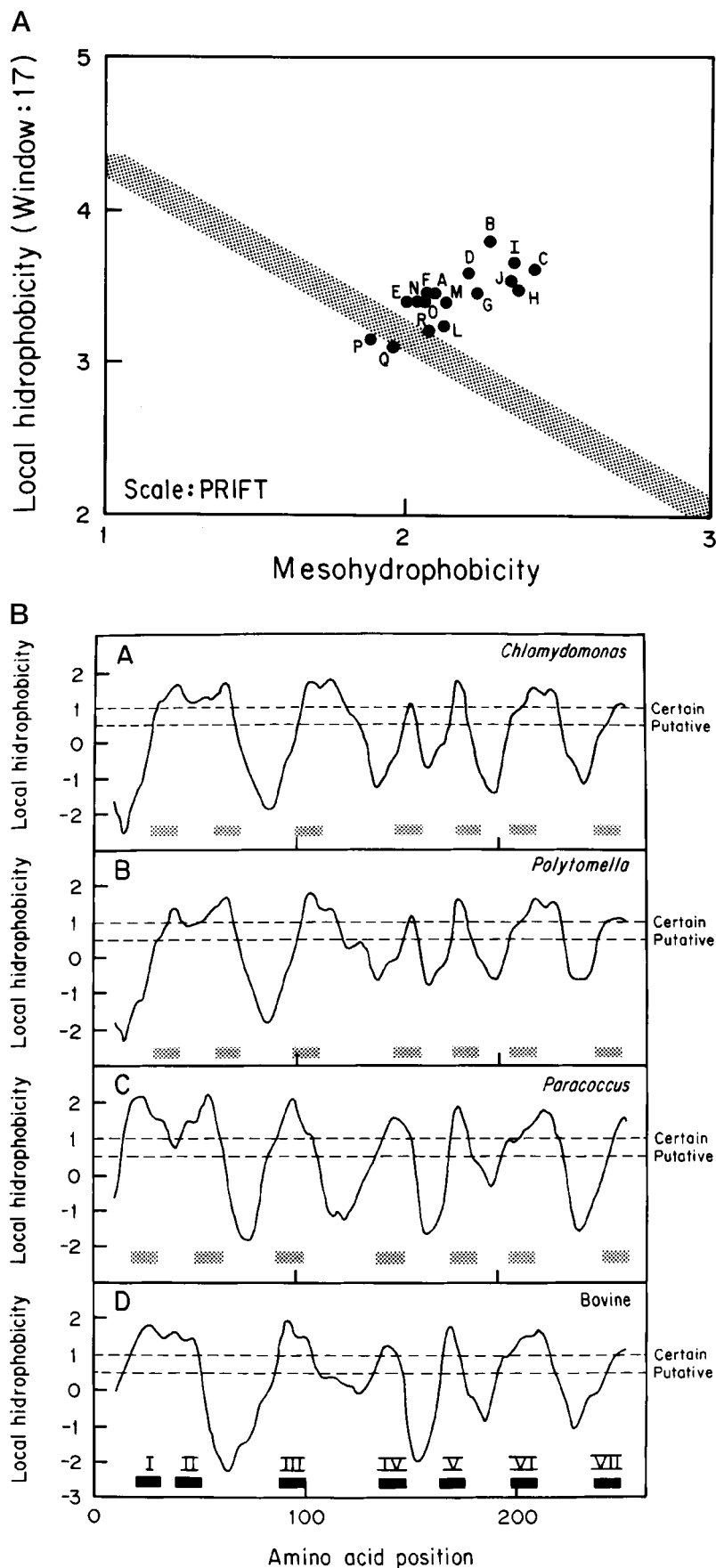
Other organisms that lack the *cox3* gene in their mitochondrial genomes are the chlorophyte alga *Pedinomonas minor* and the ciliates *Paramecium aurelia* and *Tetrahymena pyriformis* (44). It is possible that these organisms may have also transferred their *cox3* genes to the nucleus control.

Nucleotide sequences encoding putative MTS were identified in the *cox3* genes of *Polytomella* spp. and *C. reinhardtii*. The MTS of Ps-COX III and Cr-COX III show some similarities with the mitochondrial targeting sequence of the Rieske iron-sulfur protein from *C. reinhardtii* (Fig. 5). The MTS sequences from chlamydomonad algae are rich in alanines, prolines, and charged amino acids. These sequences predict an amphiphilic α -helix structure in the N-terminal region. In addition, they share a similar site for cleavage for the mitochondrial processing peptidase, which seems to recognize the consensus sequence R(A/S/T)(M/F)↓(A/S/G)S(D/H)A (45).

Characteristics of Gene Transfer from the Mitochondria to the Nucleus in the Algae of the Family Chlamydomonadaceae—The theory of the origin of mitochondria proposes that there was a gradual transfer of genes from the original bacterial endosymbiont to the nucleus (46). This transfer is an ongoing process, as exemplified by the presence of genes encoded in

³ X. Pérez-Martínez, S. Funes, E. Davidson, M. P. King, and D. González-Halphen, unpublished observations.

FIG. 6. Mesohydrophobicity and hydrophobicity plots of cytochrome *c* oxidase subunit III from different organisms. A, mesohydrophobicity versus maximal local hydrophobicity plot for cytochrome *c* oxidase subunit III from different organisms using the PRIFT scale. Proteins are distributed on the *abscissa* according to their maximum hydrophobicity value and on the *ordinate* according to the hydrophobicity of the most hydrophobic segment. The boundary was calculated as in Claros *et al.* (19). The GenBank™ accession numbers of the nuclear COX III sequences used to construct this graph were: A, *Aegilops columnaris* (U46765); B, *Candida parapsilosis* (X75679); C, *Chondrus crispus* (P48872); D, *Helianthus annuus* (X57669); E, *Magnolia grandiflora* (Z68127); F, *Zea mays* (X53055); G, *Oenothera berteriana* (X04764); H, *Pilayella littoralis* (Z37967); I, *P. wickerhamii* (Q37620); J, *Gracilaria lemaneiformis* (AF118119); K, *Oryza sativa* (X17040); L, *Schizosaccharomyces pombe* (X16868); M, *Glycine max*, soybean (X15131); N, *Vicia faba* (X51690); O, *Triticum aestivum* (X15944); P, *C. reinhardtii* (AF233515, this work); Q, *Polytomella* spp. (AF233514, this work); and R, *Homo sapiens*, human (P00414). B, hydrophathy plots comparing the deduced COX III sequences of *Polytomella* spp. (B) and *C. reinhardtii* (A) with the ones of the bovine enzyme (D) and *P. denitrificans* (C) are shown. Black boxes with roman numerals indicate the positions of certain transmembrane stretches based on the crystallographic structure of bovine COX III. Gray boxes indicate calculated transmembrane stretches.



both the mitochondrial and the nuclear genomes, *i.e.* ATP synthase subunit 9 of *Neurospora crassa* (47), and COX II of some leguminosae (48). In several species, the process of moving mitochondrial genes to the nucleus may have a selective advantage, because nuclear genes exhibit a lower mutation rate and the nucleus seems to have a more sophisticated DNA repair system than mitochondria (49). Gene transfer from organelles to the nucleus is also thought to increase its rate of recombination and reduce accumulation of deleterious mutations (50).

The *cox3* gene transferred from the mitochondria to the nucleus in the chlamydomonad algae satisfies many of the criteria necessary for a gene that has been translocated from the mitochondrial to the nuclear genome, as proposed by Brennicke *et al.* (42) and by Claros *et al.* (19). It acquired a presequence for targeting into mitochondria, changed its codon usage, acquired a polyadenylation signal, and diminished the $\langle H \rangle$ and *mesoH* of its protein product. In addition, the corresponding mitochondrial copy that presumably existed has completely disappeared, suggesting that this transfer occurred early in evolution.

The high sequence similarity found between a region of the putative MTS encoded by the *cox3* genes from *C. reinhardtii* and *Polytomella* spp. (Fig. 3A) suggests that the transfer of this gene from the mitochondria to the nucleus and the corresponding acquisition of the presequence, occurred before the *Polytomella* colorless genus diverged from the main *Chlamydomonas* photosynthetic lineage. Otherwise no conservation of the presequences would be expected. The drastic change in codon usage, which is more remarkable in these algae because of its highly biased nuclear codon usage (14), also suggests that the transfer of the *cox3* gene in these organisms occurred early in evolution, when there was a massive transfer of genes from the protomitochondrion to the nucleus (46). This process might have occurred before the Post Cretaceous era (65 or more million years ago), when the nonphotosynthetic algae are thought to have derived from the green lineages (11). The phylogenetic analysis carried out with the predicted COX III sequences (Fig. 3B) shows similar results to those obtained with classical mitochondrial proteins like COX I (15), or cytochrome *b* (36). Sequences from the algae of the genera *Polytomella* and *Chlamydomonas* tend to strongly affiliate in these phylogenetic analyses.

Importability of Nuclear-encoded Subunits into Mitochondria: Subunit III Exhibits Diminished Local $\langle H \rangle$ and Diminished mesoH—Why have some genes remained in the mitochondrial DNA? One explanation has been the variation of the genetic code in mitochondria, where the triplet UGA encodes tryptophan instead of a polypeptide chain termination signal (51). Another explanation suggests that organelle genomes have persisted by encoding structural proteins that maintain redox balance within the bioenergetic membranes (52). Alternatively, it has been proposed that the genes that remained localized in the mitochondrial genome are those that encode highly hydrophobic polytopic proteins, containing two or more helices that span the membrane (53). This is because the presence of a larger number of hydrophobic segments in a polypeptide could impair its import into mitochondria (54) or cause mistargeting to the endoplasmic reticulum (53). Moreover, the synthesis of hydrophobic polypeptides inside the mitochondria may ensure their proper insertion in the inner membrane and the correct topological arrangement required for vectorial proton translocation. Two classic examples are the cytochrome *b* gene (*cob1*), which encodes an 8-transmembrane-stretch polypeptide (55), and the cytochrome *c* oxidase subunit I (*cox1*), which encodes a protein with 12 membrane-associated helices

(2, 3). Both genes are present in all mitochondrial genomes characterized to date. Other genes that encode highly hydrophobic polypeptides are also present in the majority of mtDNAs (56), *i.e.* *atp6* (encoding 5 transmembrane helices), *atp8* (encoding 2 transmembrane helices), *nad1* (encoding 8–9 transmembrane helices), *nad2* (encoding 13–14 transmembrane helices), *nad3* (encoding 3 transmembrane helices), *nad4* (encoding 13–14 transmembrane helices), *nad4L* (encoding 3 transmembrane helices), *nad5* (encoding 15–16 transmembrane helices), *nad6* (encoding 5 transmembrane helices), *cox2* (encoding 2 transmembrane helices), and *cox3* (encoding 7 transmembrane helices).

In yeast, *in vivo* studies with cytoplasmically synthesized constructs of variable lengths of apocytochrome *b*, showed that in mitochondria, the import of polypeptides with more than three or four transmembrane helices is strongly hindered (19). Analysis of sequences from nuclear-encoded and mitochondrial-encoded mitochondrial proteins suggested that low values of *mesoH* and $\langle H \rangle$ are more useful indicators than the number of transmembrane regions in determining whether a protein could be imported into the mitochondrion. It is known that mitochondria readily import proteins with several transmembrane stretches, for example the adenine nucleotide translocator, if they possess low $\langle H \rangle$ and low *mesoH* (19). However, the import pathway of the translocator differs greatly from the “conservative intramitochondrial sorting pathway,” in which polypeptides are transferred to the mitochondrial matrix space and then sorted to its final membrane destination (57). We hypothesize that the latter may be the mechanism for the biogenesis of the COX III proteins described in this work.

Transfer of genes from organelles to the nucleus involves several steps (42): (i) the export of the nucleic acid molecule as DNA or RNA (48), (ii) integration into the nucleus by nonhomologous recombination (58) or by a common end-joining mechanism (59), (iii) acquisition of a presequence by duplication of existing targeting signals (60), (iv) acquisition of a promoter, a ribosome binding site, and a polyadenylation signal (49), (v) change in codon usage (49), (vi) modification of the nucleotide sequence to encode for a polypeptide with diminished local hydrophobicity ($\langle H \rangle$) and diminished *mesoH*, which may allow the import of the protein products into mitochondria (19), (vii) inactivation of the mitochondrial gene copy, and (viii) stepwise loss of the mitochondrial gene (61).

Our data support the hypothesis that the genes that encode proteins with high $\langle H \rangle$ and high *mesoH* have remained in the mitochondrial genome, whereas those genes that encode proteins with low values of $\langle H \rangle$ and *mesoH* have been exported to the nucleus, and their protein products imported back into mitochondria (19). The strategy used by the algae of the family Chlamydomonadaceae seems to involve the acquisition of a large and possibly bipartite MTS and a lowering of $\langle H \rangle$ and *mesoH* in the COX III polypeptides, which are requirements for the proper insertion of the protein into the mitochondrial inner membrane. We hypothesize that the limiting step in gene transfer from organelles to the nucleus has not been the differences in genetic code but hindrances to the import into the mitochondrial inner membrane polytopic proteins whose membrane topology is a critical requirement for its catalytic activity (vectorial proton pumping).

Hydropathy analysis of Cr-COX III and Ps-COX III showed the presence of seven putative transmembrane stretches. The hydrophobicity of these seven helices seems to be lower in the chlamydomonad algae when compared with the *P. denitrificans* or the bovine subunits (Fig. 6B). This is more evident in the three-dimensional model for Ps-COX III built upon the crystallographic coordinates of its bovine counterpart (3). In our model

(data not shown), shorter transmembrane stretches are observed as well as interruptions in the middle section of the membrane helices. In addition, helix VII of the Ps-COX III protein is only half the size of the corresponding helix in the bovine polypeptide and may not span the membrane bilayer. The helices that are in contact with COX I (helices I and III) do not exhibit structural modifications, suggesting that the diminished hydrophobicity of COX III is stronger in those regions of the protein that seem not to be involved in subunit-subunit interactions.

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