Curr Genet (2002) 40: 399–404
DOI 10.1007/s00294-002-0270-6

RESEARCH ARTICLE

**Xochitl Pérez-Martínez · Soledad Funes
Elena Tolkunova · Edgar Davidson · Michael P. King
Diego González-Halphen**

Structure of nuclear-localized *cox3* genes in *Chlamydomonas reinhardtii* and in its colorless close relative *Polytomella* sp.

Received: 17 November 2001 / Accepted: 21 December 2001 / Published online: 13 February 2002

Abstract Several chlorophyte algae do not have the *cox3* gene, encoding subunit III of cytochrome *c* oxidase, in their mitochondrial genomes. The *cox3* gene is nuclear-encoded in the photosynthetic alga *Chlamydomonas reinhardtii* and in the colorless alga *Polytomella* sp. In this work, the genomic sequences of the *cox3* genes of these two closely related algae are reported. The *cox3* genes of both *C. reinhardtii* and *Polytomella* sp. contain four introns in the region encoding the putative mitochondrial-targeting sequences. These four introns show low sequence identities, but their locations are conserved between these species. The *cox3* gene of *C. reinhardtii* has five additional introns in the region encoding the mature subunit III of cytochrome *c* oxidase. Sequence analysis of intron 6 of the *cox3* gene of *C. reinhardtii* revealed similarity with two sequence elements present in introns of several other nuclear genes from this green alga. In the majority of the genes, these conserved sequences are located either near the 3′ end or near the 5′ end of the introns. Based on these data, we propose that the colorless genus *Polytomella* separated from *C. reinhardtii* after the *cox3* gene was transferred to the nucleus. The data also support the evolutionary hypothesis of a recent acquisition of introns in *C. reinhardtii*.

Keywords *Chlamydomonas reinhardtii* · Cytochrome *c* oxidase · Mitochondrial-targeting signals · *Polytomella* sp.

Communicated by B.B. Sears

X. Pérez-Martínez · S. Funes · D. González-Halphen (✉)
Departamento de Genética Molecular,
Instituto de Fisiología Celular,
Universidad Nacional Autónoma de México,
Apartado Postal 70–243, México 04510, D.F., Mexico
E-mail: dhalphen@ifisiol.unam.mx

E. Tolkunova · E. Davidson · M.P. King
Department of Biochemistry and Molecular Pharmacology,
Thomas Jefferson University, 233 South 10th Street,
BLSB 308, Philadelphia, PA 19107, USA

Introduction

Members of the genus *Chlamydomonas* form a monophyletic group, but are distributed in at least six distinct lineages (Buchheim et al. 1996; Friedl 1997). The green alga *C. reinhardtii* – a model of choice in the molecular studies of photosynthesis and flagellar structure – has been grouped with members of the genus *Volvox* in several phylogenetic analyses (Buchheim et al. 1996; Nakayama et al. 1996; Rumpf et al. 1996; Friedl 1997). The colorless chlorophytes of the genus *Polytomella* are also close relatives of *C. reinhardtii*; complete 18S rDNA sequence analysis suggests that *C. reinhardtii*, *Polytomella parva*, *Polytomella* sp. and *Volvox carterii* are members of a single monophyletic clade, the so-called *Volvox* clade (Melkonian and Surek 1995; Nakayama et al. 1996). The genus *Polytomella* is believed to have evolved from a *Chlamydomonas*-like ancestor by loss of its cell wall and functional chloroplasts (Round 1980; Melkonian and Surek 1995). The close phylogenetic relationship between members of the genus *Polytomella* and *C. reinhardtii* is also supported by morphological and structural similarities (Mattox and Stewart 1984), the similarity of its nuclear encoded β-tubulin genes (Conner et al. 1989), its mitochondrial *cox1* genes (Antaramian et al. 1996), and the biochemical similarities in the α and β subunits of their mitochondrial F_1F_0-ATPases (Atteia et al. 1997).

The mitochondrial genomes of several chlorophyte algae lack some of the genes that are commonly found in the mitochondrial genome of the majority of eukaryotes. The missing genes include *cox2*, *cox3*, *atp6*, *atp8*, *atp9*, *nad3* and *nad4L* in *C. reinhardtii* (Michaelis et al. 1990), *C. eugametos* (Denovan-Wright et al. 1998), and *Chlorogonium elongatum* (Kroymann and Zetsche 1998). In the green alga *Chlamydomonas reinhardtii* and in the colorless alga *Polytomella* sp., the *cox2* and *cox3* genes encoding subunits II and III of cytochrome *c* oxidase have been transferred from the mitochondrial genome to the nucleus (Pérez-Martínez et al. 2000, 2001). The

nuclear-localized *cox2* and *cox3* genes reflect the characteristics of genes transferred from the mitochondrion to the nucleus proposed by Brennicke et al. (1993) and Claros et al. (1995): (1) they acquired a nucleotide sequence encoding a putative mitochondrial-targeting sequence (MTS), (ii) they exhibit a nuclear codon-usage pattern, (3) they acquired polyadenylation signals typical of chlamydomonad nuclear genes that differ from the consensus in other eukaryotes, and (4) their deduced protein products exhibit an overall diminished mesohydrophobicity, when compared with their counterparts encoded in the mitochondrial genome.

In this work, we describe the cloning and characterization of the genomic *cox3* genes from *C. reinhardtii* and *Polytomella* sp. This is the first description of *cox3* genes that are localized in the cell nucleus. The data suggest that transfer of the *cox3* gene to the nucleus occurred before the divergence of *Polytomella* sp. and *C. reinhardtii*. They also support the hypothesis of a late acquisition of introns in *C. reinhardtii*.

Materials and methods

Strains and culture conditions

Polytomella sp. (198.80, E.G. Pringsheim), from the Sammlung von Algenkulturen (Gottingen, Germany), was grown as described by Gutiérrez-Cirlos et al. (1994). *C. reinhardtii* (wild type, CC125 mt+) was obtained from the *Chlamydomonas* Genetic Center (Duke University) and was grown as described by Rochaix et al. (1988).

Cloning and sequencing of genomic *cox3* from *Polytomella* sp. and *C. reinhardtii*

Total DNA was obtained from *Polytomella* sp. and *C. reinhardtii* as described by Pérez-Martínez et al. (2000). All other molecular biology techniques were standard (Sambrook et al. 1989). Nucleotide sequencing was performed by the Kimmel Cancer Center DNA Sequencing Facility, Thomas Jefferson University.

The genomic sequence of the *cox3* gene of *Polytomella* sp. was obtained by PCR amplification with Hot Start Taq polymerase (Qiagen), using primers designed to anneal to the 5′ and 3′ noncoding regions of the *cox3* cDNA. The forward primer was 5′-CGT TTT TGG TCA AGT TGA AA-3′ and the reverse primer was 5′-CGC ATA ACG CGA AGT CAC TAC -3′. Total DNA was denatured for 5 min at 94 °C and subjected to 30 cycles of: 45 s denaturation at 94 °C, 1 min annealing at 50 °C, and 2.5 min extension at 72 °C. PCR products were cloned into the pGEM-T easy vector system (Promega) and sequenced.

C. reinhardtii cox3 genomic sequence was obtained by amplification of three overlapping PCR products, using Hot Start Taq polymerase (Qiagen). The sets of primers used were: forward 1: 5′-AGC GCG ACC GGT GAA ACC AG-3′, reverse 1: 5′-TGG AAG GGG TGG CGC TTG CCG-3′, forward 2: 5′-CCA AGG AGT TCT ACA TGG AGC AC-3′, reverse 2 :5′-CCT TGG CCA CCA TGG CCA CGT TGG-3′, forward 3: 5′-GTG GCG CTG CAG ATG CAG TGG C-3′, reverse 3: 5′-CTG CCA CAC ACA CCC GTC ATA CG-3′. Total DNA was denatured for 5 min at 94 °C, and subjected to 30 cycles of: 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 2.5 min extension at 72 °C. PCR products were cloned into the pGEM-T easy vector system from Promega.

The genomic sequences of the *cox3* genes of *Polytomella* sp. and *C. reinhardtii* are not shown, but are available in the DDBJ/EMBL/GenBank data banks under accession numbers AF286057 and AF286058, respectively.

Results and discussion

The *cox3* genomic sequences of chlamydomonad algae contain several introns

Based on the sequences of the *cox3* cDNA clones from *Polytomella* sp. and *C. reinhardtii* (Pérez-Martínez et al. 2000), deoxyoligonucleotide primers were designed and used for PCR amplification of the corresponding genomic coding regions. When total genomic DNA from *Polytomella* sp. was used as template for amplification, a 2.0-kb product was obtained. In contrast, when cDNA from the same alga was used as template, the predicted 1.2-kb product was obtained. When genomic DNA from *C. reinhardtii* was used as template to amplify the complete coding region with three different primer pairs, the genomic sequence was estimated to be 3.2 kb. The mRNA gave rise to three amplicons, reflecting a total size of 1.2 kb. The PCR fragments obtained from amplification using genomic DNA from the two algae were cloned and sequenced. A comparison of the sequences with the *cox3* cDNA sequences previously obtained (Pérez-Martínez et al. 2000) confirmed that they represented *cox3* genes and allowed identification of introns in the genomic sequence. Figure 1 shows the overall genomic organization of the two *cox3* genes.

The two chlamydomonad *cox3* genes have conserved intron positions in the regions encoding the putative MTS

The boundaries, phases and sizes for the introns of both *Polytomella* sp. and *C. reinhardtii cox3* genes are shown in Table 1. The *cox3* gene of *Polytomella* sp. has four introns (numbered 1–4) that range in size over 71–444 nucleotides. All are located in the region encoding the putative MTS that is thought to direct and insert subunit COX III into the mitochondrial inner membrane. This MTS is not present in the mature COX III protein of *Polytomella* sp. (Pérez-Martínez et al. 2000). In contrast, the genomic sequence of *cox3* of *C. reinhardtii* is interrupted by nine introns (numbered 1–9) ranging in size over 133–323 nucleotides. The first four introns, like those of *Polytomella* sp., are located in the region encoding the putative MTS of the *C. reinhardtii* COX III polypeptide. Introns 5–9 are distributed along the nucleotide sequence that encodes the mature COX III subunit.

An alignment of the MTSs encoded by both *cox3* genes indicates that the location and the phases of introns 1, 3, and 4, have been conserved between the two species (Fig. 2). Intron 2 also has a conserved phase, but its position is displaced by three nucleotides. This suggests that all four introns were acquired after the *cox3*

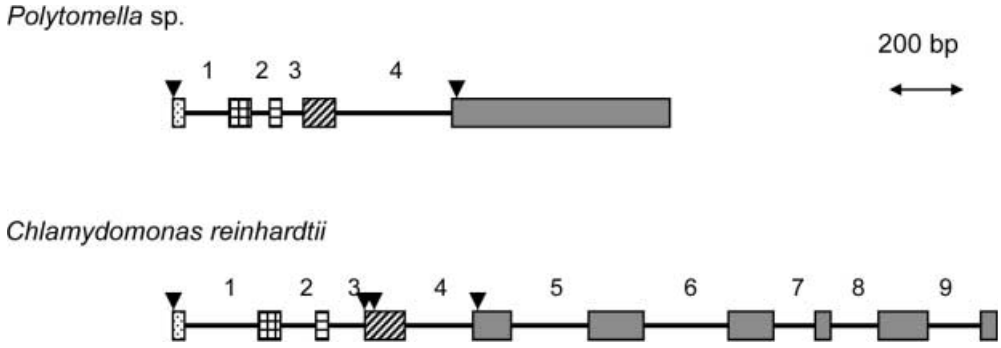

Fig. 1. Overall organization of exons and introns in *Polytomella* sp. and *Chlamydomonas reinhardtii cox3* genes. *Rectangles* show exons and *lines* represent introns. *Gray rectangles* show the region encoding the mature protein. *Rectangles* marked with *dots*, *squares*, *horizontal lines* and *dashed lines* represent homologous exons encoding the mitochondrial-targeting sequence (MTS) found in both algae. *Black triangles* indicate the relative positions of methionines in the encoded MTS and the first methionine in the encoded COX III subunit

gene was transferred to the nucleus in the common ancestor, but before *Polytomella* diverged from *C. reinhardtii*. An alternative scenario would imply intron insertion in preferential sites of *cox3* sequences. When other intron-containing mitochondrial *cox3* genes were explored, it was found they all exhibit introns that are localized in different positions. The bryophytes *Pellia epiphyla* (Malek et al. 1996) and *Marchantia polymorpha* (Oda et al. 1992) contain one and two introns, respectively, in the mitochondrial *cox3* gene. These introns are present in different positions from those found in the *Chlamydomonas* and *Polytomella* nuclear *cox3* genes. The plant *Lycopodium squarrosum* (Hiesel et al. 1994) and the yeast *Yarrowia lipolytica* (Matsuoka et al. 1994) c*ox3* genes each contain a single, non-conserved intron. The position of these introns is also different from that found in the *Chlamydomonas* and *Polytomella* nuclear *cox3* genes. These comparisons indicate the absence of a single set of preferential intron insertion sites in *cox3* sequences.

Although the regions of the *cox3* genes encoding the MTSs of both chlamydomonad algae share a conserved location of introns, the nucleotide sequences of these introns are poorly conserved (less than 38% identity). This is likely due to the rapid rate of nucleotide substitutions in introns of volvocine algae, which is ten-fold higher than in exons (Liss et al. 1997). Nevertheless, the presence of conserved intron locations in the highly conserved regions encoding the MTS in the *cox3* genes of *Polytomella* sp. and *C. reinhardtii* indicates that these algae acquired the region encoding the MTS and their respective introns before speciation. The presence of five additional introns in the *C. reinhardtii cox3* gene also suggests that these introns originated during the period that occurred after the divergence of these two species. Alternatively, but less likely, they were present in the common ancestor and then lost from the *cox3* gene of *Polytomella* sp. This last scenario would imply a selective loss of introns in regions encoding the *Polytomella* mature COX III subunit, without loss of introns in the region encoding the MTS.

Splicing junctions and internal conserved sequences in the *cox3* gene introns

All the introns in chlamydomonad *cox3* genes show orthodox splice sites, exhibiting GT at the 5′ end and AG at the 3′ end (Table 1). In *Chlamydomonas*, consensus-conserved sequences surrounding the splicing sites have been identified: (C/A)(A/C)G↓GTG(A/C)G for the 5′ splice site and (G/A)CAG↓(G/A) for the 3′ splice site (Silflow 1998). The splice junctions of the introns of the *cox3* gene of *C. reinhardtii* also conform to these consensus-conserved sequences. The sequences

Table 1. Phases, sizes, and flanking sequences of 5′ and 3′ splice sites for *Chlamydomonas reinhardtii* (*Cr*) and *Polytomella* sp. (*Ps*) *cox3* introns

	Phase		5′ splice site		Size (bp)		3′ splice site	
Intron	*Ps*	*Cr*	*Ps*	*Cr*	*Ps*	*Cr*	*Ps*	*Cr*
1	1	1	CTG/GTAAGA	CAG/GTGTGT	167	267	TGTAG/GCT	TGCAG/GCT
2	2	2	GAG/GTAAGA	CGG/GTGAGC	71	133	TTTAG/GAA	CGCAG/CCG
3	0	0	AAG/GTAATT	AAG/GTAAGG	85	139	TATAG/ACT	CGCAG/ATG
4	2	2	CAG/GTAAGA	CCG/GTGAGG	444	256	TATAG/GTC	TGCAG/CAC
5	–	1	–	TGG/GTGAGC	–	290	–	CGCAG/GCA
6	–	2	–	GCT/GTGAGT	–	323	–	CGCAG/CTG
7	–	0	–	AAG/GTGGGG	–	159	–	CGCAG/GTG
8	–	2	–	CCA/GTGAGT	–	180	–	CGCAG/GTA
9	–	2	–	CTG/GTGGGT	–	204	–	CACAG/GCA

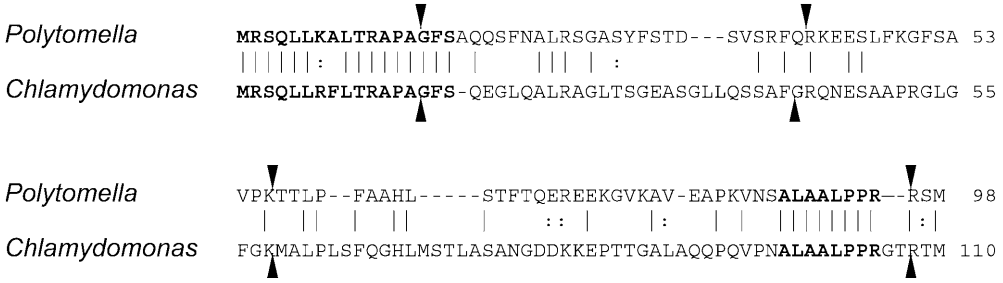

Fig. 2. Amino acid sequence alignment of MTS of *Polytomella* sp. and *C. reinhardtii cox3* and comparison of intron positions. *Arrows* indicate the positions of introns 1–4 in the corresponding gene of each alga. An *arrow* over an amino acid means that the intron is not in phase with the open reading frame (orf). Only intron 3 is in phase with the orf

surrounding splicing sites in the *cox3* gene of *Polytomella* sp. show some differences from the *C. reinhardtii* consensus splicing sites; and they conform more to the sites (A/T)G↓GTAA for the 5′ splice site and TAG↓(G/A) for the 3′ splice site. Additional genomic sequences of *Polytomella* sp. genes are needed to establish canonical splicing sites in this organism.

A general feature of *Chlamydomonas* genes is that introns are often small, ranging from 57 bp to 1,318 bp, with an average size of 219 bp (Silflow 1998). The *Chlamydomonas* introns in the *cox3* gene range from 133 bp to 323 bp. *C. reinhardtii* contains a high intron density in its nuclear genome, estimated at 3.9–4.2 introns/kb of genomic sequence (Silflow 1998; Funke et al. 1999). For the *C. reinhardtii cox3* gene, a frequency of 2.9 introns/kb is observed. In contrast, the mitochondrial genomes of chlamydomonad algae exhibit a lower density of introns, as compared with the nuclear genome. Introns are present in the *cob* gene of *C. smithii* (Colleaux et al. 1990), the *cox1*, *nad5*, and *cob* genes of *C. elongatum* (Kroymann and Zetsche 1998), and in the *cox1*, *nad1*, *nad5*, *cob*, and ribosomal RNA coding regions of *C. eugametos* (Denovan-Wright et al. 1998). A degenerate group II intron is also present in the intronless mitochondrial genome of *C. reinhardtii* (Nedelcu and Lee 1998).

Introns 5–9 of the *cox3* gene of *C. reinhardtii* are distributed along the sequence encoding the mature COX III polypeptide (Fig. 1). Intron 6, the largest intron in the *C. reinhardtii cox3* gene, shows two internal conserved sequences (ICS) found in several introns of *C. reinhardtii* and in members of the genus *Volvox* (Table 2). In the majority of these *Chlamydomonas* and *Volvox* genes, these ICS are located near the 5′ splicing junctions (ICS 1), or near the 3′ splicing junctions (ICS 2). The ICSs reported in this work differ from most other *Chlamydomonas* ICS in that they lack the consensus sequence NCTAG located 15–51 bp upstream of the 3′ splice site (Lee et al. 1997). The ICS described here also differ from ICS found in yeast, where the great majority of the introns found in its entire genome (214 out of 231) contain a 7-nucleotide ICS, with the consensus sequence TACTAAC (Spingola et al. 1999).

The chlamydomonad ICS seem to be present in the larger introns of a gene or in introns not smaller that 212 bp. This observation is consistent with the fact that two ICS are present only in intron 6 of the *Chlamydomonas cox3* gene, which is the largest intron (323 bp). It is important to note that the relative position of ICS 1 within the intron is usually conserved, being near the 5′ end. In some examples in Table 2, the sequence of ICS 1 is inverted with respect to the *cox3* intron 6 sequence. In these cases, the relative position of ICS 1 within the intron is also inverted and is found near the 3′ end of the intron. The relative position of ICS 2 within the intron is also conserved and is usually found near the 3′ end of the intron. When the sequence of ICS 2 is inverted, the position within the intron is also inverted and it is present near the 5′ end of the intron. These ICS are not present in all *Chlamydomonas* introns, but they may play an important role in the processing of particular introns in this alga. ICS 2 was previously described by Funke et al. (1999), when comparing an intron from the gene encoding acetolactate synthase (*ALS*) with an intron of the gene encoding carbonic anhydrase (*CAH3*). Those authors proposed that this nucleotide stretch might represent a sequence encompassing a branch site of these introns. Or, it might represent a signal of yet unknown importance in the RNA-splicing process or in the expression of genes in *C. reinhardtii* (Lumbreras et al. 1998).

This is the first description of the sequences of *cox3* genes localized in the nucleus. Based on these sequences, it is proposed that *cox3* genes were transferred to the nucleus in a common ancestor of *Polytomella* sp. and *C. reinhardtii*. This process was accompanied by the loss of the mitochondrial *cox3* gene copy. The inactivation of the original mitochondrial gene and its subsequent disappearance likely occurred once the nuclear *cox3* gene copy became active. It is also proposed that the acquisition of an additional nucleotide sequence encoding a MTS and its corresponding introns occurred before the divergence of these two species. Nucleotide insertions, deletions and substitutions have occurred in introns 1–4, which changed their sequence and size, while retaining their original positions. Although it is difficult to distinguish between evolutionary scenarios, intron-loss against intron-gain for chlamydomonad *cox3* genes, the simplest interpretation is that intron acquisition in *C. reinhardtii* occurred after its separation from the

Table 2. Internal conserved sequences (ICS 1, ICS 2) of *C. reinhardtii* introns. *C. reinhardtii cox3* intron 6 contains an ICS 1 and an ICS 2 that are present in other *Chlamydomonas* and *Volvox* introns. *Superscript a* denotes ICS 1 or ICS 2 sequence that is inverted with respect to *cox3* intron 6. GenBank accession numbers are given *in parentheses*

Intron	Size (bp)	Gene name	Location in the intron
ICS 1: GGGAGGGGGGAGAGG			
6	323	*C. reinhardtii cox3*	31 bp from 5′
8	443	*C. reinhardtii* acetolactate synthase gene, *ALS* (AF047459)	6 bp from 5′
14	288	*C. reinhardtii* variable flagellar number protein gene, *VFL1* (AF154916)	16 bp from 5′
2	253	*C. reinhardtii EYE2* gene (AF233430)	22 bp from 5′
2[a]	228	*C. reinhardtii* actin-related protein gene, *ACT2* (U68060)	21 bp from 3′
5[a]	454	*Volvox carteri* argininosuccinate lyase gene, *VASL* (AF233374)	5 bp from 3′
1	748	*V. spermatosphera* UTEX 2273 GTPase Ypt4p gene (U55929)	2 bp from 5′
ICS 2: ATCATGAATGTAACCCC			
6	323	*C. reinhardtii cox3*	25 bp from 3′
3	493	*C. reinhardtii* class II DNA photolyase gene, *PHR2* (AF129458)	177 bp from 3′
8	694	*C. reinhardtii* class II DNA photolyase gene, *PHR2*	17 bp from 3′
2	480	*C. reinhardtii* phosphatidylinositol 3-kinase gene (U97663)	21 bp from 3′
5[a]	500	*C. reinhardtii* acetolactate synthase gene, *ALS*	2 bp from 5′
9	582	*C. reinhardtii* acetolactate synthase gene, *ALS*	34 bp from 3′
4	287	*C. reinhardtii* carbonic anhydrase α-type gene, *CAH3* (U73856)	26 bp from 3′
8[a]	490	*C. reinhardtii* argininosuccinate lyase gene, *ARG7* (X16619)	27 bp from 5′
8	212	*C. reinhardtii* nitrate reductase gene, *NIT1* (AF203033)	20 bp from 3′
2	281	*C. reinhardtii zys1B* gene (AB001486)	32 bp from 3′

colorless genus *Polytomella*. This proposal is in agreement with the suggestion of a late occurrence of multiple intron-insertion events in the evolution of eukaryotic genes (Palmer and Logsdon 1991) and also with the proposal of a late acquisition of introns in the genes of *C. reinhardtii* and its multicellular relative, *V. carteri* (Funke et al. 1999). A test of the late-acquisition hypothesis could be accomplished through a comparative study of intron distribution among a sampling of chlamydomonad taxa that includes exemplars from all of the known lineages (Buchheim et al. 1996; Friedl 1997). In particular, it will be important that future investigations include those taxa currently regarded as sister to *C. reinhardtii* (e.g., *C. globosa*, *C. incerta*, *C. zebra*, *V. carteri*).

Acknowledgements The authors are indebted to Dr. E. Harris (Duke University) for providing the *C. reinhardtii* wild-type strain, to M. Vázquez-Acevedo for technical assistance, and to Drs. A. Atteia (IFC, UNAM), D.W. Krogmann (Purdue University), A. Lazcano-Araujo (Facultad de Ciencias, UNAM), and F. Recillas-Targa (IFC, UNAM) for reviewing the manuscript. We are indebted to M.A. Buchheim (University of Tulsa) for helpful suggestions and critical review of the manuscript. This work was supported by grants TW01176 from Fogarty International Center at NIH and HL59646 from NHLBI, NIH, USA, grant 27754 N from the Consejo Nacional de Ciencia y Tecnología, Mexico, and grant IN202598 from the Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México, Mexico.

References

Antaramian A, Coria R, Ramírez J, González-Halphen D (1996) The deduced primary structure of subunit I from the cytochrome *c* oxidase suggests that the genus *Polytomella* shares a common mitochondrial origin with *Chlamydomonas*. Biochim Biophys Acta 1273:198–202

Atteia A, Dreyfus G, González-Halphen D (1997) Characterization of the α and β subunits of the F_0F_1-ATPase from the alga *Polytomella* sp., a close relative of *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1320:275–284

Brennicke A, Grohmann L, Hiesel R, Knoop V, Schuster W (1993) The mitochondrial genome on its way to the nucleus: different stages of gene transfer in higher plants. FEBS Lett 325:140–145

Buchheim MA, Lemieux C, Otis C, Gutell RR, Chapman RL, Turmel M (1996) Phylogeny of the Chlamydomonadales (Chlorophyceae): a comparison of ribosomal RNA gene sequences from the nucleus and the chloroplast. Mol Phylogenet Evol 5:391–402

Claros MG, Perea J, Shu Y, Samatey FA, Popot JL, Jacq C (1995) Limitations to in vivo import of hydrophobic proteins into yeast mitochondria. The case of a cytoplasmically synthesized apocytochrome *b*. Eur J Biochem 228:762–771

Colleaux L, Michel-Wolwertz M-R, Matagne RF, Dujon B (1990) The apocytochrome *b* gene of *Chlamydomonas smithii* contains a mobile intron related to both *Saccharomyces* and *Neurospora* introns. Mol Gen Genet 223:288–296

Conner TW, Thompson MD, Silflow CD (1989) Structure of the three β-tubulin-encoding genes of the unicellular alga, *Polytomella agilis*. Gene 84:345–358

Denovan-Wright EM, Nedelcu AM, Lee RW (1998) Complete sequence of the mitochondrial DNA of *Chlamydomonas eugametos*. Plant Mol Biol 36:285–295

Friedl T (1997) The evolution of green algae. Plant Syst Evol [Suppl] 11:87–101

Funke RP, Kovar JL, Logsdon JM Jr, Corrette-Bennet JC, Straus DR, Weeks DP (1999) Nucleus-encoded, plastid-targeted acetolactate synthase genes in two closely related chlorophytes, *Chlamydomonas reinhardtii* and *Volvox carteri*: phylogenetic origins and recent insertion of introns. Mol Gen Genet 262:12–21

Gutiérrez-Cirlos EB, Antaramian A, Vázquez-Acevedo M, Coria R, González-Halphen D (1994) A highly active ubiquinol-cytochrome *c* reductase (bc_1 complex) from the colorless alga *Polytomella* sp., a close relative of *Chlamydomonas*. J Biol Chem 269:9147–9154

Hiesel R, Haeseler A von, Brennicke A (1994) Plant mitochondrial nucleic acid sequences as a tool for phylogenetic analysis. Proc Natl Acad Sci USA 91:634–638

Kroymann J, Zetsche K (1998) The mitochondrial genome of *Chlorogonium elongatum* inferred from the complete sequence. J Mol Biol 47:431–440

Lee VD, Stapleton M, Huang B (1997) Genomic structure of *Chlamydomonas* caltractin. Evidence for intron insertion suggests a probable genealogy for the EF-hand superfamily of proteins. J Mol Biol 22:175–191

Liss M, Kirk DL, Beyser K, Fabry S (1997) Intron sequences provide a tool for high-resolution phylogenetic analysis of volvocine algae. Curr Genet 31:214–227

Lumbreras V, Stevens D, Purton S (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. Plant J 14:441–447

Malek O, Lattig K, Hiesel R, Brennicke A, Knoop V (1996) RNA editing in bryophytes and a molecular phylogeny of land plants. EMBO J 15:1403–1411

Matsuoka M, Matsubara M, Kakehi M, Imanaka T (1994) Homologous maturase-like proteins are encoded within the group I introns in different mitochondrial genes specifying *Yarrowia lipolytica* cytochrome *c* oxidase subunit 3 and *Saccharomyces cerevisiae* apocytochrome *b*. Curr Genet 26:377–381

Mattox KR, Stewart KD (1984) Classification of the green algae. In: Irvine DEG, John DM (eds) Systematics of the green algae. Academic Press, London, pp 29–72

Melkonian M, Surek B (1995) Phylogeny of the Chlorophyta: congruence between ultrastructural and molecular evidence. Bull Soc Zool Fr 120:191–208

Michaelis G, Vahrenholz C, Pratje E (1990) Mitochondrial DNA of *Chlamydomonas reinhardtii*: the gene for apocytochrome *b* and the complete functional map of the 15.8 kb DNA. Mol Gen Genet 223:211–216

Nakayama T, Watanabe S, Mitsui K, Uchida H, Inouye I (1996) The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rRNA sequence data. Phycol Res 44:47–55

Nedelcu A, Lee RW (1998) A degenerate group II intron in the intronless mitochondrial genome of *Chlamydomonas reinhardtii*: evolutionary implications. Mol Biol Evol 15:918–922

Oda K, Yamato K, Ohta E, Nakamura Y, Takemura M, Nozato N, Kohchi T, Ogura Y, Kanegae T, Akashi K, Ohyama K (1992) Gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* mitochondrial DNA. A primitive form of plant mitochondrial genome. J Mol Biol 223:1–7

Palmer JD, Logsdon JM (1991) The recent origins of introns. Curr Opin Genet Dev 1: 470–477

Pérez-Martínez X, Vázquez-Acevedo M, Tolkunova E, Funes S, Claros MG, Davidson E, King MP, González-Halphen D (2000) Unusual location of a mitochondrial gene: subunit III of cytochrome *c* oxidase is encoded in the nucleus of chlamydomonad algae. J Biol Chem 275:30144–30152

Pérez-Martínez X, Antaramian A, Vázquez-Acevedo M, Funes S, Tolkunova E, d'Alayer J, Claros MG, Davidson E, King MP, González-Halphen D (2001) Subunit II of cytochrome c oxidase in chlamydomonad algae is a heterodimer encoded by two independent nuclear genes. J Biol Chem 276:11302–11309

Rochaix JD, Mayfield S, Goldschmidt-Clermont M, Erickson J (1988) Plant molecular biology. In: Shaw CH (ed) Molecular biology of *Chlamydomonas*. IRL Press, Oxford, pp 253–275

Round FE (1980) The evolution of pigmented and unpigmented unicells – a reconsideration of the Protista. Biosystems 12:61–69

Rumpf R, Vernon D, Schreiber D, Birky CW Jr (1996) Evolutionary consequences of the loss of photosynthesis in Chlamydomonadaceae: phylogenetic analysis of *Rrn18* (18S rRNA) in 13*Polytoma* strains (Chlorophyta). J Phycol 32:119–126

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Silflow C (1998) Organization of the nuclear genome. In: Rochaix JD, Goldschmidt-Clermont M, Merchant S (eds) The molecular biology of chloroplasts and mitochondria in *Chlamydomonas*. Kluwer, Rotterdam, pp25–40

Spingola M, Grate L, Haussler D, Ares M Jr (1999) Genome-wide bioinformatic and molecular analysis of introns in *Saccharomyces cerevisiae*. RNA 5:221–234