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FACULTAD DE QUIMICA

LOCALIZACIÓN ATÍPICA DE DOS GENES MITOCONDRIALES.

LOS GENES QUE CODIFICAN PARA LAS SUBUNIDADES II Y III DE LA CITOCROMO *c* OXIDASA SE ENCUENTRAN EN EL NÚCLEO DE LAS ALGAS CHLAMYDOMONADACEAS.

T E S I S

QUE PARA OBTENER EL GRADO DE

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P R E S E N T A:

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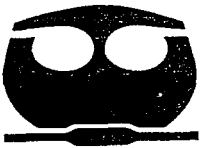

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RESUMEN

Los genes que codifican para las subunidades II (*cox2*) y III (*cox3*) de la citocromo *c* oxidasa están presentes en el DNA mitocondrial de la gran mayoría de los organismos eucariotes descritos a la fecha. Estas proteínas hidrofóbicas se sintetizan en la matriz y se integran en la membrana interna mitocondrial. Un caso de interés son las algas de la familia de las Chlamydomonadaceas, en que el genoma mitocondrial carece de los genes *cox2* y *cox3*. Esto puede sugerir que las proteínas no forman parte de la citocromo *c* oxidasa y que los genes no existen, ó alternativamente, que las proteínas si están presentes en el complejo, pero los genes que las codifcan han sido transferidos al núcleo. En este trabajo se purificó a la citocromo *c* oxidasa del alga incolora *Polytomella* spp., y se demostró que ambas subunidades están presentes en el complejo enzimático.

La subunidad III se identificó mediante el uso de un anticuerpo monoclonal contra la subunidad III de *Saccharomyces cerevisiae*. Se estimó una masa molecular de 29.6 KDa para esta proteína y se obtuvo la secuencia de su extremo amino terminal. A continuación, se obtuvo la secuencia genómica y de cDNA del gen *cox3* de *Polytomella* spp. De igual manera, se clonó el gen *cox3* de *C. reinhardtii*, otro miembro de la familia de las Chlamydomonadaceas. Se demostró que el gen *cox3* de ambas algas es nuclear. Este gen predice la existencia de una presecuencia que podría dirigir a la proteína desde el citosol a la mitocondria. De igual manera, el gen predice una proteína con 7 cruces transmembranales, con una estructura muy similar a la encontrada para la subunidad III en la estructura cristalina de la citocromo *c* oxidasa de bovino. Se predice que la proteína posee una hidrofobicidad general disminuida que, junto con una posible presecuencia mitocondrial, podrían ser factores importantes para la integración exitosa de la subunidad III en la citocromo *c* oxidasa de estas algas.

Por otro lado, la subunidad II de la citocromo *c* oxidasa de *Polytomella* spp. se identificó mediante la secuencia del extremo amino terminal y mediante una secuencia interna de la proteína. La banda identificada como la subunidad II migra en geles de poliacrilamida con una masa molecular de 18.6 KDa, la cual es menor a la esperada para la mayoría de las subunidades II (29- 30 KDa). Se demostró que tanto en *Polytomella* spp. como en *C. reinhardtii* esta subunidad está codificada por dos genes en vez de uno, y que ambos genes tienen una localización nuclear. El gen *cox2a* codifica para la región amino terminal y los dos cruces transmembranales de la proteína, y predice la existencia de una presecuencia mitocondrial. Adicionalmente, el gen predice una extensión de 20 aminoácidos en el extremo carboxilo terminal de la proteína que posee una gran cantidad de residuos cargados y que no está presente en ninguna otra subunidad II. El gen *cox2b* codifica para la región soluble del extremo carboxilo terminal y para el sitio de unión a cobre. Este gen predice una extensión de 44 aminoácidos no conservados, en el extremo amino terminal de la proteína. Esta extensión no tiene características de presecuencia mitocondrial que se procesa por peptidasas de matriz mitocondrial. Es una región con muchos aminoácidos cargados que permanece en la proteína madura. No fue posible detectar un transcrito maduro, producto de un procesamiento alternativo, que contenga al gen *cox2a* y al gen *cox2b*. Nuestros resultados sugieren que ambos genes se transcriben por separado y se traducen en dos proteínas diferentes, una codificada por el gen *cox2a* y la otra por el gen *cox2b*. Las extensiones predichas por cada gen, podrían mediar una interacción electrostática para conformar a la subunidad II activa en el complejo maduro de la citocromo *c* oxidasa mitocondrial.

ABSTRACT

The genes encoding subunits II (*cox2*) and III (*cox3*) of the cytochrome *c* oxidase are present in the mitochondrial DNA of the vast majority of the eukariotic organisms described to date. This hydrophobic proteins are synthesized in the matrix and assembled in the mitochondrial inner membrane. An interesting example are the algae of the Chlamydomonad family, in which the mitochondrial genome lack the genes *cox2* and *cox3*. This can suggest that the proteins are not present at all in the cytochrome *c* oxidase complex and that the corresponding genes don't exist, or alternatively, that this proteins are present in the complex, but the genes encoding them have been transferred to the nucleus. In this work, the cytochrome *c* oxidase from the colorless algae *Polytomella* spp. was purified. It was shown that both subunits COXII and COXIII are present in the mature enzymatic complex.

Subunit III was identified with monoclonal antibodies directed against *Saccharomyces cerevisiae* subunit III. A molecular mass of 29.6 KDa was calculated for this protein, an its amino terminal sequence was obtained. The genomic and the cDNA sequences of the *Polytomella* spp. *cox3* gene were obtained. The complete *cox3* gene from *C. reinhardtii*, another member of the Chlamydomonad algae, was also cloned. It was shown that the *cox3* gene in both algae is nuclear. This gene predicts a presequence that could target the protein to the mitochondria. It predicts a protein with seven transmembrane segments, similar to the three-dimensional crystal structure of the subunit III from the bovine cytochrome *c* oxidase obtained by X-ray crystalography. The gene predicts a protein with diminished hydrophobicity that, along with a putative mitochondrial targeting sequence, could be essential for the successful assembly of the subunit III in the mature cytochrome *c* oxidase from these algae.

The cytochrome *c* oxidase subunit II from *Polytomella* spp. was identified by the amino terminal and internal sequence of the protein. The band identified as subunit II migrates as a 18.6 KDa protein in SDS-PAGE. This size is smaller than the observed one for all the subunits II characterized to date (29- 30KDa). It was shown that in *Polytomella* spp. and in *C. reinhardtii*, this subunit is encoded by two different genes, and that both genes are nuclear-localized. The *cox2a* gene encodes for the amino terminal region and the two transmembrane stretches of the protein, and predicts a mitochondrial targeting sequence with high similarity to thylacoid transit peptides. Additionally, the gene predicts a 20 residues extension at the carboxy end of the protein. This extension is highly charged and is not found in any other subunit II. The *cox2b* gene encodes for the carboxy soluble end of the protein and for the copper binding site. The gene predicts a non conserved 44 residues extension in the amino terminal end of the protein. This extension has no processed mitochondrial targeting sequence characteristics. It is a highly charged region that is present in the mature protein. No trans-spliced product, which could include *cox2a* and *cox2b* messengers, was detected. Our data suggest that these genes are transcribed independently and translated into two different proteins: one encoded by the *cox2a* gene, and the other by the *cox2b* gene. The predicted extensions from both genes could have a central role in a electrostatic interaction between the two proteins to assemble the mature subunit II in the cytochrome *c* oxidase.

ABREVIATURAS

cox3	gen que codifica para la subunidad III de la citocromo *c* oxidasa.
cox2a	gen que codifica para la región transmembranal de la subunidad II de la citocromo *c* oxidasa
cox2b	gen que codifca para la región carboxilo terminal de la subunidad II de la citocromo *c* oxidasa
COXIIa	subunidad IIa de la citocromo *c* oxidasa
COXIIb	subunidad IIb de la citocromo *c* oxidasa.
Cr-COX III	subunidad III de la citocromo *c* oxidasa de *Chlamydomonas reinhardtii.*
DDBJ/EMBL	DNA DataBank of Japan / European Molecular Biology Laboratory , bases de datos internacionales para secuencias de nucleótidos.
DEAE	dietil-aminoetil-
DEPC	dietil-pirocarbonato
DNA	ácido desoxirribonucléico.
dNTP	desoxinucleotidos de trifosfato
Hmax	hidrofobicidad máxima.
<H>	hidrofobicidad promedio de un segmento en una secuencia.
IPTG	isopropil tio-β-D-galactósido, siglas en inglés.
LB	medio de cultivo Luria-Bertani
MALDI-TOF	Espectrometria de masas (matrix-assisted laser desorption ionization–time of flight)
*meso*H	mesohidrofobicidad
μH	momento hidrofóbico
mtDNA	DNA mitocondrial.
MTS	mitochondrial targeting sequence, siglas en inglés de presecuencia mitocondrial
PCR	polymerase chain reaction, siglas en inglés de reacción en cadena de la polimerasa.
p(DFM)	discriminant function for mitochondrial proteins, siglas en inglés de función discriminante para proteínas mitocondriales.
PMSF	fluoruro de fenilmetilsulfonilo
Ps-COX III	subunidad III de la citocromo *c* oxidasa de *Polytomella* spp.
RACE	rapid amplification of cDNA ends, siglas en inglés de amplificación rápida de extremos de cDNA
RP-HPLC	reverse phase-high precision liquid chromatography, siglas en inglés de cromatografia liquida de alta precisión en fase reversa.

RNA	ácido ribonucléico.
RT	reverse transcriptase, siglas en inglés de transcriptasa reversa.
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis, siglas en inglés de electroforesis en geles de poliacrilamida-dodecil sulfato de sodio.
TLCK	Nα-*p*-tosil-L-lisin-cloro-metil cetona, siglas en inglés.
TMPD	N,N,N',N'-tetrametil-*p*-fenilén-diamina, siglas en inglés
x-Gal	5-Bromo-4-cloro-3-indolil-β-$_D$-galactósido, siglas en inglés.

INTRODUCCIÓN

1. La mitocondria.

En este organelo se genera la mayor parte de la energía de la célula eucariote. Esto ocurre mediante el proceso conocido como fosforilación oxidativa, en el cual los electrones son transferidos a través de una serie de complejos enzimáticos que conforman a la cadena respiratoria. Estos electrones son donados por el NADH o succinato, que provienen de la oxidación de nutrimentos como la glucosa, y son transferidos a través de los complejos respiratorios hasta el oxigeno molecular, su aceptor final. La cadena respiratoria convierte la energía libre de las reacciones redox a un gradiente electroquímico de protones que se establece a través de la membrana interna mitocondrial. Este gradiente contiene la energía necesaria para procesos como la síntesis de ATP, el transporte de substratos o la movilidad de la célula

La cadena respiratoria está formada por cuatro complejos enzimáticos presentes en la membrana interna mitocondrial con una orientación especifica. Los complejos de la cadena respiratoria incluyen a la NADH deshidrogenasa o complejo I y a la succinato deshidrogenasa o complejo II, ambos reciben electrones de substratos reducidos y los transfieren a una poza de quinonas que se encuentra en la membrana. De esta poza recibe electrones el complejo bc_1 o complejo III el cual los transfiere vía citocromo *c* soluble a la citocromo *c* oxidasa o complejo IV. Este último complejo contiene al sitio que cataliza la reducción de O_2 a H_2O (Capaldi, 1991, Trumpower y Gennis, 1994; Saraste, 1999). De especial interés para este trabajo es la citocromo *c* oxidasa o complejo IV

2. La citocromo *c* oxidasa

Esta enzima constituye la parte final de la cadena respiratoria de mitocondrias y de muchas bacterias. Se encarga de reducir al oxigeno molecular y transformarlo en agua a partir de los electrones que recibe del citocromo *c* soluble presente en el espacio intermembranal. Acoplada a esta reacción hay una translocación de protones desde la matriz mitocondrial hasta el espacio intermembranal (Calboun y col., 1994). La reacción puede expresarse como:

$$4\ \text{cit. } c_{(red)} + O_2 + 8H^+_{(matriz)} \rightarrow 4\ \text{cit. } c_{(ox)} + 2H_2O + 4H^+_{(espacio\ intermembranal)}$$

La composición de subunidades de la oxidasa es variable, aunque en todos los organismos aerobios se ha visto que la composición mínima es de 3 subunidades: subunidad I (COX I), subunidad II (COX II) y subunidad III (COX III). Se ha reportado que en mamíferos hay 13 subunidades (Kadenbach y col., 1983), en levadura hay 9 (Poyton y col., 1995) y en *Paracoccus denitrificans* hay 4 (Iwata y col., 1995).

En la mayoria de los organismos, COX I, COX II y COX III están codificadas en el DNA mitocondrial. Las subunidades supernumerarias, que no son muy conservadas y tienen un cruce transmembranal o ninguno, están codificadas en el DNA nuclear, se sintetizan en el citosol y se importan a la mitocondria (Capaldi, 1990; Grossman, y Lomaz, 1997). En la actualidad se conocen las estructuras cristalinas a 2.8 Å de resolución de la citocromo c oxidasa de *Paracoccus denitrificans* (Iwata y col., 1995) y de bovino (Tsukihara y col., 1996). En estas estructuras se observa que las 3 subunidades básicas de la enzima tienen un arreglo topológico muy similar entre ambos organismos. Podemos decir que tanto las secuencias como las estructuras de las subunidades COXI, COXII y COXIII están conservadas a lo largo de la escala filogenética. En la **figura 1** se muestran las estructuras cristalinas de estas subunidades en la citocromo c oxidasa de *P. denitrificans* (Iwata y col., 1995).

COX I: Es una subunidad altamente hidrofóbica, con 12 cruces transmembranales, y se encuentra muy conservada entre las especies. Aquí se encuentran los centros metálicos que se encargan de reducir al oxígeno molecular. Tiene un hemo *a* que no interactúa directamente con el oxígeno, pero que transfiere los electrones provenientes de la subunidad II a un centro binuclear con hemo a_3 y cobre (Cu_B). Esta subunidad está también involucrada en el bombeo de protones a través de la membrana interna mitocondrial que contribuye a la formación del gradiente electroquímico de protones (Michel, 1998; Yoshikawa, 1999; Verkhovsky y col., 1999).

COX II: Es una proteína compuesta por tres segmentos: una región pequeña amino terminal que se localiza en el espacio intermembranal, dos hélices transmembranales y una región en el extremo C-terminal que es hidrofílica y que se localiza en el espacio intermembranal. Esta última sección contiene a los residuos que unen al centro bimetálico de cobre conocido como Cu_A, el cual recibe a los electrones provenientes del citocromo c reducido. Se ha reportado que existe una región aromática altamente conservada después del segundo cruce transmembranal, cuya función es importante para la actividad de la enzima (Overholtzer y col., 1996). Esta región participa en la transferencia de electrones desde el citocromo c hasta los centros metálicos de la subunidad I. Witt y col. (1998) demostraron que uno de los triptófanos presentes en esta región es el aceptor inicial de los electrones provenientes del citocromo c reducido. Tanto la hélice II como ciertas regiones del extremo C-terminal tienen un alto grado de conservación (Holm y col., 1987).

COX III: Es una proteína altamente hidrofóbica, con 7 cruces transmembranales. De las 3 subunidades, ésta es la menos conservada, siendo la hélice III y parte de la VII las que presentan mayor conservación. No se conoce con certeza la función de esta subunidad, ya que no posee grupos metálicos que pudieran participar en las reacciones redox.

Hay evidencias que sugieren que no es importante en el bombeo de protones, pero que si participa en la biogénesis del complejo (Hallia y col., 1991) y en el transporte del oxigeno molecular hasta el sitio catalitico en la subunidad I (Riistama y col., 1996).

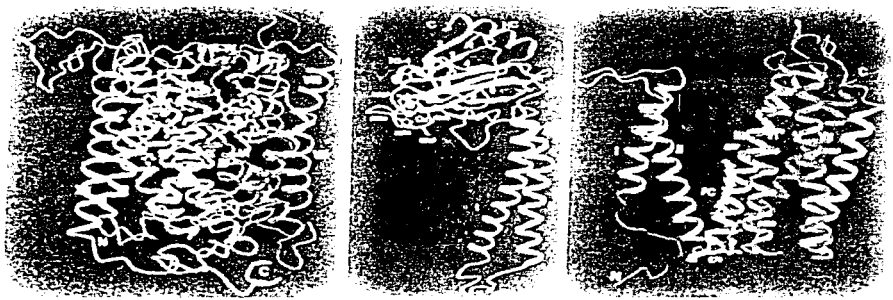

Figura 1. Estructura cristalina de las subunidades I, II y III de la citocromo *c* oxidasa de *P. denitrificans* (Iwata y col., 1995).

3. El genoma mitocondrial.

De acuerdo a la teoria del endosimbionte, la mitocondria se originó de ancestros bacterianos cercanos a la familia de las Rickettsias (Andersson y col., 1998), que se incorporaron a una célula huésped. En la actualidad la mitocondria es un organelo semiautónomo, porque tiene su propio genoma que se replica y expresa, pero es incapaz de tener existencia independiente ya que a lo largo de la evolución parte de su genoma ha sido exportado e integrado al DNA nuclear.

Los genes que aún están en la mitocondria representan información genética retenida desde el endosimbionte original (Gray, 1989a; Gray, 1989b; Leblanc y col., 1997). A lo largo de la evolución, casi toda la información genética del ancestro mitocondrial se ha transferido al genoma nuclear y en la actualidad hay evidencia de que este proceso de transferencia aún continúa, ya que en algunas plantas y hongos existen ejemplos de intermediarios de este evento (Brennicke y col., 1993; Adams y col., 1999; Figueroa y col., 1999; Kubo y col., 1999)

El DNA mitocondrial tiene una gran diversidad en cuanto a estructura, contenido y organización de genes así como diferentes modos de expresión y replicación en diferentes organismos. En mamíferos es una genoma compacto, entre 16 y 20 Kb, y sin intrones, mientras que en plantas puede ser de 10 a 100 veces más grande con una mayor cantidad de genes retenidos y con muchas regiones intergenicas e intrones (Attardi, 1988; Schuster y Brennicke, 1994)

Es importante hacer notar que los genes que codifican para proteínas muy hidrofóbicas con varios cruces transmembranales como el citocromo *b* del complejo bc; o COX I, COX II y COX III de la citocromo *c* oxidasa han permanecido en el genoma mitocondrial de la gran mayoría de los organismos eucariotes descritos a la fecha (Unseld y col., 1997; Gray y col., 1998; Gray y col., 1999).

4. Importación de proteínas a la mitocondria.

Como se mencionó con anterioridad, la mayoría de las proteínas de la mitocondria se codifican en el genoma nuclear, se sintetizan en el citosol y se importan a la mitocondria. Estas proteínas contienen señales que las dirigen a la mitocondria. Estas señales pueden ser extensiones en el extremo amino terminal de la proteína precursora o pueden ser secuencias de aminoácidos presentes en el interior de la proteína madura. Las extensiones amino terminal, conocidas como presecuencias, son regularmente eliminadas mediante proteasas mitocondriales. De esta manera se produce una proteína madura. El proceso de importación de proteínas a la mitocondria requiere una maquinaria proteica compuesta de por lo menos tres complejos. Uno se localiza en la membrana externa y otros dos en la membrana interna mitocondrial. Estos complejos de translocación actúan en conjunto con proteínas solubles del citosol, espacio intermembranal y matriz mitocondrial. La translocación de precursores que contienen presecuencia se lleva a cabo mediante interacciones electrostáticas de la presecuencia con los componentes del aparato de importacion. La translocación de estas proteínas a través de la membrana interna utiliza el gradiente electroquímico de protones y ATP. En la matriz mitocondrial se localizan chaperonas y proteasas que permiten que la proteína se dirija a su localización final en la mitocondria (Voos y col., 1999).

Por otro lado, las proteínas de membrana interna que forman parte de la familia de acarreadores de metabolitos (como la translocasa de adenín nucleótidos), interactúan con otro complejo de espacio intermembranal y se insertan directamente en la membrana interna con la ayuda de un segundo complejo de translocación de proteínas (Endres y col., 1999). Una característica importante de las presecuencias del primer grupo de proteínas que se importan a la mitocondria es que, a pesar de que éstas no contienen secuencias de aminoácidos conservados, la estructura secundaria es muy importante para su función. Estas suelen tener α-hélices anfifílicas con varios aminoácidos cargados positivamente en el extremo amino terminal (von Heijne y col., 1989).

5. El alga verde *Chlamydomonas reinhardtii*.

Chlamydomonas reinhardtii es un alga verde unicelular biflagelada que pertenece a la familia de las *Chlamydomonadaceae*. Se conoce la secuencia completa del genoma mitocondrial de este organismo (Michaelis y col., 1990). A diferencia del genoma mitocondrial de plantas superiores, que es circular y grande (200-2400 Kb), el DNA mitocondrial de *C. reinhardtii* es lineal y muy compacto, de solamente 15.8 Kb. Tiene regiones no codificantes muy pequeñas y no contiene intrones. Codifica para ocho proteínas que incluyen al citocromo *b* del complejo bc$_1$, a COX I de la citocromo *c* oxidasa, cinco subunidades de la NADH deshidrogenasa y una transcriptasa reversa que no presenta homología con ningún gen mitocondrial de hongos, mamíferos ni plantas superiores. No se conoce la función de esta posible transcriptasa reversa. En este genoma se codifican únicamente tres RNAs de transferencia, mientras que en la mayoría de las especies descritas están presentes alrededor de 20 tRNAs (Unseld y col., 1997). Codifica también para dos RNA ribosomales que se encuentran fragmentados y dispersos en una región del genoma mitocondrial (Nedelcu y col., 1996).

La cantidad de genes que codifican para proteínas de la cadena respiratoria en este genoma es muy reducida. Están ausentes todos los genes que codifican para la ATPasa, incluyendo a aquellos que normalmente se encuentran presentes en el genoma mitocondrial de la mayoría de las especies, como es el caso de los genes que codifican para ATP6 y ATP8. De igual manera, están ausentes varios genes que codifican para subunidades hidrofóbicas del complejo I como NAD3 y NAD4L.

De particular interés para el presente trabajo es el hecho de que los genes que codifican para las subunidades COXII (*cox2*) y COXIII (*cox3*) de la citocromo *c* oxidasa no están presentes en el DNA mitocondrial de *C. reinhardtii*.

6. El alga incolora *Polytomella* spp.

El segundo organismo que nos interesa es *Polytomella* spp. Es un alga unicelular, incolora y sin pared celular que también pertenece a la familia de las Chlamydomonadaceas (Melkonian, 1990) . A pesar de que carece de aparato fotosintético y pared celular, *Polytomella* comparte una gran cantidad de caracteristicas a nivel morfológico, bioquímico y molecular con *C. reinhardtii* (Brown y col., 1976; Mattox y Stewart, 1984; Conner y col., 1989; Melkonian y Surek, 1995; Antaramian y col., 1996; Nedelcu y col., 1996, Atteia y col . 1997; Antaramián y col . 1998; el presente trabajo). De acuerdo a la hipótesis de Round, (1980), *Polytomella* y *Chlamydomonas* compartieron el mismo evento endosimbiótico que dió origen a la mitocondria. La pérdida de pared celular y cloroplastos en *Polytomella* fue un evento secundario más reciente en la evolución.

PLANTEAMIENTO DEL PROBLEMA

Como se dijo con anterioridad, del conocimiento de la secuencia completa del DNA mitocondrial de *C. reinhardtii* es claro que los genes que codifican para COX II y COX III de la citocromo *c* oxidasa están ausentes. Debido a que ambas son proteínas muy hidrofóbicas, de acuerdo a von Heijne (1986) sería de esperarse que, al igual que en la gran mayoría de los organismos descritos actualmente, estos genes se hubieran quedado retenidos en el genoma mitocondrial. Más aún, se sabe que de igual manera, estos genes se encuentran ausentes de los genomas mitocondriales de otros miembros de la familia de las Chlamydomonadaceas, como es el caso de *Chlamydomonas moewusii* (Lee, 1991), *Chlorogonium elongatum* (Kroymann y Zetsche, 1998) y *Chlamydomonas eugametos* (Denovan-Wright y col. 1998). Estos resultados sugieren que la ausencia de los genes *cox2* y *cox3* del genoma mitocondrial es una característica que comparten todos los miembros de la familia de las Chlamydomonadaceas, incluyendo a aquellos miembros incoloros como *Polytomella* spp.

En este trabajo se pretende demostrar que las proteínas COXII y COXIII de la citocromo *c* oxidasa de *C. reinhardtii* y *Polytomella* spp. forman parte del complejo mitocondrial activo, y que los genes que las codifican han sido transferidos del DNA mitocondrial al nuclear.

Hasta el momento ha sido muy difícil llevar a cabo la purificación y caracterización de los complejos mitocondriales de *C. reinhardtii* debido a que las preparaciones mitocondriales presentan serios problemas de contaminación por proteínas del cloroplasto (Atteia y col., 1992; Atteia 1994). Por otro lado, *Polytomella* spp. presenta mitocondrias que pueden aislarse fácilmente debido a la ausencia de pared celular y de proteínas del cloroplasto. De este organismo ha sido posible purificar al complejo bc- (Gutiérrez-Cirlos y col., 1994) y a la ATPasa (Atteia y col., 1997). En este trabajo, se presenta la purificación de la citocromo *c* oxidasa de *Polytomella* spp. y se demuestra que las subunidades II y III de este complejo sí están presentes. A partir de la secuencia polipeptídica obtenida de las subunidades purificadas se aislaron los genes que las codifican tanto en *Polytomella* spp. como en *C. reinhardtii*, y se demostró su localización nuclear.

OBJETIVOS

1. **Demostrar que las proteínas COXII y COXIII forman parte de la citocromo *c* oxidasa mitocondrial de *Polytomella* spp.** Para ello, se purificará a la citocromo *c* oxidasa de *Polytomella* spp. y se identificarán a las subunidades II y III. Para ello se emplearán anticuerpos generados contra las subunidades II y III de levadura y se obtendrá la secuencia de aminoácidos de los extremos amino terminales de estas subunidades.

2. **Demostrar que los genes que codifican para COXIII de *Polytomella* spp. y *C. reinhardtii* son nucleares y no mitocondriales.** Para ello, se clonará el gen *cox3* de *Polytomella* spp., empleando la secuencia del extremo amino terminal de la proteína para diseñar desoxioligonucleótidos degenerados. El gen *cox3* de *Polytomella* spp. será empleado como sonda para obtener el gen *cox3* de *C. reinhardtii* de una biblioteca de cDNA.

3. **Demostrar que los genes que codifican para COXII de *Polytomella* spp. y *C. reinhardtii* son nucleares y no mitocondriales.** Para ello se clonará el gen *cox2* de *Polytomella* spp. Se empleará la secuencia amino terminal obtenida de la proteína para diseñar desoxioligonucleótidos degenerados. Una vez que se tenga clonado el gen completo de *Polytomella* spp., éste se empleará como sonda para clonar el gen *cox2* de *C. reinhardtii*.

4. **Analizar las adaptaciones que sufrieron estos genes para una transferencia exitosa al núcleo.** Se analizarán algunas características como el cambio en uso de codones, la adquisición de señales de poliadenilación y de posibles presecuencias mitocondriales. Adicionalmente se analizarán las propiedades de hidropatía de las proteínas predichas por los genes *cox2* y *cox3*.

RESULTADOS

El capítulo **5** presenta el artículo "**Unusual location of a mitochondrial gene. Subunit III of cytochrome *c* oxidase is encoded in the nucleus of chlamydomonad algae**". En este trabajo se demuestra que la subunidad III de la citocromo *c* oxidasa del alga incolora *Polytomella* spp. si está presente en el complejo enzimático. Se obtuvo la secuencia de cDNA del gen *cox3* que codifica para esta proteína al igual que el gen *cox3* de *C. reinhardtii*. Se demostró que *cox3* está codificado en el DNA nuclear y no en el mitocondrial, y se analizaron algunas de las modificaciones que este gen sufrió al exportarse al núcleo y que deben contribuir a facilitar la importación de la proteína a la mitocondria. En este trabajo se cubre parte de los objetivos 1, 2 y 4.

En el capítulo **6** se presenta el artículo en proceso "**Structure of the nuclear *cox3* genes in green and colorless chlamydomonad algae**". En este trabajo se analiza la estructura genómica del gen *cox3* de *Polytomella* spp. y *C. reinhardtii*. Las características de los genes reportados apoyan la localización nuclear en estas algas. El análisis sugiere que la transferencia del gen *cox3* al núcleo, así como la adquisición de una presecuencia mitocondrial se llevaron a cabo en el ancestro común de *Polytomella* spp.y *C. reinhardtii*. En este trabajo se cubre parte de los objetivos 2 y 4.

En el capítulo **7** se presenta el artículo en proceso "**Subunit II of cytochrome *c* oxidase in chlamydomonad algae is a heterodimer encoded by two independent nuclear genes**". En este capítulo se demuestra que la subunidad II de la citocromo *c* oxidasa del alga *Polytomella* spp. si está presente en el complejo enzimático. En este trabajo se clonaron los genes que codifican para la subunidad II tanto en *Polytomella* spp. como en *C. reinhardtii*. Se demostró que en estas algas, la subunidad II está formada por dos proteínas diferentes, COXIIa y COXIIb, que se codifican por dos genes con una localización nuclear. Se analizaron algunas de las modificaciones que estos genes sufrieron al integrarse a este genoma. En este trabajo se cubre una parte de los objetivos 1, y 4 y se cubre el objetivo 3.

RESULTADOS I:

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Unusual Location of a Mitochondrial Gene

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

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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 *atp1* and *atp8* (encoding subunits *a* and *A6L* of the F_o portion of ATP synthase). These genes are also absent in the mtDNAs of the closely related algae *Chlamydomonas smithii* (6), *Chlamydomonas eugametos* (7), *Chlamydomonas moewusii* (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 as reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession numbers AF233511 and AF233515.

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[1] The abbreviations used are: mtDNA, mitochondrial DNA; Cy-COX III, cytochrome *c* oxidase subunit III protein from *C. reinhardtii*; *m*soH, mean hydrophobicity; 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; $\langle H \rangle$, mean hydrophobicity of a sequence segment; μH, 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 *meso*H (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 (Göttingen, 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 $MgSO_4$, 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 $MgSO_4$, 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 Microséquençage 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 Biochemicals) 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 WHFMVDVVWL: 5′-AG CCA (G/A)AC (G/A)AC (G/A)TC (G/A)AC (C/G)A(C/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/adapter as first strand cDNA primer (oligo dT/adapter, 5′-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT-3′). For 3′ end cDNA cloning, oligo dT/adapter 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/adapter and B5 (5′-CAA CGG ATC CGA ACA ACA AGG-3′) with adapter 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 × 10^4 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/adapter, and reverse, 5′-TGC TCC ATG TAG AAC TCC TTG G-3′. The sequences for nested PCR were: forward, oligo adapter, 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 *meso*H. 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*).

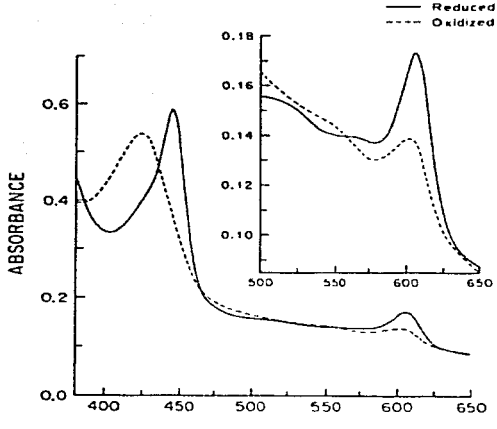

FIG. 1. **Visible spectra of cytochrome *c* oxidase from *Polytomella* spp.** The cytochrome *c* oxidase was diluted in 50 mM Tris·HCl (pH 8.0) containing 1 mM $MgSO_4$ and 0.1 mg/ml of lauryl maltoside. *Broken lines*, oxidized sample, as obtained. *Continuous line*, cytochrome *c* oxidase fully reduced 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 μmol O_2/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 *aa*$_3$ 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. 2*A*). 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. 2*B*, *lane 3*). This band had a molecular mass similar to that of the corresponding subunit III of cytochrome *c* oxidase from yeast (Fig. 2*B*, *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. 2*B*, *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 obtained 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 λgt10 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. 3*A*) 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 down-stream one predicts a presequence of 40 amino acids. It is known that the sequence surrounding start codon affects the efficiency of translation, in *C. reinhardtii* there is a consensus of (A/C)(A/C)(A/C)ATG(G/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

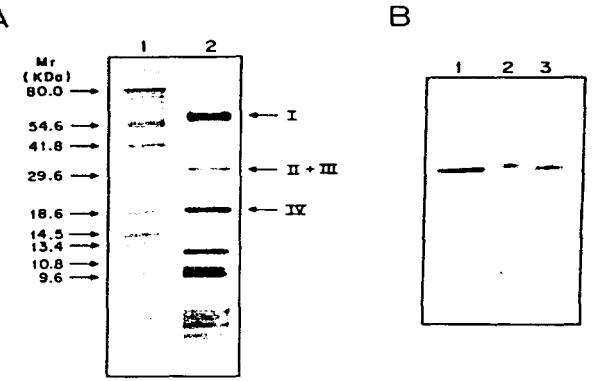

FIG. 2. **Subunit composition and immunoblot analysis of the cytochrome complex from *Polytomella* spp.** *A*, the cytochrome *c* oxidase preparation was analyzed on a 16% acrylamide gel stained with Coomassie Brilliant Blue (23) and compared with the bovine enzyme. *Lane 1*, cytochrome *c* oxidase from *Polytomella* spp. (20 μg of protein). *Lane 2*, cytochrome *c* oxidase from beef-heart mitochondria (30 μg 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 μg of protein per lane). *Lane 2*, purified COX III from *Polytomella* spp. *Lane 3*, isolated cytochrome *c* oxidase from *Polytomella* spp. (20 μg of protein/lane).

values are much higher when the first 17 residues MRSQLLK/RIALTRAPAGFS 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. 3*B*). 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 polyadenilation signals TGTAA (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. 4*A*) *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. 4*B*). 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 Chlamydomonad 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. 6*A* 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.

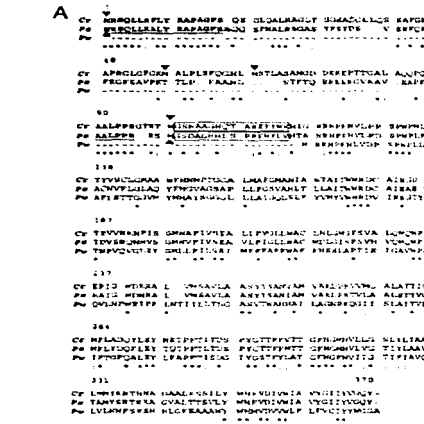

FIG. 3. **Sequence alignment and phylogenetic analysis of cytochrome *c* oxidase subunit III sequences.** *A*, sequence alignment of COX III from *Polytomella* spp (*Ps*), *C. reinhardtii* (*Cr*), and *P. wickerhamii* (*Pw*) (62). Numbering is referred to the *Polytomella* spp sequence. *Black triangles* 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. *Two dots* indicate similar residues. Sequence comparisons made without considering the putative presequences showed 60.5% identity and 74.9% 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.

TABLE 1

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 *Cyte1* 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	cox3	Res	Codon	Mito	Nuc	cox3
Gly	GGG	8	0	0	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	Cys	TGT	43	0	0
·	·	·	·	·	Cys	TGC	57	100	100
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
Glu	GAA	53	5	0	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
Val	GTA	34	0	0	Leu	TTA	28	1	0
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	Phe	TTC	30	92	90
·	·	·	·	·	·	·	·	·	·
Ala	GCT	5	5	·	·	·	·	·	·
Ala	GCA	14	1	2	·	·	·	·	·
Ala	GCT	66	16	51	·	·	·	·	·
Ala	GCC	15	51	47	Ser	TCC	8	69	39
·	·	·	·	·	·	·	·	·	·
Arg	AGA	4	4	11	·	·	·	·	·
Arg	AGA	1	0	·	Arg	CGA	21	0	0
Ser	AGT	19	1	·	·	·	41	52	49
·	AGC	32	1	11	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
Lys	AAG	12	97	8	·	·	41	95	100
Lys	AAA	68	3	10	Gln	CAA	59	4	0
Asn	AAT	51	11	·	·	·	·	·	·
Asn	AAC	43	89	100	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·
Met	ATG	100	100	100	·	·	·	·	·
Ile	ATA	25	2	0	Leu	CTA	12	0	0
·	ATC	75	98	·	·	·	17	24	24
·	ATT	47	1	·	Leu	CTC	5	47	38
·	·	·	·	·	·	·	·	·	·
Thr	ACA	1	0	·	·	·	·	·	·
Thr	ACA	33	0	0	Pro	CCA	39	0	6
·	·	·	·	·	·	·	·	·	·
·	·	·	·	·	·	·	·	·	·

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 (MH 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. 6*B*). This suggests a structure of these polypeptides similar to the ones determined by

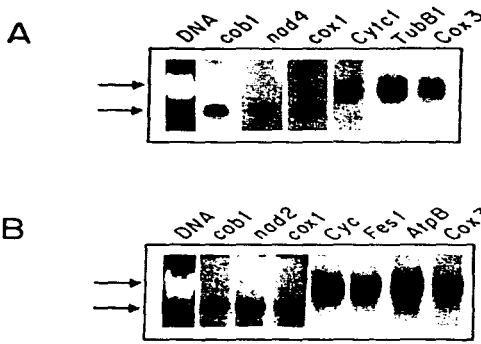

FIG. 4. **The gene *cox3* is nuclear-localized in *Polytomella* spp. and in *C. reinhardtii*.** *A*, 30 μg of total DNA from *Polytomella* spp. was run in a 0.7% agarose gel. The gel transferred to a nylon membrane and hybridized with different nuclear and mitochondrial probes described in the text.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

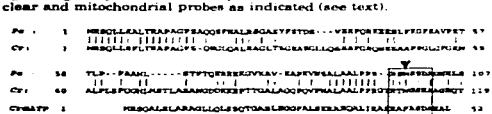

FIG. 5. **Comparison of presequences in nuclear encoded mitochondrial proteins in *Polytomella* spp. and *C. reinhardtii*.** Identical and similar residues are denoted with *straight vertical lines* and *double dots*, respectively. Only the sequences from *Polytomella* spp. (*Ps*) and *C. reinhardtii* (*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 (*CraATP*) and the Rieske iron sulfur protein (*CrFeS*) of *C. reinhardtii* were taken from Nurani and Franzén (63) and from Atta 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 Pa-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 TGTAA 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 *meso*H of the predicted protein product (Fig. 6*A*), 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.

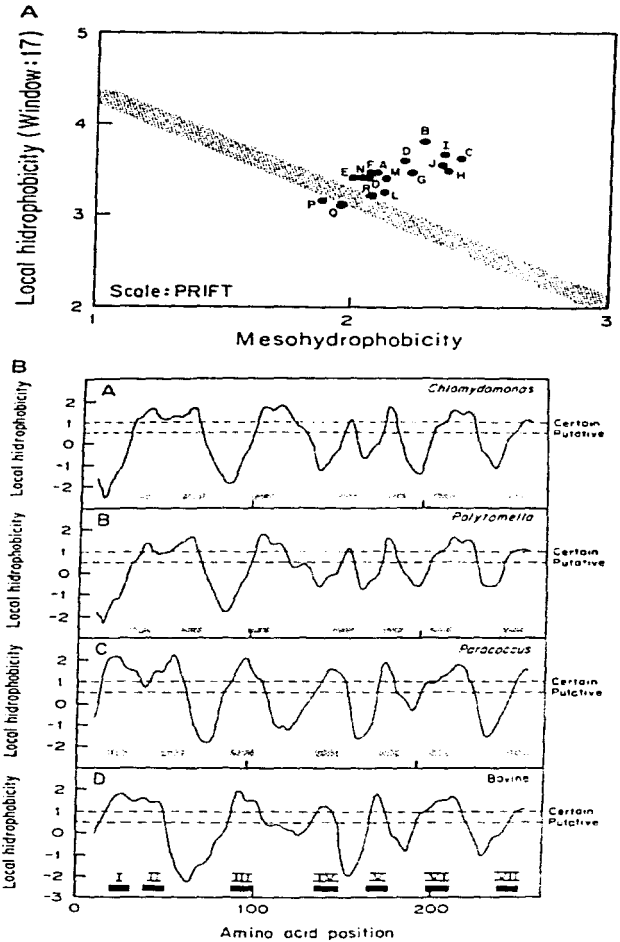

FIG. 6. Mesohydrophobicity and hydrophobicity plots of cytochrome *c* oxidase subunit III from different organisms. *A*, mesohydrophobicity *versus* maximal local hydrophobicity plot for cytochrome *c* oxidase subunit III from different organisms using the PRIFT scale. Proteins are distributed on the *abcissa* 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*, *Pisavella littoralis* (Z37967); *I*, *P. wickerhamii* (Q37620); *J*, *Gracilaria lemaneiformis* (AF118119); *K*, *Oryza sativa* (X17040); *L*, *Schizosaccharomyces pombe* (X16868); *M*, *Glycine max*, soybean (X15133); *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*, hydropathy 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 <H> 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. 3*A*) 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. 3*B*) 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 <H> 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 (*cob*), 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 <H> 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 posses low <H> 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: (*i*) the export of the nucleic acid molecule as DNA or RNA (58), (*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 (<H>) 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 <H> and high *mesoH* have remained in the mitochondrial genome, whereas those genes that encode proteins with low values of <H> 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 <H> 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. 6*B*). This is more evident in the three-dimensional model for Ps-COX III built upon the crystallographic coordinates of its bovine counterpart (30). 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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RESULTADOS II:

Artículo en proceso.

STRUCTURE OF THE NUCLEAR *cox3* GENES IN GREEN AND COLORLESS CHLAMYDOMONAD ALGAE.

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Running heads: Genomic *cox3* sequences of chlamydomonad algae.

Este artículo se enviará a la revista "Gene".

ABSTRACT

The chlorophyte algae of the family Chlamydomonadaceae share as a common feature the lack of the *cox3* gene, encoding subunit III of cytochrome *c* oxidase, in their mitochondrial genomes. Recently, it was shown that the *cox3* gene is nuclear encoded in two members of this family: the photosynthetic alga *Chlamydomonas reinhardtii* and the colorless alga *Polytomella* spp. 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* spp. contain 4 introns in the region encoding the putative mitochondrial targeting sequences. These 4 introns show low identities but their locations were conserved in both *cox3* genes. The *cox3* gene of *C. reinhardtii* has 5 additional introns in the region encoding the mature polypeptide of subunit III of cytochrome *c* oxidase. Sequence analysis of intron 6 of the *cox3* gene of *C. reinhardtii* revealed similarity with two stretches of nucleotides present in introns of several nuclear genes from this green alga. In the majority of the explored sequences, these internal conserved sequences are located 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 the main photosynthetic lineage of *Chlamydomonas* 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* / evolution of colorless algae / introns / mitochondrial targeting signals / *Polytomella* spp.

INTRODUCTION

Chlorophyte algae of the family Chlamydomonadaceae include several photosynthetic organisms of the genera *Chlamydomonas*, *Chlorogonium*, *Chloromonas*, *Lobomonas*, *Brachiomonas*, and *Spermatozopsis*, and two genera of colorless algae, *Polytoma* and *Polytomella* (Melkonian, 1990). The close phylogenetic relationship between the genera *Chlamydomonas* and *Polytomella* is supported by a large body of morphologic, genetic, and biochemical evidence (Mattox and Stewart, 1984; Conner *et al.*, 1989; Melkonian, 1990; Melkonian and Surek, 1995; Antaramian *et al.*, 1996; Nedelcu *et al.*, 1996; Atteia *et al.*, 1997; Pérez-Martínez *et al.*, 2000). The colorless alga is thought to derive from the main *Chlamydomonas* lineage by a secondary evolutionary process in which the photosynthetic apparatus was lost (Round, 1980).

The mitochondrial genomes of all the members of the Chlamydomonadaceae family that have been characterized to date 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*, *nad3* and *nad4L* (Gray and Boer, 1988; Michaelis *et al.*, 1990; Colleaux *et al.*, 1990; Turmel *et al.* 1993; Denovan-Wright *et al.* 1998; Kroymann and Zetsche, 1998). Recently, we have shown that in the green alga *C. reinhardtii* and in the colorless alga *Polytomella* spp. the *cox3* gene had been transferred from the mitochondria to the nucleus (Pérez-Martínez *et al.*, 2000). We first confirmed by protein purification and sequencing that the COX III protein was present in the cytochrome *c* oxidase complex of *Polytomella* spp. We then cloned and sequenced cDNAs encoding COX III and showed that the corresponding genes were nuclear-localized. The characteristics of the nuclear localized *cox3* genes are in accordance with the different stages of gene transfer from

the mitochondrion to the nucleus proposed by Brennicke *et al.* (1993), Claros *et al.* (1995), and Martin and Herrmann (1998),: *i*) they acquired a nucleotide sequence encoding a putative mitochondrial targeting sequence (MTS), *ii*) they exhibit a codon usage pattern which is typically nuclear, *iii*) they acquired polyadenylation signals typical of chlamydomonad nuclear genes that differ from the consensus in other eukaryotes, and *iv*) their deduced protein products exhibit an overall diminished mesohydrophobicity when compared with their counterparts encoded in the mitochondrial genome (Perez-Martinez *et al.*, 2000).

In this work, we describe the cloning and characterization of the genomic sequences of the *cox3* genes from *C. reinhardtii* and *Polytomella* spp. This is the first report of the genomic sequences of *cox3* genes that are localized in the cell nucleus.

MATERIALS AND METHODS

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

Nucleic acids preparation - Total DNA was obtained from broken cells of *Polytomella* spp. and *C. reinhardtii* as described (Pérez-Martinez *et al.*, 2000). All other standard molecular biology techniques were as reported in Sambrook *et al.*, (1989), and nucleotide sequencing was performed by the Kimmel Cancer Center DNA Sequencing Facility, Thomas Jefferson University.

Cloning and sequencing of genomic cox3 from Polytomella spp. - The genomic sequence of the *cox3* gene of *Polytomella* spp. was obtained by PCR amplification using Hot Start Taq Polymerase (QIAGEN), and primers designed to hybridize on the start and ending regions of the coding sequence of the *cox3* cDNA. The forward primer was 5'-CGT TTT TGG TCA AGT TGA AA-3', and the reverse primer was 5'-GCT CAT GTA ACT ATG CCA CAA GAC-3'. Total DNA was denatured for 5 min. at 94 °C, and subjected to 30 cycles of 45 sec. denaturation at 94 °C, 1 min. annealing at 50 °C, and 2.5 min. extension at 72 °C. Five different single-band PCR products were cloned into pGEM-T easy Vector System from Promega and sequenced.

Cloning and sequencing of genomic cox3 from C. reinhardtii - *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 pGEM-T easy Vector System from Promega.

The genomic sequences of the *cox3* genes of *Polytomella* spp. and *C. reinhardtii* are not shown, but are available in the DDBJ/EMBL/GenBank data bank under accession numbers AF286057 and AF286058 respectively, and for referees in **Figures A** and **B** of additional material.

RESULTS AND DISCUSSION

The cox3 genomic sequences of chlamydomonad algae contain several introns.- Based on the sequences of the *cox3* cDNA clones from *Polytomella* spp. and *C. reinhardtii* (Pérez-Martínez *et al.*, 2000), deoxyoligonucleotide primers were designed and used for PCR amplification of the corresponding genomic coding regions (data not shown). When *Polytomella* spp. cDNA was used as template for amplification, the predicted 1.2 kb product was obtained. In contrast, when total genomic DNA was used as template, a larger 2.0 kb product was obtained. When *C. reinhardtii* cDNA was used as template to amplify the complete coding region with 3 different primer pairs, an estimated size of 1.2 kb for the mRNA was obtained, while genomic DNA gave rise to 3 amplicons reflecting a total size of 3.2 kb for the genomic sequence. These results suggested the presence of introns in these genes. The PCR fragments obtained from amplification using genomic DNA 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 position of some introns are conserved in the two chlamydomonad cox3 genes.- The intron boundaries, phases and sizes for both *cox3* genes are shown in **Table 1**. The *cox3* gene of *Polytomella* spp. shows the presence of four introns, numbered 1 to 4, that range in size from 71 to 444 nucleotides; all are located in the region encoding the presequence that functions as a MTS, that is thought to direct and insert subunit COX III into the mitochondrial inner membrane. This presequence is not present in the mature COX III protein of *Polytomella* spp. (Pérez-Martínez *et al.*, 2000). In contrast, the genomic sequence of the *cox3* of *C. reinhardtii* is interrupted by 9 introns, numbered 1 to 9, ranging in size from 133 to 323 nucleotides. The first 4 introns are also located in the region encoding the MTS of the *C. reinhardtii* COX III polypeptide. Introns 5 to 9 are distributed along the nucleotide sequence that encodes the mature COX III subunit.

Figure 2 shows an alignment of the MTSs encoded by both *cox3* genes to indicate the positions of introns between these regions. The location and the phases of introns 1, 3, and 4, show that these introns have been conserved between the two species. Intron 2 has also a conserved phase, but its position is displaced by three nucleotides. This suggests that all these introns were acquired after the *cox3* gene was transferred to the nucleus in the common ancestor, but before *Polytomella* diverged from the main *Chlamydomonas* lineage. In contrast to the conserved location of the introns in the MTS encoding region, their nucleotide sequences are poorly conserved between these algae (less than 38% identity). This is likely due to the rapid rate of nucleotide substitutions in introns, whose nucleotide substitution rate is estimated to be ten-fold higher than that in exons (Lisa *et al.*, 1997). It is believed that the evolutionary separation of the genus *Polytomella* from the main *Chlamydomonas* lineage occurred about 65 million years ago (Round, 1980). We estimate that this period of time is sufficient to account for the divergence of sequence similarity between the *cox3* introns of *Polytomella* and *Chlamydomonas*.

The presence of similar intron positions and similar MTS encoding regions in the two *cox3* genes of chlamydomonad algae, strongly supports the hypothesis that the *cox3* genes were transferred from the mitochondria to the nucleus before the genus *Polytomella* diverged from the main *Chlamydomonas* lineage. The two *cox3* genes of these chlamydomonad algae must have acquired the region encoding the MTS and their respective introns before speciation, and before the separation of the colorless genus from the photosynthetic lineage.

The presence of 5 extra introns in the *Chlamydomonas cox3* gene 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* spp. One general feature of *Chlamydomonas* genes is that introns are often small, with an average size of 219 bp and ranging from 57 bp to 1318 bp. (Silflow, 1998). The *Chlamydomonas* introns in the *cox3* gene range from 133 to 323 bp.

The vast majority of the mtDNA-encoded *cox3* genes reported to date lack introns. We examined the locations of the introns in the exceptions to determine if there was any evidence of conservation with the sites of the Chlamydomonad introns. 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 in the *Chlamydomonas* and *Polytomella* nuclear *cox3* genes. Other examples are the plant *Lycopodium squarrosum* (Hiesel *et al.*, 1994) and the yeast *Yarrowia lipolytica* (Matsuoka *et al.*, 1994) *cox3* genes, which contain a single intron in positions not conserved between them nor with the *Chlamydomonas* and *Polytomella* genes. The overall data suggest that introns were inserted in the nuclear encoded *cox3* genes after the divergence of the chlorophyte algae from the evolutionary line of plants.

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 I). The splice junction also conforms to the classical sequences AG/GT at the 5' end, and AG/GC at the 3' end described for the majority of eukaryotes (Lee *et al.*, 1991; LeDizet and Piperno, 1995). 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 surrounding splicing sites in the *cox3* gene of *Polytomella* spp. show some differences with the *C. reinhardtii* consensus splicing sites, and 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* spp. genes are needed to establish canonical splicing sites in this organism.

This is the first description of nuclear genomic sequences of *cox3* genes. In the case of *Polytomella* spp., it is also the first nuclear sequence reported for this colorless algae. *C. reinhardtii* is known to contain a high intron density in its nuclear genome; an estimate of 3.9 to 4.2 introns per kb of genomic sequence has been calculated (Silflow, 1998; Funke *et al.*, 1999). A value of 2.9 introns per kb for the *C. reinhardtii cox3* gene was observed. In contrast, the mitochondrial genomes of Chlamydomonad algae usually are not interrupted by introns, with the exception of the *cob* gene in *C. smithii* (Colleaux *et al.*, 1990); *cox1*, *nad5*, and *cob* genes in *C. elongatum* (Kroymann and Zetsche, 1998); *cox1*, *nad1*, *nad5*, *cob*, and ribosomal RNA coding regions of *C. eugametos* (Denovan Wright *et al.*, 1998); and a degenerate group II intron in the intronless mitochondrial genome of *C. reinhardtii* (Nedelcu and Lee, 1998). The partial sequence of the mtDNA of *Polytomella* spp. also indicates the absence of introns in this alga (Funes *et al.*, unpublished results).

Introns 5 to 9 found in the *cox3* gene of *C. reinhardtii* are distributed along the sequence encoding the mature COX III polypeptide (**Figure 1**). Except for intron number 6, these introns do not show significant sequence similarity with other introns in the database (**Figure 3**). Intron 6, the largest one in the *C. reinhardtii cox3* gene, shows two internal conserved sequences (ICS) found in several

introns of *C. reinhardtii* and of members of the genus *Volvox* (**Table II** and **Table III**). 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). ICS 2 was previously observed 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). These authors proposed that this nucleotide stretch might represent a sequence encompassing a branch site of these introns. Otherwise, it may represent a signal of yet unknown importance in the RNA splicing process or in the expression of genes in *C. reinhardtii*. The ICSs reported in this work differ from another ICS previously observed in that they lack the consensus sequence NCTAG located 15-51 bp upstream of the 3' splice site in 56 nuclear introns of *Chlamydomonas* (Lee *et al.*, 1991).These 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 these 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 II**, the sequence of the ICS 1 is inverted with respect to the *cox3* intron 6 sequence. In these cases, the relative position of the ICS 1 within the intron is also inverted and is found near the 3' end of the intron.

The same is observed for ICS 2 (**Table III**). In this case, the sequence is preferentially found near the 3' end of the intron. When the sequence of the ICS 2 is inverted compared to the *cox3* intron 6 sequence, the position within the intron is also inverted and it is present near the 3' end of the intron. These ICS are not present in all *Chlamydomonas* introns, but they may play an important role for intron processing and gene expression. The presence of ICS in introns is also a common feature in yeast. Although relatively few introns are present in the fully-sequenced genome of *Saccharomyces cerevisiae* (Ares *et al.*, 1999), the great majority of them (214 out of 231) contain a 7-nucleotide ICS, with the consensus sequence TACTAAC (Fouser and Friesen, 1986; Spingola *et al.*, 1999).

A model for the separation of *Polytomella* from the main *Chlamydomonas* photosynthetic lineage and the late acquisition of introns in the *Chlamydomonas cox3* gene is shown in **Figure 4**. Although it is impossible to ascertain an intron-loss against an intron-gain evolutionary scenario for chlamydomonad *cox3* genes, the data strongly suggest a late intron sequence acquisition in *C. reinhardtii* that occurred after the separation of the colorless genus *Polytomella* from the main *Chlamydomonas* photosynthetic lineage. The data are in agreement with the suggestion of a late occurence of multiple intron insertion events in the evolution of eukaryotic genes (Palmer and Logsdon, 1991), and also with the proposal of Funke *et al.* (1999), for the late acquisition of introns in acetolactate synthase genes in *C. reinhardtii* and its multicellular relative *Volvox carteri*. The presence of intronic sequences may be required for the adequate expression of some genes in *C. reinhardtii* (Lumbreras *et al.*, 1998). The comparison of *cox3* sequences of *Polytomella* spp. and *C. reinhardtii* also suggests that intron acquisition in *C. reinhardtii* occurred relatively recently in evolution.

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Figure legends:

Table I. Sizes, phases, and flanking sequences of 5' and 3' splice sites for *C. reinhardtii* (*Cr*) and *Polytomella* spp. (*Ps*) *cox3* introns.

Table II. Internal conserved sequence (ICS 1) found in *C. reinhardtii cox3* intron 6 that is present in other chlorophyte introns. GeneBank accesion numbers and references of *C. reinhardtii* nuclear genes used to construct this Table: *AF047459* [*C. reinhardtii* acetolactate synthase (ALS) gene, Funke *et al.*, 1997a]; *AF154916* [*C. reinhardtii* variable flagellar number protein, (VFL1), Tam and Lefebvre, 1993]; *AF233430* [*C. reinhardtii* (EYE2) gene, Roberts,DGW, Lamb MR, Dieckmann CL, 2000, unpublished]; *U68060* [*C. reinhardtii* actin-related protein (ACT2) gene, Lee *et al.*, 1997]; *AF233374* [*Volvox carteri* argininosuccinate lyase (VASL) gene, Heimrich O and Mages W, 2000, unpublished]; *U55929* [*Volvox spermatosphaera* UTEX 2273 GTPase Ypt4p (ypt4) gene, intron 6, Liss *et al.*, 1997].

Table III. Internal conserved sequence (ICS 2) found in *C. reinhardtii cox3* intron 6 that is present in other *C. reinhardtii* introns. GeneBank accesion numbers and references of *C. reinhardtii* nuclear genes used to construct this Table: *AF129458* [*C. reinhardtii* class II DNA photolyase (PHR2) gene, Petersen *et al.*, 1999]; *U97663* (*C. reinhardtii* phosphatidylinositol 3-kinase gene, Molendijk and Irvine, 1998); *AF047459* [*C. reinhardtii* acetolactate synthase (ALS) gene, Funke *et al.*, 1997a]; *U73856* (*C. reinhardtii* carbonic anhydrase, alpha type CAH3 gene, Funke et al., 1997b); *X16619* (*C. reinhardtii* ARG7 gene for argininosuccinate lyase, Debuchy et al., 1989); *AF203033* (*C. reinhardtii* nitrate reductase, NIT1 gene, Zhang D, LaVoie MP, Christenson S, Lefebvre PA, 1999, unpublished); *AB001486* (*C. reinhardtii ycs*1B gene, Uchida *et al.*, 1993).

Figure 1. Overall organization of exons and introns in *Polytomella* spp. and *C. reinhardtii cox3* coding gene. 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 conserved exons in the mitochondrial targeting sequence found in both algae. Black triangles indicate methionines positions in the presequences.

Figure 2. Amino acid sequence alignment of MTS of *Polytomella* spp. and *C. reinhardtii cox3* and comparison of intron positions. The arrows indicate positions of introns 1 to 4 in the corresponding gene of each alga. If the arrow is over one amino acid, it means that the intron is not in phase with the *orf*. Only intron 3 is in phase with the *orf*. Bold characters indicate amino acids that are highly conserved between the two sequences.

Figure 3. Nucleotide sequence of intron 6 of the *cox3* gene of *C. reinhardtii*. ICS 1and ICS 2 are indicated in the boxed regions. ICS 1 is present near the 5' end of the intron and ICS 2 is located near the 3' end of the intron.

Figure 4. Model suggesting the transfer of the *cox3* gene from the mitochondrion to the nucleus and its relationship with speciation. Intron positions are indicated by arrows. 1. Transfer of the *cox3* gene from mitochondria to the nucleus. 2. Acquisition of a nucleotide sequence encoding a MTS (shaded box). 3. Acquisition of introns in the nucleotide sequence encoding the MTS (steps 2 and 3 may have ocurred simultaneously). 4. Separation of the colorless genus *Polytomella* from the green *Chlamydomonas* lineage. 3nd acquisition of additional introns (arrows) in the *cox3* gene of *C. reinhardtii*. Nucleotide additions, deletions and substitutions may have occurred in introns 1-4, which changed their sequence and size, while retaining their original positions. 5. Loss of the mitochondrial *cox3* gene copy.

The inactivation of the original mitochondrial gene and its subsecuent dissapearance must have ocurred once the nuclear *cox3* gene copy became active. For simplicity, the mitochondrion (mt) and the nucleus (nu) are shown out of the cell context.

Table 1.

	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/GT/AGA	CGG/GTGAG T	71	133	TTTAG/GAA	CGCAG/CCG
3	0	0	AAG/GTAATT	AAG/GTAAGG	85	139	TATAG/ACT	CGCAG/ATG
4	2	2	CAG/GTAAGA	GCG/GTGAG G	444	256	TGCAT/GTG	TGCAG/CAC
5	-	1		TGG/GT AAGC		290		CGCAG/GCA
6	-	2		GCT/GTGAG T		323		CGCAG/CTG
7	-	0		AAG/GTGTGT		159		CGCAG/GTG
8	-	2		CTA/GTGAG T		180		CGCAG/GTA
9	-	2		CTG/GTGGGT		204		CACAG/GCA

Table II

ICS 1: GGGAGGGGGGAGAGG		
Intron/ Size(bp)	Gene name	Location in the intron
6 / 323	*C. reinhardtii cox3*	31 bp from 5'
8 / 443	*C. reinhardtii* acetolactate synthase (ALS)	6 bp from 5'
14/ 288	*C. reinhardtii* variable flagellar number protein	16 bp from 5'
2 / 253	*C. reinhardtii* EYE2 gene	22 bp from 5'
2*/ 228	*C. reinhardtii* actin-related protein (ACT2)	21 bp from 3'
5*/ 454	*Volvox carteri* argininosuccinate lyase (VASL)	5 bp from 3'
/ 748	*Volvox spermatosphera* UTEX 2273 GTPase Ypt4p gene, intron	2 bp from 5'

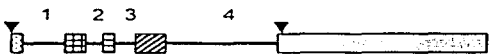
* The sequence is inverted with respect to *cox3* intron 6

Table III.

ICS 2: ATCATGAATGTAACCCC		
Intron/ Size(bp)	Gene name	Location in the intron
6 / 323	*C. reinhardtii cox3*	25 bp from 3'
3 / 493	*C. reinhardtii* class II DNA photolyase	177 bp from 3'
8 / 694	*C. reinhardtii* class II DNA photolyase	17 bp from 3'
2 / 480	*C. reinhardtii* phosphatidylinositol 3-kinase	21 bp from 3'
5*/ 500	*C. reinhardtii* acetolactate synthase (ALS)	2 bp from 5'
9 / 582	*C. reinhardtii* acetolactate synthase (ALS)	34 bp from 3'
4 / 287	*C. reinhardtii* carbonic anhydrase (CAH3)	26 bp from 3'
8*/ 490	*C. reinhardtii* argininosuccinate lyase (ARG7)	27 bp from 5'
8 / 212	*C. reinhardtii* nitrate reductase (NIT1)	20 bp from 3'
2 / 281	*C. reinhardtii* zys1B gene	32 bp from 3'

* The sequence is inverted with respect to *cox3* intron 6.

Polytomella spp.



Chlamydomonas reinhardtii

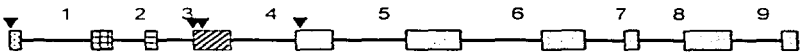


Figure 1.

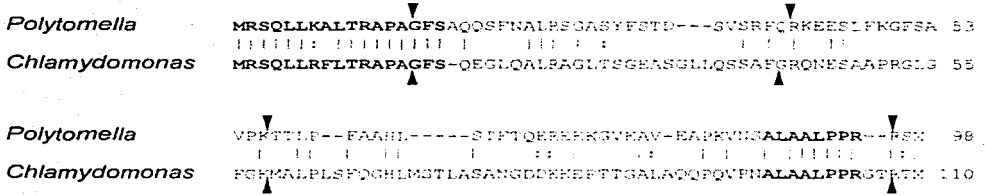


Figure 2

GTGAGTACGGCAGCGTGCGCTAGGAGCGAAGGGAGGGGGCAGAGGAGGGTGGTCCTGAGG
AAGGTCAGGCACCAACGCCGTGCGGGATGGAGCATGGATTGAGAAGAGCTGCGGCAGCAC
CGATACCGATACTAGGTTGCATAGCAGCCACCGGGGCCCCTGGTGCACAAACCATCATTC
CCCCTGCATCCCTGTGTCTTGCATCCCCGTCTATTTTACTTGTGACCTCCTTCTTGCCCT
TCTCCCCCAAGATTGCTTACCGTATTTCCATTTCTTGGCTAATCATGAATGTAACCCCTC
CCCCTTCCGCCTCGCCTCCGCAG

Figure 3

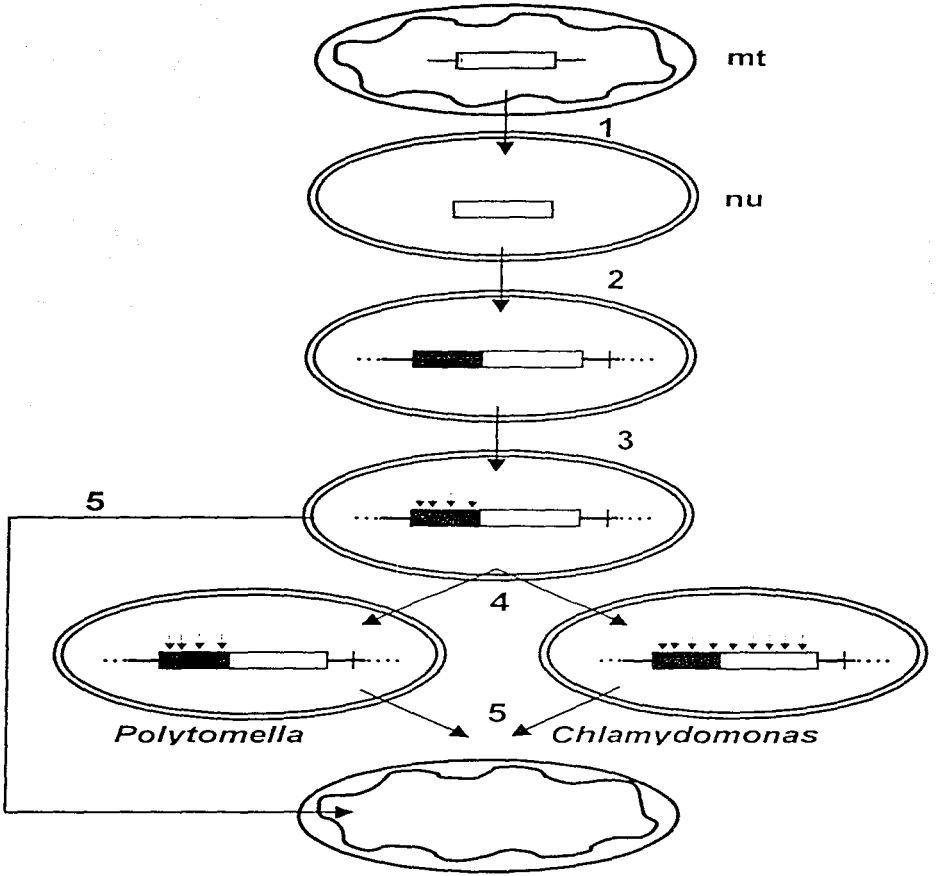


Figure 4

RESULTADOS III:

Artículo en proceso.

SUBUNIT II OF CYTOCHROME *c* OXIDASE IN CHLAMYDOMONAD ALGAE IS A HETERODIMER ENCODED BY TWO INDEPENDENT NUCLEAR GENES

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Running heads: *Cox2a* and *Cox2b* genes in chlamydomonad algae.

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ABBREVIATIONS

*meso*H	mesohydrophobicity
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting sequence
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
<H>	mean hydrophobicity of a sequence segment
μH	hydrophobic moment
p(DFM)	discriminant function for mitochondrial proteins

ABSTRACT

The mitochondrial genomes of chlamydomonad algae lack the genes *cox2* and *cox3* that encode essential constituents of cytochrome *c* oxidase (subunits COX II and COX III). Recently, it was shown that the *cox3* gene has been transferred to the nucleus in two species of these algae. In this work, the partial primary structure of COX II of *Polytomella* spp. was obtained by amino-terminal sequencing and internal sequence analysis after proteolysis. The partial sequences enabled us to clone the genes encoding COX II in *Polytomella* spp. and *Chlamydomonas reinhardtii*. Evidence is provided that in these chlamydomonad algae the *cox2* genes are independent and localized in the nucleus. The *cox2a* gene encodes the amino terminal portion of subunit II of cytochrome *c* oxidase (COX IIA), and the *cox2b* gene encodes the carboxy terminal region of this protein (COX IIB). The *cox2a* and *cox2b* genes are independently transcribed into messenger RNAs and translated into separate polypeptides. Direct protein sequencing suggests that these two independent proteins assemble non-covalently in the inner mitochondrial membrane to form the mature COX II subunit.

Keywords: *Chlamydomonas reinhardtii* / mesohydrophobicity / mitochondrial targeting signals / *Polytomella* spp. / transfer of mitochondrial genes to the nucleus /

INTRODUCTION

Mitochondria are thought to be descended from free-living α-protobacteria (Gray *et al.*, 1998). After the endosymbiotic event there was a massive transfer of genes from the protomitochondrion to the nucleus (Gray, 1992). Few genes remain in mitochondrial genomes. Those that remain, encode essential components of the protein synthesis machinery and a limited set of polypeptide subunits of proton translocating complexes in oxidative phosphorylation (Attardi and Schatz, 1988). These highly hydrophobic polytopic proteins contain two or more transmembrane stretches (von Heijne, 1986; Popot and de Vitry, 1990). The classic set of genes encoding oxidative phosphorylation components that exist in the mitochondrial genomes include *cob* (encoding cytochrome *b* of the bc_1 complex), *cox1*, *cox2* and *cox3* (encoding subunits I, COX II and COX III of cytochrome *c* oxidase), *atp6* and *atp8* (encoding subunits a and A6L of the F_0 portion of ATP synthase) and *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5* and *nad6* (encoding subunits 1, 2, 3, 4, 4L, 5 and 6 of NADH: ubiquinone oxidoreductase).

The transfer of mitochondrial genes to the nucleus is an ongoing process (Palmer, 1991), as exemplified by the presence of genes encoded in both the mitochondrial and the nuclear genomes, such as subunit 9 of ATP synthase from *Neurospora crassa* (van den Boogaart *et al.*, 1982), or COX II in the family leguminosae (Covello and Gray, 1992; Adams *et al.*, 1999).

The algae of the family Chlamydomonadaceae lack in their mitochondrial genome some of the classical genes that encode essential components of oxidative phosphorylation: *nad3*, *nad4L*, *cox2*, *cox3*, *atp6*, and *atp8* (Michaelis *et al.*, 1990; Denovan-Wright *et al.* 1998; Kroymann and Zetsche, 1998). In two members of this family, *C. reinhardtii* and *Polytomella* spp., it has been shown that the *cox3* gene was transferred to the nucleus (Pérez-Martínez *et al.*, 2000), and that the corresponding mitochondrial copy had been lost.

Several mitochondrial complexes have been isolated and characterized from the colorless alga *Polytomella* spp., a close relative of *Chlamydomonas* (Gutiérrez-Cirlos *et al.*, 1994; Atteia *et al.*, 1997). Recently, an active, cyanide-sensitive cytochrome *c* oxidase preparation was obtained from *Polytomella* spp. (Pérez-Martínez *et al.*, 2000). In this work, the presence of COX II in this complex was established by N-terminal and internal sequence analysis. Evidence is provided that in both *Polytomella* spp. and *C. reinhardtii*, COX II is encoded by two different and separate nuclear genes, which were named *cox2a* and *cox2b*. These genes are independently localized in the nuclear genome, *cox2a* encoding a protein equivalent to the amino terminal portion of subunit II of cytochrome *c* oxidase (COX IIA), and *cox2b* encoding a protein equivalent to the carboxy terminal region of the same subunit (COX IIB). We hypothesize that these separate, independent, and active genes give rise to an heterodimeric COX II subunit that results from the non-covalent assembly of the COX IIA and COX IIB polypeptides in the inner mitochondrial membrane.

RESULTS

Subunit II is present in the cytochrome c oxidase complex from Polytomella spp. and exhibits a lower apparent molecular mass than its counterparts in other organisms.- Cytochrome *c* oxidase from *Polytomella* spp. was isolated as previously described (Pérez-Martínez *et al.*, 2000). The four largest polypeptides with apparent molecular masses of 54.6, 29.6, 18.6 and 14.5 kDa were subjected to Edman degradation (**Figure 1**). The 54.6 kDa polypeptide was identified as subunit I because its apparent molecular mass is close to the molecular mass predicted from the mitochondrial *cox1* gene sequence (54,781 Da) (Antaramian *et al.*, 1996). Subunit I of *Polytomella* spp. (Ps-COX I) exhibited a blocked N-terminus, not susceptible to Edman degradation. The N-terminal sequence of the 29.6 kDa polypeptide has been previously identified as subunit III (Ps-COX III) (Pérez-Martínez *et al.*, 2000). The N-terminal sequence of the 14.5 kDa polypeptide was also determined, but did not show similarity with other sequences from cytochrome *c* oxidase in the data bank.

The N-terminal sequence of the 18.6 kDa polypeptide LAPVAWQLGFQDSATS QAQA was markedly similar to COX II from other species. Thus, the 18.6 kDa polypeptide was identified as subunit II of cytochrome *c* oxidase (Ps-COX II), although it exhibited a smaller apparent molecular mass than COX II from mammals and yeast, which have apparent molecular masses of 29-30 kDa. For the same 18.6 kDa polypeptide, a second sequence MDAIPGR(R L)NQIWI HNREG was determined in the same reaction of Edman degradation. This sequence represented less than 50% of the protein present in the 18.6 kDa band. It also showed a great similarity with a sequence of other COX II polypeptides near the consensus copper binding site. It was thus identified as an internal fragment of Ps-COX II. We therefore concluded that Ps-COX II was partially cleaved during Edman degradation.

Cloning and sequencing of the cox2b gene from Polytomella spp. and C. reinhardtii. - On the basis of the amino acid sequences of Ps-COX II, two degenerate oligodeoxynucleotides were designed. A PCR-amplification product of 300 bp was obtained using total DNA from *Polytomella* spp. as a template. The backward oligodeoxynucleotide B1, designed based on the internal sequence MDAIPGR L(L)NQ, hybridized with the corresponding region of the gene. In contrast, the forward deoxyoligonucleotide F1, based on the N-terminal sequence LAPVAWQLGF hybridized unspecifically with an unexpected region of the same gene. This gave rise to a 300 bp amplicon of a putative *cox2* gene lacking the

region that encodes the N-terminal sequence of Ps-COX II determined by Edman degradation.

The amplified fragment was used as a probe to screen a minilibrary of total DNA of *Polytomella* spp. constructed from *Pst*I digests of the appropiate size. A 2 kb positive clone was identified, isolated, and sequenced. The genomic sequence obtained encodes a partial sequence of Ps-COX II, comprising 153 residues of the carboxy terminal portion of this subunit. The overall organization of the gene, named *cox2b* is shown in **Figure 2**. The N-terminal sequence of the Ps-COX II determined by Edman degradation was not found in the deduced protein sequence. Nevertheless, the predicted protein contains the consensus sequence GQCSEICGG, known to be the binuclear copper binding site of COX II (Tsukihara *et al.*, 1996).

Using primers based on the genomic clone sequenced above a fragment of the corresponding cDNA was amplified from total RNA from *Polytomella* spp. using RT-PCR. The remainder of the cDNA sequence was obtained following 5'-RACE. The cDNA obtained was 568-bp long and contained a deduced reading frame of 153 amino acids. The last 110 residues showed homology with the carboxyl termini of many COX II proteins. In contrast, the 43 residues of the amino terminal region lacked homology to COX II, but residues 96 to 114 were identical to the minor sequence MDAIPGRGR LDNQIWLTINREG obtained from direct protein sequencing. The cDNA sequence confirmed the genomic sequence obtained for the *cox2b* gene, and indicated the absence of introns in this gene.

The PCR amplified fragment of the *cox2b* gene of *Polytomella* spp. was also used to screen a λgt10 cDNA library of *C. reinhardtii* and 10 positive plaques were identified, isolated, and sequenced. This cDNA *cox2b* sequence exhibited 85% identity and 92% similarity with the one obtained from *Polytomella* spp., and represents a gene encoding the C-terminal portion of COX II from *C. reinhardtii* (Cr-COX IIB). A scheme of the structure of the *cox2b* cDNA from *C. reinhardtii* is shown in **Figure 2.** In addition, the *cox2b* cDNA from *C. reinhardtii* was used to screen a BAC genomic library of this green alga. Three positive clones were identified, and one of them was sequenced. The genomic sequence of the *cox2b* gene of *C. reinhardtii* showed the presence of one intron of 187 bp, which is located at 21 nucleotides upstream of the stop codon. Altogether, these data suggested that the *cox2* gene had been split into two genes in *Polytomella* spp. and in *C. reinhardtii*, and therefore that another gene, encoding for the N-terminus of the protein, remained to be found.

Cloning and sequencing of the cox2a gene from Polytomella spp. and C. reinhardtii - In order to clone the gene that encoded the N-terminal region of Ps-COX II, nested PCR amplification was carried out with primers based on the N-terminal sequence of the protein, QDSATSQAQA (E3 primer), and on an internal conserved sequence in several COX II polypeptides, PSEAITYS (B4 primer). Using cDNA of *Polytomella* spp. as a template, a 250 bp PCR product was obtained; it encodes a sequence which exhibits high similarity with other *cox2* genes (results not shown). Using 5' and 3' -RACE, the complete sequence of the corresponding cDNA was obtained. This cDNA encoded the N-terminal sequence FAPVAWQLGF determined by Edman degradation for the 18.6 kDa polypeptide. This cDNA contained a full reading frame, with a stop codon and a putative polyadenylation signal. This cDNA was named *cox2a*, since it encoded the N-terminal portion of the Ps-COX II protein (Ps-COX IIA), its overall organization is shown in **Figure 2**. The predicted mature protein encoded by the *cox2a* gene contains two putative transmembrane stretches from residue 28 to 48 and from 69 to 89, and the highly conserved sequence

GRQWYWSY present in all sequences of COX II subunits known to date.

The *Polytomella* spp. cDNA of the *cox2a* gene contains an open reading frame of 816 bp, predicted to encode a protein of 271 amino acids. Our identification of the amino terminus of the mature protein as amino acid 131, allows us to predict the presence of a MTS.

Primers were designed on the 5' and 3' ends of the *cox2a* cDNA sequence of *Polytomella* spp., and used to amplify the corresponding gene using total DNA or cDNA from the colorless alga as a template. The corresponding genomic PCR product of 1583 bp was cloned and sequenced. The genomic *cox2a* gene contained 6 introns.

The 1047 bp PCR amplified cDNA fragment of the *cox2a* gene of *Polytomella* spp. was also used to screen a λgt10 cDNA library of *C. reinhardtii*. The largest positive plaque was subcloned into pBluescript, and sequenced. The complete sequence was obtained by 5'-RACE. The *C. reinhardtii* cDNA *cox2a* gene encodes the Cr-COX IIA polypeptide; its predicted amino acid sequence exhibits 53.9% identity with the sequence predicted for the COX IIA protein from *Polytomella* spp. The overall organization of the *cox2a* cDNA from *C. reinhardtii* is shown in **Figure 2.** The *C. reinhardtii* cDNA of the *cox2a* gene contains an open reading frame of 852 bp, encoding a protein of 284 residues. The open reading frame predicts 3 methionine residues that could correspond to the initiation of the MTS. When the third methionine was considered to be the first translated residue, the protein exhibited a possible mitochondrial localization [p(DFM)=0.77)], and also predicted a bipartite MTS.

The cox2a and cox2b genes of Polytomella spp. and C. reinhardtii are nuclear-localized, single-copy genes, that are functionally expressed. - Southern blot analysis was carried out to ascertain that the *cox2a* and *cox2b* genes were nuclear encoded. **Figure 3A,** shows one lane of an agarose gel where total DNA isolated from *Polytomella* spp. was run. The mtDNA readily separated as a discrete band running below the major band representing nuclear DNA (Perez-Martinez *et al.*, 2000). The mitochondrial band hybridized with a *cox1* gene probe (Antaramian *et al.*, 1996), while the nuclear DNA hybridized with the gene of β-tubulin from *Polytomella agilis* (Conner *et al.*, 1989). The *cox2a* and *cox2b* genes of *Polytomella* spp. 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* **(Figure 3A, right panel).** The smaller band hybridized with the *cox1* mitochondrial probe from *C. reinhardtii* (Gray and Boer, 1988). In contrast, nuclear DNA hybridized with the gene encoding cytochrome *c* of *C. reinhardtii* (Amati *et al.*, 1988), and the *cox2a* and *cox2b* genes of the same alga. We conclude that the *cox2a* and *cox2b* genes are nuclear encoded in both *Polytomella* spp. and *C. reinhardtii*.

To determine if the *cox2a* and *cox2b* genes were present as single copies in the genomes of *Polytomella* spp. and *C. reinhardtii*, additional southern blot analysis was performed. Total DNA of *Polytomella* spp. and of *C. reinhardtii* were digested with a battery of different restriction enzymes and transferred to nylon membranes. The southern blot membranes were probed with the radioactively labelled *cox2a* and *cox2b* PCR fragments of each alga. The results obtained are shown in **Figure 3. Panel B**. Single hybridization bands were obtained for the *cox2a and the cox2b* genes of *Polytomella* spp. and of *C. reinhardtii*. This suggested that both

genes were present as single copy genes in these chlamydomonad algae.

To examine the expression of the *cox2a* and *cox2b* genes of *Polytomella* spp. and *C. reinhardtii*, total RNA was extracted from both algae and subjected to northern hybridization. The *cox2a* and *cox2b* genes of both algae generated transcripts of the size predicted by the corresponding cDNA sequences (**Figure 3, Panel C**). It was not possible to detect any trans-spliced mRNA produced by the processing of *cox2a* and *cox2b* messengers by northern blot analysis or by RT-PCR (results not shown). The *cox2b* gene of *Polytomella* spp. exhibited a double band. It is likely that those bands correspond to two mRNAs populations with polyadenine chains located in two different positions. In fact, the genomic sequence of this *cox2b* gene exhibited three possible putative polyadenylation sites in the 3' non-coding region.

An additional evidence for the nuclear localization and expression of the *cox2a* and *cox2b* genes is deduced from their patterns of codon utilization, which are typical of nuclear genes from these algae. There is a significant bias in each codon family; this is because triplets that end in A are scarcely present in these genera of algae (Conner *et al.*, 1989). In addition, the conserved polyadenylation signals TGTAA (Silflow, 1998), present in the vast majority of nuclear genes in the chlamydomonad family, were found towards the 3' end of the cDNA sequences of *cox2a* and *cox2b*.

Primary structures of COX IIA and COX IIB polypeptides encoded by the cox2a and cox2b genes of Polytomella spp. and C. reinhardtii.-

Translation of the DNA sequences of the *cox2a* genes from both algae predict a mature COX IIA protein of 141 residues (16,223 Da for *Polytomella* spp. and 16,543 Da for *C. reinhardtii*). The alignment of these two predicted amino acid sequences revealed an identity of 72% and a similarity of 81% (**Figure 4**). Both sequences also exhibit highest similarity with the N-terminal portion of the COX-II polypeptide encoded by the mitochondrial *cox2* gene from the chlorophyte alga *Prototheca wickerhamii* (Wolff *et al.*, 1994) (see **Figure 4**). The 20 residue-stretches located at the C-termini of Ps-COX IIA and Cr-COX IIA, highly enriched in charged residues, exhibited high similarity between them, but no similarity with COX II sequences from *P. wickerhamii*, *Vigna unguiculata* or from other organisms. Therefore, this highly-charged C-terminal extension of 20 residues in these COX IIA proteins seems to be unique to chlamydomonad algae.

Both *cox2b* genes have open reading frames that predict COX IIB proteins of 153 residues for both algae (17,259 Da for *Polytomella* spp. and 17,250 Da for *C. reinhardtii*). The alignment of these two predicted amino acid sequences revealed an identity of 85% and a similarity of 92%. Both sequences exhibit highest similarity with the C-terminal fragment of COX II encoded by the mitochondrial *cox2* gene from the chlorophyte alga *P. wickerhamii* (Wolff *et al.*, 1994) (**Figure 4**). The predicted Ps-COX IIB and Cr-COX IIB contain the eight invariant residues G(Q/C)SE(I/L)CG that are known to be the ligands of the binuclear copper center (Tsukihara *et al.*, 1995). In contrast, the first 43 residues of the predicted proteins, highly enriched in charged residues, exhibited high similarity between the two chlamydomonad sequences, but no similarity with COX II sequences from other organisms. This highly-charged N-terminal extension of 43 residues in Ps-COX IIB and Cr-COX IIB seems also to be unique to *Polytomella* spp. *and C. reinhardtii*, nevertheless, it doesn't seem to conform to a conventional MTS (see below).

We had previously obtained protein sequence data from the region of a polyacrylamide gel corresponding to a stained protein with a molecular weight of approximately 18.6 kDa. This sequence corresponded to the predicted amino acid sequences from both *cox2a* and *cox2b* genes from *Polytomella* spp. Given that the predicted molecular weights of the mature COX IIA subunit is 16,223 Da and that of the full length COX IIB is 17,219 Da, it is likely that the band we analysed previously contained both subunits. Due to the presence of new sequences in COX IIA and COX IIB that are not present in other COX II subunits from other organisms, it was necessary to ascertain which sequences predicted from both *cox2a* and *cox2b* genes were present in the mature COX IIA and COX IIB proteins. The 18.6 kDa band was purified from polyacrylamide gels and subjected to trypsin digestion and HPLC separation. The tryptic products were loaded onto HPLC columns, and several of the resulting fractions were subjected to amino terminal sequence analysis (see Materials and Methods). Sequences for both Ps-COX IIA and Ps-COX IIB were found, indicating the comigration of these two proteins in the 18.6 kDa band. The data obtained is summarized in **Figure 5**. The purified 18.6 kDa Ps-COX II polypeptide was also subjected to endolysin-C digestion and SDS-PAGE. The digestion pattern gave rise to 7 polypeptides, with apparent molecular masses of 16.0, 15.0, 13.2, 9.0, 6.0, 5.0 and 4.5 kDa (data not shown). All 7 polypeptides were subjected to Edman degradation. N-terminal sequences could be obtained for all the endolysin C-derived fragments, except for the 16.0 kDa polypetide, which exhibited a blocked N-terminus. Several overlapping sequences were obtained for the tryptic products and for the endolysin-C fragments of the protein. The overall data is summarized in **Figure 5**. A total of 154 residues out of the 294 residues predicted by the *cox2a* and *cox2b* genes were identified in the 18.6 kDa Ps-COX II polypeptide by direct amino acid sequence analysis. No difference was found between the predicted sequences and the sequences obtained by Edmann degradation. We therefore propose that the *cox2a* and *cox2b* genes encode for two different regions of the COX II subunit, COX IIA, a N-terminal part of 16,223 Da, and COX IIB, a C-terminal part of 17,219 Da. These two polypeptides are unresolved by SDS-PAGE, and run together as a band with an apparent molecular mass of 18.6 kDa in denaturing gels. In addition, the COX IIB polypeptide encoded by the *cox2b* gene must have a blocked N-terminus (MSADKDQL), since only the major N-terminal sequence EAPVAWQLGL of COX IIA was detected by direct amino terminal sequence analysis of the 18.6 kDa polypeptide transferred to PVDF membranes. The N-terminal sequence of the Ps-COX IIB was never detected, most probably due to the presence of a blocked N-terminus. Since the Ps-COX IIB sequence was obtained starting at amino acid Asp5 of one of the tryptic fragments, D-Q-L-K, this indicates that the Ps-COX IIB protein does not have a cleavable MTS.

To confirm that Ps-COX II is constituted by two independent polypeptides, the purified cytochrome *c* oxidase complex of *Polytomella* spp. was subjected to HPLC separation. A 17 kDa polypeptide was selected and subjected to trypsinization and mass spectrometry analysis. The trypsinized fragments exhibited molecular masses that are almost identical to the theoretical molecular masses expected for Ps-COX IIB, as shown in **Table 1**.

The overall data predict two polypeptides of 141 and 153 residues, which would assemble together. The normal COX II subunit exhibits two transmembrane stretches, and a hydrophylic region with the consensus copper binding site. Hydropathy profile analysis, carried out with the TopPred II algorithm and with different scales, predicted two classical transmembrane stretches for the

COX IIA polypeptides from both algae (data not shown). This suggests a structure similar to the one determined by X-ray crystallography of the bacterial and mammalian COX II subunits (Iwata *et al.*, 1995; Tsukihara *et al.*, 1996). We hypothesize that the unique carboxy-terminal extension of COX IIA may interact with the unique N-terminal extension of the COX IIB protein. This interaction may give rise to the proper assembly of the two COX II subunit regions in the mitochondrial inner membrane. A model based on this hypothesis is shown in **Figure 6**. According to this model, the extra loop formed by the interaction of the N-terminal and C-terminal extensions, not described before for any subunit II of other eukaryotic or prokaryotic organisms, is topologically distant from the site of interaction of soluble cytochrome c with the COX II subunit, and is therefore thought not to interfere with the activity of the cytochrome c oxidase complex.

Importability characteristics of nuclear-encoded subunit II of cytochrome c oxidase from chlamydomonad algae - The predicted Ps-COX IIA, Ps-COX IIB, Cr-COX IIA, and Cr-COX IIB proteins were tested for their hydrophobic characteristics and for their possible importability into mitochondria. Both Ps-Cox IIA and Cr-COX IIA proteins, containing two putative transmembrane stretches, exhibited mesohydrophobicity patterns similar to other COX II polypeptides from other organisms that are synthesized in mitochondria (data not shown).

Ps-COX IIB and Cr-COX IIB, that contain no transmembrane stretch, exhibited very low <H> and *meso*H values, and were predicted to be imported readily into mitochondria (data not shown). Nevertheless, the existence of numerous negatively charged amino acids did not allow a clear prediction of a mitochondrial targeting sequence. This is because they do not follow the main characteristics of MTSs: the paucity of acidic residues and abundance of basic residues (Claros et al., 1997). However, from position 19 to 44, the N-terminal region of Ps-COX IIB posseses a positively charged amphiphilic alpha-helix with a high hydrophobic moment (μH from 5.98 to 7.42) and a very hydrophobic face (from 3.55 to 6.35). In a similar way, the N-terminal region of Cr-COX IIB posseses a putative amphiphilic helix from residues 14 to 32 with μH ranging from 7.93 to 8.92 and a hydrophobic face (from 4.70 to 6.44). The amphiphilicity is essential for the function of these sequences as MTS (Roise *et al.*, 1988).

DISCUSSION.

Cytochrome c oxidase subunit II is present as two proteins in the isolated complex from Polytomella spp. - The existence of COX II in the cytochrome c oxidase complex from *Polytomella* spp. was evidenced by N-terminal and internal sequencing of a region of a polyacrylamide gel corresponding to a mass of 18.6 kDa. This is a notably lower apparent molecular mass as judged by SDS-PAGE as compared to 29-30 kDa in yeast and mammalian enzymes. We showed that the function of COX II in this organism is taken by two proteins Ps-COX IIA and Ps-COX IIB of similar apparent molecular masses in SDS-PAGE. COX II is conserved in cytochrome c oxidases throughout the phylogenetic scale; along with COX I and COX III it constitutes the main structural core of the complex. Cytochrome c oxidase from *C. reinhardtii* has been purified and partially characterized (Atteia,1994; Bennoun *et al.*, 1995). In those works, a 14.5 kDa band in this preparation was identified immunochemically as COX II, and N-terminal sequence was obtained. The large differences reported for COX II subunits (between 14.5 and 18.6 kDa) exemplify the differences in mobility of these subunits in different SDS-PAGE systems. In addition, the data suggest that the minimum unit of cytochrome c oxidase, i.e., the complex formed by COX I, COX II, and COX III, is conserved

all along the phylogenetic scale (Saraste, 1990), including those organisms that do not contain the *cox2* and *cox3* genes in their mtDNAs.

COX II is nuclear encoded in the algae of the family Chlamydomonadaceae - This work describes the sequences of 4 new genes of the *cox2* family, belonging to two chlamydomonad algae. The genes *cox2a* and *cox2b* from *Polytomella* spp. and *C. reinhardtii* were found to be nuclear-localized, as evidenced by southern blot hybridization (**Figure 3**), a biased codon usage typical of nuclear-localized genes in chlamydomonad algae and the presence of a polyadenylation signal TGTAA usually found in the nuclear-localized genes of these algae (**Figure 2**). Several partial overlapping sequences of the cox2a and cox2b genes from *C. reinhardtii* reported in this work have been recently deposited in the Expressed Tagged Sequences data base (Asamizu *et al.*, 1999; Grossman, A., Davies, J., Federspiel, N., Harris, E., Lefebvre, P., Silflow,C., Stern, D., and Surzycki, R., unpublished results).

Other examples of functional gene transfer of *cox2* genes to the nucleus have been reported in several members of the leguminosae family (Adams, 1999). Other organisms whose mitochondrial genomes have been completely sequenced and lack the *cox2* gene, are the apicomplexan protozoan *Plasmodium falciparum* (Feagin *et al.*, 1992) and the chlorophyte alga *Pedinomonas minor* (Gray *et al.*, 1998). It is likely that these organisms may have transferred their *cox2* genes to nuclear control.

COX II is encoded by two distinct and separate genes in the algae of the family Chlamydomonadaceae - The COX II subunits of both *Polytomella* spp. and *C. reinhardtii* were found to be encoded by two distinct and separate genes: *cox2a* encodes the N-terminal part of the subunit, including two putative transmembrane stretches and the highly conserved aromatic region GRQWYWSY, and *cox2b* encodes the C-terminal part of the same polypeptide and the highly conserved GQCSE(I/L)CG signature that constitutes the consensus binding site of the binuclear copper center in COX II (Tsukihara *et al.*, 1995). The deduced primary sequences of Ps-COX IIA and Cr-COX IIA, and of Ps-COX IIB and Cr-COX IIB, were found to be homologous. No evidence for the presence of higher length mRNAs was found, that could suggest a transplicing mechanism giving rise to a larger-size mature mRNA that would be transcribed into a full-length COX II subunit. We conclude that the *cox2a* and *cox2b* genes encode separate regions of the chlamydomonad COX II. Therefore, we suggest that in these algae, the COX II subunit is encoded by two genes, synthesized as two independent polypeptides, imported into mitochondria, and assembled in the mitochondrial inner membrane. The COX IIA protein of chlamydomonad algae contains a 20 residue extension towards the carboxy-terminus that exhibits a high density of charged amino acids and that seems to be unique to these algae. The COX IIB polypeptide contains a 43 residue extension in the amino terminus that also exhibits a high density of charged amino acids and that is also unique to these algae. We hypothesize that these two extensions are necessary to maintain an interaction between the COX IIA and the COX IIB polypeptides in the inner mitochondrial membrane.

The presence of a gene divided into two independently transcribed reading frames, has also been described for the mitochondrial *nad1* genes of *Tetrahymena pyriformis* and *Paramecium aurelia* (Edqvist *et al.*, 2000), and for the rapeseed mitochondrial gene encoding a homologue of the bacterial protein Ccl1 (Handa et al., 1996). This data has led us to hypothesize that two independent *cox2a* and *cox2b* genes already existed in the mitochondrial genome of the common ancestor of chlamydomonad algae, and that these genes

were transferred from the mitochondria to the nucleus before the separation of the colorless genera of *Polytomella* from the main photosynthetic lineage of *Chlamydomonas*. Recently, the mitochondrial genome of the chlorophyceaan alga *Scenedesmus obliquus* was completely sequenced (Nedelcu et al., 2000). The gene content and the fragmentation pattern of the ribosomal RNA genes suggested that this genome represents an intermediate stage between the *Prototheca*-like green algae and the *Chlamydomonas*-like green algae. The genes *cox1*, *cox2* and *cox3*, encoding the three major cytochrome *c* oxidase subunits were found to be present in this genome. Nevertheless, the *cox2* gene was found to be truncated, and thought to represent a pseudogene. This truncated gene exhibits high similarity with the *cox2a* genes from *Polytomella* spp. and *C. reinhardtii*, and its predicted protein product also contains a unique C-terminal extension similar to the ones described in this work. Most likely, the mitochondrial *cox2* gene from *S. obliquus* may be an active gene, while the other fragment of the gene may have been transferred to the nucleus. This scenario may represent and intermediate stage of green algal evolution in which the *cox2b* gene (encoding for a highly hydrophyllic polypeptide) has been transferred to the nucleus, while the *cox2a* gene (encoding a more hydrophobic polypeptide) is retained in the mitochondrial genome.

The peptides coded by cox2a and cox2b have been adapted to be imported into mitochondria.- Since the *cox2a* and *cox2b* genes are nuclear-localized, they would be expected to encode MTSs that direct the import of the corresponding protein products into mitochondria. Putative presequences have been identified in the nuclear-encoded *cox2* genes from cowpea (Nugent and Palmer, 1991) and soybean (Covello and Gray, 1992). The open reading frame of the *cox2a* gene from *Polytomella* spp. predicts four ATG codons that could correspond to the initiation of the MTS. The upstream methionine predicts a presequence of 130 aminoacids, the second methionine predicts a 113 residues MTS, the third one predicts a 107 aminoacids MTS, and the downstream one predicts a presequence of 74 aminoacids. In addition, the 4 possible presequences end with the three residues ASA, which are consensus for the peptidases in the mitochondrial intermembrane space (Pérez-Martínez *et al.*, 2000). The four possible MTS were analyzed using the program MitoProt II. The 130 amino acid MTS renders a polypeptide that is not predicted to be mitochondrial [p(DEM)= 0.43], although the presence of an amphiphilic alpha-helix from residues 10 to 30 could explain its mitochondrial localization. When considering MTS's of 113 or 107 amino acids, the predicted protein cannot be considered mitocondrial at all [p(DEM)= 0.09 for both proteins]. However, if the fourth Met is considered the first translated aminoacid, the protein is considered clearly mitochondrial [p(DEM)= 0.76] and predicts a typical MTS with two amphiphilic alpha-helices, one from residues 6 to 24, and another from residues 19 to 24. In addition, a typical cleavage site is predicted between positions 33 and 34. In such a situation, the hypothetical hydrophobic domain ranging from 54 to 74 residues can be considered a signal peptide that redirects the protein from the mitochondrial matrix to the mitochondrial inner membrane. This suggests that the predicted Ps-COX IIA protein may contain a bipartite presequence. The predicted presequences of the Ps-COX IIA and Cr-COX IIA show some similarities with the MTSs of other nuclear-encoded mitochondrial subunits from *C. reinhardtii* (Perez-Martinez *et al.*, 2000). The MTSs from chlamydomonad algae are rich in alanines, prolines, and charged amino acids. In contrast, the *cox2b* genes do not seem to encode a canonical MTS, nevertheless, they encode short stretches that exhibit a high density of positive and negative charged residues.

The first 43 residues in the predicted Ps-COX IIB and Cr-COX IIB encoded by the *cox2b* genes, although exhibiting high similarity between them, show no similarity with other known COX II sequences; therefore they seem to be unique to the chlamydomonad algae. Although the overall amino acid composition is not appropriate for a clear prediction of a MTS, it is possible that the 43 stretch may function as a non-cleavable MTS, which remains as an integral part of the mature COX II subunit after import and assembly, as was shown from direct sequence of triptic fragments of the Ps-COXII. We hypothesize that the COX IIB polypeptides may be imported directly into the mitochondrial intermembrane space, as described for soluble cytochrome *c*.

The present work suggests a novel strategy utilized by chlamydomonad algae that allowed functional transfer of a mitochondrial genes to the nucleus. It involves the division of the original gene into two new and separate genes that encode two independent proteins. The low hydrophobicity parameters exhibited by the protein encoded by the *cox2b* gene may greatly facilitate their import into mitochondria. In addition, the presence of unique and highly-charged sequences in the C-terminus of the polypeptide encoded by the *cox2a* gene and in the N-terminus of the polypeptide encoded by the *cox2b* gene, supports the hypothesis of an interaction of these two heterodimeric proteins in the inner mitochondrial membrane that give rise to a fully-functional COX II subunit.

MATERIALS AND METHODS

Strain and culture conditions - *Polytomella* spp. (198.80, E.G. Pringsheim) from the Sammlung von Algenkulturen (Gottingen, Germany), was grown as previously described (Gutiérrez-Cirlos *et al.*, 1994).

Purification of cytochrome c oxidase and protein determination.-Cytochrome *c* oxidase from *Polytomella* spp. was obtained as previously described (Pérez-Martínez *et al.*, 2000). Protein was determined as described by Markwell *et al* , (1978).

Polyacrylamide gel electrophoresis.- Polyacrylamide gel electrophoresis was performed as in Schägger *et al.* (1986), 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 apparent molecular masses of bovine cytochrome *c* oxidase (Capaldi *et al.*, 1988). For tryptic digestion analysis, gels were stained with Amido Black and the polypeptide of interest was excised from the gel.

Sequencing of subunits by Edman degradation - The isolation of polypeptides for N-terminal sequencing was done as previously described (Gutiérrez-Cirlos *et al.*, 1994). N-terminal sequencing was carried out on an Applied Biosystems Sequencer at the Laboratoire de Microséquençage des Protéines, Institut Pasteur, Paris (France).

Trypsinolysis and sequence analysis of polypeptides - A 18.6 kDa polypeptide was purified from polyacrylamide gels and subjected to tryptic digestion. The tryptic products were loaded into two different HPLC columns: DEAE-C14 and DEAE-C18. From the DEAE-C18 HPLC column 32 main peaks were resolved. Peaks 4, 7, 9, 10, 11, 13, 14, 17, 21, 22, 23, 24, 29, 31 and 32 (numbered progressively in the order of elution) were subjected to amino terminal sequence analysis. From the DEAE-C14 HPLC column 18 peaks were resolved. Two tryptic fragments, peaks 13 and 14, were selected for N-terminal sequence analysis.

Nucleic acids preparation - Total DNA and total RNA from *Polytomella* spp. and *C.*

reinhardtii were obtained as previously described (Pérez-Martínez *et al.*, 2000). Sequencing was carried out by the Kimmel Cancer Center DNA Sequencing Facility, Thomas Jefferson University and at the Unidad de Biología Molecular, Instituto de Fisiología Celular, UNAM, Mexico.

Cloning and sequencing of the cox2b gene from Polytomella spp. - Genomic *Polytomella* spp. *cox2b* fragment was amplified by PCR using two degenerate deoxyoligonucleotides. The first one was based on the internal sequence of the protein QDSATSQAQA (F1): 5'- CA(A+G) GA(C+T) AG(C+T) GC(C+T) AC(A+T) AG(C+T) CA(G+A) GC(C+T) CA(A+G) G -3'. The second one was based on the internal sequence of the protein MDAIPGRENQ (B1): 5'-TG (G+A)TT (C+T)AA (A+G)CG (A+T)CC (A+T)GG (A+G)AT (A+G)GC (A+G)TC CAT-3'. For PCR amplification, total DNA was denatured for 5 min at 94 °C, and subjected to 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C, and 1 min extension at 72°C. The resulting 300 bp product containing the *cox2b* gene was cloned in pMOS blue-t (Amersham). Southern blot analysis with total DNA from *Polytomella* spp. digested with *Pst*I was carried out and a 2 kb band was identified after probing with the 300 bp fragment. *Polytomella* DNA was digested with Pst I and, after agarose gel electrophoresis, fragments of approximately 2kb were purified and cloned into pBluescript vector. This sized library was probed with the 300 bp fragment to identify clones containing the *cox2b* gene. All standard molecular biology techniques were as described (Sambrook et al., 1989).

Cloning and sequencing of the cox2b cDNA from Polytomella spp. - The *cox2b* cDNA sequence from *Polytomella* spp was obtained with 5' RACE-PCR (Frohman, 1993) using primers based on the genomic sequence obtained above. First strand cDNA templates were prepared from 1-2 µg of total RNA with M-MLV reverse transcriptase from Promega. A poly dT tail was added to the 5' end of the cDNA with a terminal transferase from Boehringer. Forward primer was oligo dT-adapter: 5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT-3', and reverse primer (B2) was 5'-AGC TGT TTA AGA CCA TGA CTT C-3'. A 550 nt PCR product was obtained and cloned into pGEM-T easy (Promega).

Cloning and sequencing of cox2a cDNA and genomic sequences of Polytomella spp. - The cDNA sequence of the *Polytomella* spp. *cox2a* gene was obtained by PCR amplification. First strand cDNA templates were prepared from 1-2 µg of total RNA with superscript II reverse transcriptase from Gibco BRL and by using oligo dT-adapter as the first strand cDNA primer. A *cox2a* fragment was amplified using two degenerate deoxyoligonucleotides. The first one was based on the amino terminal sequence of the protein EAPVAWQLG (F2): 5'-GA(G+A) GC(T+C) C(G+T+C) GT(T+C) GC(T+C) TGG CAG C(T+C+G) GG-3'. The second one was based on the internal conserved sequence KAGHQWYW (B3): 5'-CCA (A+G)TA CCA C(T+C) (A+G)TG (A+G+T)CC (A+G)AT (A+G)GC C-3'. Nested PCR was done with degenerate primers based on the amino terminal sequence of the protein QDSATSQAQA (F3): 5'-CAG GA(T+C) TCT(T+C) GC(T+C) ACT(T+C) TCT(T+C) CAG GCT(T+C) CAG G-3', and on the internal conserved sequence PSEALLYS (B4): 5'-GA(A+G) TA(A+G) AG(A+G) AG(A+G) GC(A+G) AA(A+G) GA(A+G) GG-3'. For PCR amplification, samples were denatured for 5 min at 94°C, and subjected to 30 cycles of 45 sec denaturation at 94 °C, 1 min annealing at 50 °C, and 2 min extension at 72°C. A 250 nt product containing a fragment of the *cox2a* gene was obtained and cloned into pGEM-T easy (Promega). The complete cDNA sequence from the *Polytomella* spp. *cox2a* gene was obtained with 5'or 3' RACE-PCR (Frohman, 1993) using primers

based on the cDNA sequence obtained above. For 3' end cDNA cloning, oligo dT/adapter and primer F4: 5'-TCC TCT ACC ACA TCG CCA CC-3' were used. For nested PCR, adapter (5'-GAC TCG AGT CGA CAT CGA -3') and primer F5: 5'-ACT ACA CTA AGC AAG CTC TCC CTG-3' were used. A 600 nt PCR product containing the 3' end of the *cox2a* cDNA was obtained. For 5'-end cloning, a poly(A) tail was attached to 5' end with terminal transferase from Boehringer. For PCR amplification primers B5, 5'-TCA GGG AGA GCT TGC TTA GTG TAG-3' with oligo dT/adapter and B6, 5'-TTG GTG GCA ATG TGG TAG AGG-3' with adapter for nested PCR were used. A 750 nt PCR product was obtained. Both RACE products were cloned into pGEM-T easy (Promega). Primers F6, 5'-AAT GCT CGC CCA GCG TAT C-3' and B7, 5'-AAA CCT TCA CAC ACC CAT AGG C-3' were used to amplify the complete cDNA of the *cox2a* gene. For PCR amplification, samples were denatured for 5 min at 94°C, and subjected to 30 cycles of 45 sec denaturation at 94 °C, 1 min annealing at 50°C, and 2.5 min extension at 72 °C. A 850 PCR product was obtained.

The genomic sequence of the *Polytomella* spp. *cox2a* gene was obtained by PCR amplification with the same primers and conditions used above to obtain the complete *cox2a* cDNA gene. A 1.6 Kb PCR product was obtained. Both cDNA and genomic sequences of the complete *cox2a* gene from *Polytomella* spp. were cloned into pMOS blue-T (Amersham).

Cloning and sequencing of the cDNA of the cox2b gene of C. reinhardtii - The cDNA sequence from the *cox2b* gene was obtained by screening a *C. reinhardtii* cDNA library in λgt10 (Franzén and Falk, 1992). The *Polytomella spp.* 300 bp PCR product corresponding to a portion of the genomic *cox2b* gene was used as probe. Ten positive clones were obtained from 5×10^4 pfu 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.

A BAC genomic library from *C. reinhardtii* (Lefebvre and Silflow, 1999) was screened at Genome Systems using as probe the *C. reinhardtii cox2b* cDNA sequence obtained above. Three positives clones were obtained and plasmid DNA was used for direct sequencing of one of the clones. The primers used for direct sequencing were: 5'-GAT GGA CAT CCA GAC CTG GTT CAG G-3'; 5'-GTC CTT GAG CTC AGC ACG GAA CG-3'; 5'-CGA GCA GCA GCA GAG ACA CTT GG-3'; 5'-CTA CAT GCT CAC GGA GGT GCA GC-3'; 5'-CTT GCC TTC AGC ATT CTT CAT CGG-3'; and 5'-TCT GGA CAG GAG AGC AGC G GT C-3'.

Cloning and sequencing of cox2a cDNA and genomic sequences of C. reinhardtii - A *C. reinhardtii* cDNA library in λgt10 (Franzén and Falk, 1992) was screened using the *Polytomella spp.* 850 bp PCR product corresponding to the cDNA *cox2a* gene. Seven positive clones were obtained from 5×10^4 pfu 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 *cox2a* gene was subcloned into pBluescript. The 5' end of cDNA was completed by RACE PCR (the primers used were oligo dT/adapter and 5'-CAA TCA TGG CCT GAG CAG TGG -3'. For nested PCR the adapter primer and the primer 5'- TGG TGG TCA GGC CCA GAG C-3' were used.

Sequence analysis - Mitochondrial targeting sequences were analyzed and predicted using MitoProt II (Claros, 1995), including calculations of hydrophobic moment (μH). The same program was used to calculate the segments with high local hydrophobicity (<H>) in a distance comprising 13 to 17 residues, and the *mean*H was estimated by scanning each sequence for a maximum average hydrophobicity measured in windows from 60 to 80 residues and averaging the values (Claros and Vincens, 1996). More hydrophobicity scales were included to reduce the possibility of bias. Protein transmembrane stretches were predicted using the program TodPred II (Claros and von Heijne, 1994). Three dimensional structure modelling was carried out using SWISS-MODEL (Guex and Peitsch, 1997).

Data base accession numbers - The nucleotide sequences discussed in this paper will appear in the DDBJ/EMBL/GeneBank nucleotide sequence database under the accession numbers AF305078, AF305079, AF305080, AF305540, AF305541, AF305542, and AF305543.

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LEGENDS TO FIGURES

FIGURE 1. *N-terminal sequences of subunits of cytochrome c oxidase from Polytomella spp.*- N-terminal sequences of the 29.6 kDa, 18.6 kDa, and 14.5 kDa polypeptides. The minor internal sequence obtained for the 18.6 kDa polypeptide is also indicated.

FIGURE 2. *Gene organization of the cox2a and cox2b genes of chlamydomonad algae.* **A)** Overall structure of the *cox2a* cDNA from *Polytomella* spp. and *C. reinhardtii*. **B)** Overall structure of the *cox2b* cDNA from *Polytomella* spp. and *C. reinhardtii*. The introns found in the corresponding genomic sequence of each cDNA are shown as arrows. Putative polyadenylation signals are indicated as black bars, and putative MTS as striped boxes. Highly charged sequences unique to the chlamydomonad *cox2* genes are indicated as dotted bars. The highly conserved regions GRQWYWSY for *cox2a* and GQCSELCGS for *cox2b* are shown.

FIGURE 3. *The cox2a and cox2b genes are single copy, nuclear-localized, and functionally expressed in chlamydomonad algae.* **Panel A.** 30 µg of total DNA from *Polytomella* spp. and *C. reinhardtii* were run in a 0.7% agarose gel. DNA blot analysis of the same gel transferred to a nylon membrane and hybridized with different nuclear and mitochondrial probes indicated (see text). **Panel B.** total DNA from *Polytomella* spp. and from *C. reinhardtii* were digested with restriction enzymes, and DNA blot analysis was carried out with the radioactive probes *cox2a* and *cox2b*. The restriction enzymes used were: *Eco*RV (lane1) and *Pvu*II (lane 2) for *Polytomella cox2a*; *Eco*RV (lane1) and *Eco*RI (lane 2) for *Polytomella cox2b*. *Kpn*I (lane1) and *Xba*I (lane2) for *C. reinhardtii cox2a*; *Kpn*I (lane 1) and *Hind*III (lane2) for *C. reinhardtii cox2b*. **Panel C.** northern blot analysis of the transcription products of the *cox2a* and *cox2b* genes from *Polytomella* spp. and *C. reinhardtii*.

FIGURE 4. *Sequence comparison of the COX IIA and COX IIB polypeptides of Polytomella spp. and C. reinhardtii with the COX II subunit of P. wickerhamii and V. unguiculata.* Numbers indicate the first and last residue for each fragment. Asterisks denote identical residues, and two dots indicate similar residues. Abbreviations used: *Cr* (*C. reinhardtii*), *Ps* (*Polytomella* spp.), *Pw* (*P. wickerhamii*), *Vu* (*V. unguiculata*). Putative transmembrane stretches are indicated as gray boxes. The middle black bar indicates the separation of the COX IIA protein predicted by the *cox2a* gene, from the COX IIB protein predicted by the *cox2b* gene. The black triangle indicates the mature protein N-terminal sequence determined for Ps-COXII. The highly conserved regions G(R/H)QWYWSY for *cox2a*, and GQCSELCGS for *cox2b* are underlined.

FIGURE 5. *Sequence analysis of the main tryptic fragments derived from the COX IIA and COX IIB polypeptides of Polytomella spp.* Boxed region: regions identified by N-terminal sequence analysis of the 18.6 kDa polypeptide of cytochrome c oxidase. Bold-italic characters: residues identified by N-terminal sequence analysis.

FIGURE 6. *Model suggesting a possible mode of interaction of COX IIA with COX IIB to conform the mature COX II subunit of chlamydomonad algae.* This model, designed on the bovine three dimensional structure of the COX II from the bovine enzyme using the *Polytomella* spp. sequence, shows a posible interaction between the two COX II fragments that constitute the mature COX II subunit in the inner mitochondrial membrane.

TABLE I. *Mass spectrometry analysis of the major trypsin fragments for the C-terminal poprtion of the COX II subunit.* Experimental values obtained are compared with the molecular masses for the trypsin fragments predicted from the *cox2b* gene.

SEQUENCE OF THE TRYPTIC PEPTIDE	THEORETICAL MOLECULAR MASS (Da)	EXPERIMENTAL MOLECULAR MASS (Da)
AFLTEYVK	970.12	970.448
LVLPTNTLVR	1125.37	1125.649
LNQIWLTINR	1270.48	1270.702
LLVTASDV.....AVPSLGIK	2106.47	2123.172
VPASQPIQ.....DVQPGQLR	2854.18	2854.383
EGVFYGQC.....VVEAISPR	3141.56	3140.432

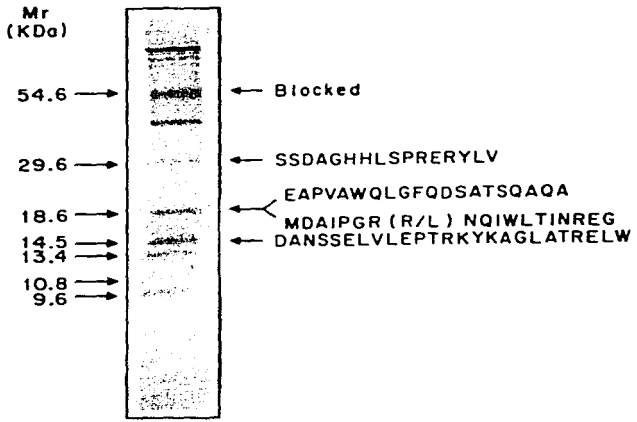
Mr
(KDa)
54.6
29.6
18.6
14.5
13.4
10.8
9.6
Blocked
SSDAGHHLSPRERYLV
EAPVAWQLGFQDSATSQAQA
MDAIPGR (R/L) NQIWLTINREG
DANSSELVLEPTRKYKAGLATRELW

Figure 1

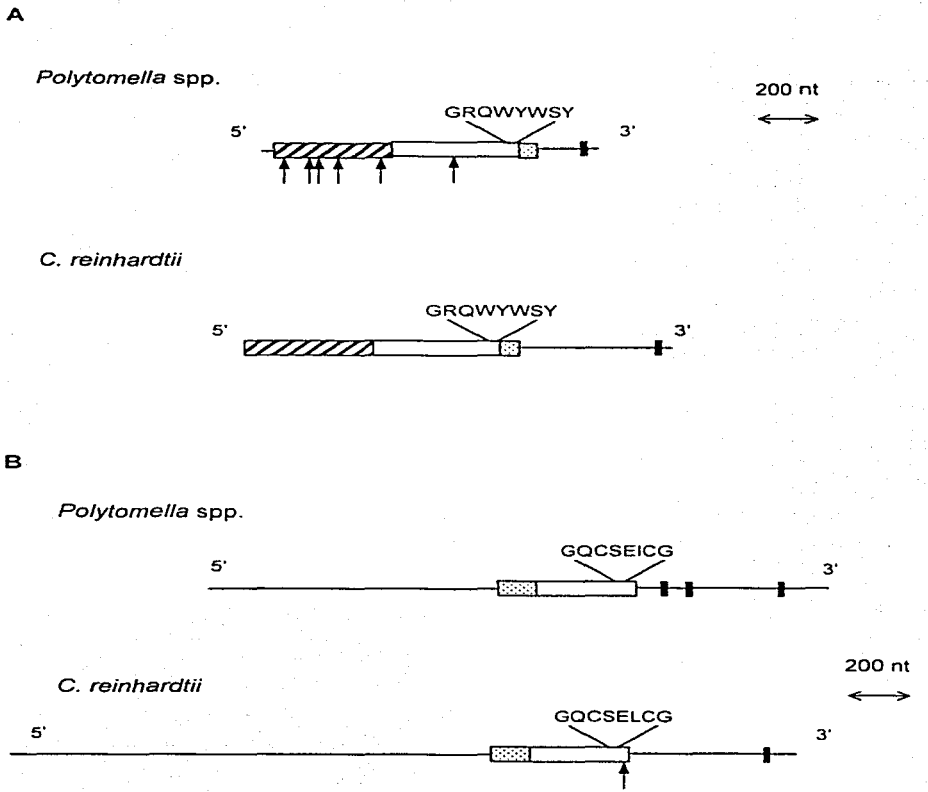
A
Polytomella spp.
200 nt
GRQWYWSY
5'
3'
C. reinhardtii
GRQWYWSY
5'
3'
B
Polytomella spp.
GQCSEICG
5'
3'
200 nt
C. reinhardtii
GQCSELCG
5'
3'

Figure 2

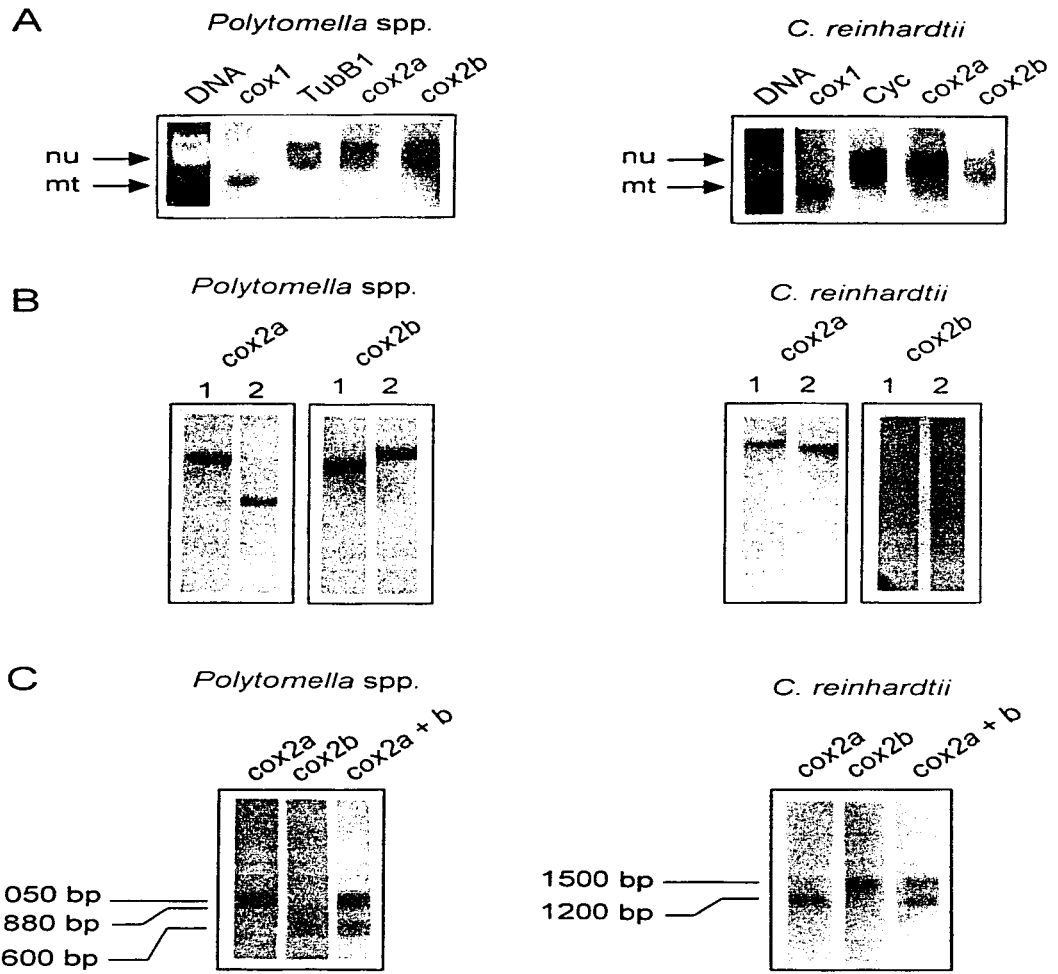
A
Polytomella spp.
DNA
cox1
TubB1
cox2a
cox2b
nu
mt
C. reinhardtii
DNA
cox1
Cyc
cox2a
cox2b
nu
mt
B
Polytomella spp.
cox2a
cox2b
1 2
1 2
C. reinhardtii
cox2a
cox2b
1 2
1 2
C
Polytomella spp.
cox2a
cox2b
cox2a + b
1050 bp
880 bp
600 bp
C. reinhardtii
cox2a
cox2b
cox2a + b
1500 bp
1200 bp

Figure 3

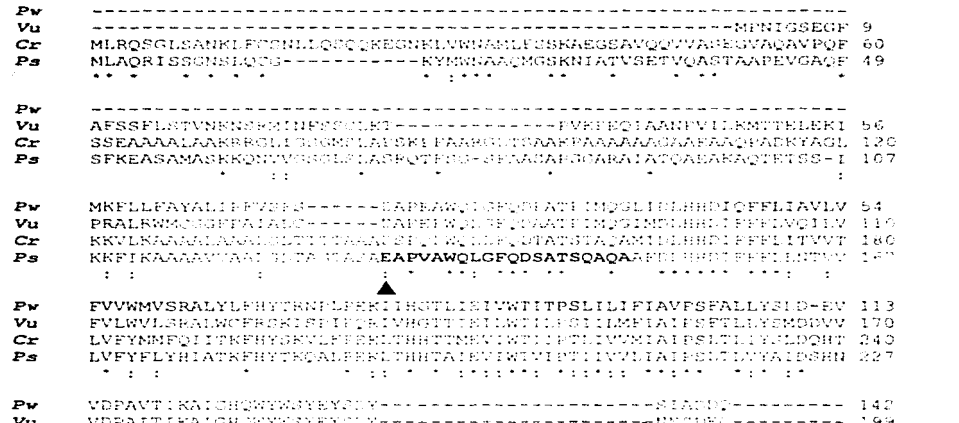

Figure 4

N-terminal region of the Ps-COX II subunit (encoded by the *cox2a* gene):

*EAPVAWQLGFQDSAT*SQAQAAFDLHHDIFFFLLNTVVL
VFYFLYHIATK↓*FHYTK*↓*QALPE*K↓LTHHTAIEVIWTVIPTII
VVLIAIPSLTLVYAIDSHNDK↓PGLTVK↓VIGR↓*QWYWSYE*
MHDHLQHK↓LLDADR↓LVAIAEK↓TITK

C-terminal region of the Ps-COX II subunit (encoded by the *cox2b* gene):

MSADK↓*DQLK*↓*EQLK*↓*ASPSFR*↓AELK↓DK↓LK↓*AALLSK*↓*VPA*
SQPIQYNFDSYMVTDVQPGQLR↓*MLEVDER*↓*LVLPTNTL*
VR↓*LLVTASDVIHSW*AVPSLGIK↓*MDAIPGR*↓*LNQIWLTIN*
R↓*EG VFYGQ*CSEICGANHSFMPIVVEAISPR↓*AFLTEYV*
K↓*K*↓*WIQ*

Figure 5

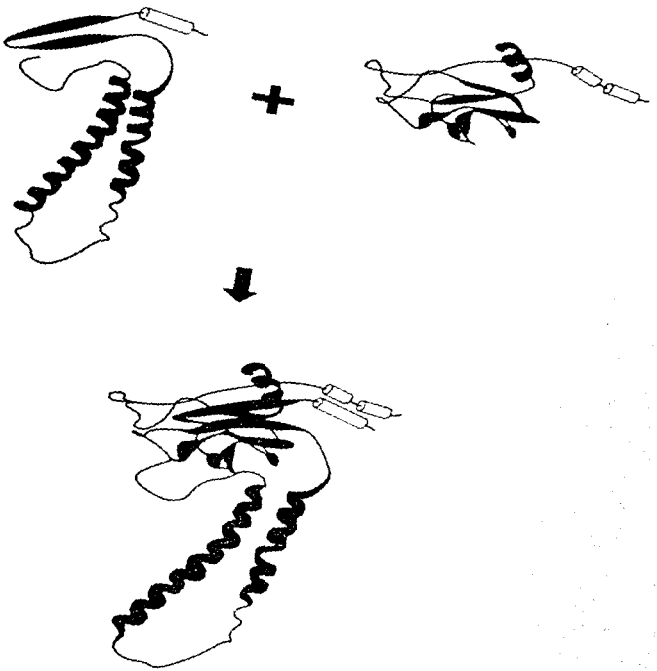

Figure 6

DISCUSIÓN

1. Las subunidades II y III de la citocromo *c* oxidasa de *Polytomella* spp. están presentes en el complejo enzimático.

En este trabajo se obtuvo una preparación de citocromo *c* oxidasa del alga incolora *Polytomella* spp. Esta enzima tiene un espectro UV-visible característico de una citocromo *c* oxidasa tipo aa_3. Presenta tanto actividad de TMPD-ascorbato oxidasa como de citocromo *c* oxidasa, y esta actividad es abatida totalmente por los inhibidores clásicos cianuro de potasio y azida de sodio. En esta preparación es posible identificar al menos 7 subunidades como constituyentes de la citocromo *c* oxidasa con masas moleculares aparentes de 54.6, 29.6, 18.6, 14.5, 13.4, 10.8 y 9.6 kDa. Adicionalmente se obtuvieron dos bandas de 80.0 y 41.8 kDa que se consideraron contaminantes de la preparación, ya que están presentes en todas las fracciones obtenidas de la columna de intercambio iónico. Estas bandas no se estudiaron más. La banda de 54.6 kDa corresponde a la masa molecular de la subunidad I deducida de la secuencia del gen (Antaramián y col., 1995), y en geles de poliacrilamida tiene una migración similar a la que presenta la subunidad I de bovino.

La subunidad II se identificó como una banda que migra con una masa molecular de 18.6 KDa cuando se corre en geles desnaturalizantes de poliacrilamida. Este tamaño es mucho menor al esperado para otras subunidades II (29-30 kDa). La identidad de esta banda se confirmó mediante la obtención de la secuencia de su extremo amino terminal. Así mismo, fue posible obtener la secuencia de aminoácidos de un fragmento interno de la proteína. Se sugiere que la menor masa molecular observada para esta banda se debe a que en realidad son dos las proteínas que conforman a la subunidad II: COXIIa y COXIIb. De la secuencia deducida de ambas proteínas a partir de los genes que las codifican, se espera que la masa molecular de COXIIa sea de 16,241 Da, y la de COXIIb sea de 16, 756 Da. Esto sugiere que la resolución en los geles desnaturalizantes de poliacrilamida no es suficiente, y que ambas proteínas migran como una sola banda.

Por otro lado, la subunidad III de la citocromo *c* oxidasa de *Polytomella* spp. se identificó como una proteína de 29.6 kDa a través de una reacción cruzada con un anticuerpo monoclonal contra COXIII de *Saccharomyces cerevisiae*. En este caso, la masa molecular estimada corresponde a lo observado para otras COXIII. A continuación se obtuvo la secuencia del extremo amino terminal de la proteína. Esta secuencia no mostró similitud con ninguna otra COXIII cuando se comparó en una base de datos.

2. Los genes que codifican para las subunidades II y III de la citocromo *c* oxidasa se transfirieron al núcleo en *Polytomella* spp. y *C. reinhardtii*.

Muchas de las proteínas de la mitocondria provienen del ancestro endosimbionte bacteriano, pero ahora los genes que las codifican residen en los cromosomas nucleares y no en el DNA mitocondrial. La transferencia de genes mitocondriales al núcleo tiene una velocidad diferente entre las familias de eucariotes. En el protista *Reclinomas americana* aún permanecen 97 genes codificados en el DNA mitocondrial (Land y col., 1997), mientras que en *Plasmodium falciparum* sólo permanecen 5 (Feagin y col., 1992). En algunos miembros de las algas Chlamydomonadaceas se ha caracterizado su genoma mitocondrial (Michaelis y col., 1990; Denovan-Wright y col., 1998; Kroymann y Zetsche, 1998). Una característica que presentan en común es el pequeño tamaño de sus genomas, entre 16 y 23 kb. Estos genomas codifican para solo 8 proteínas de la cadena respiratoria. Los genes que codifican para las subunidades II y III de la citocromo *c* oxidasa son algunos de los genes que no están presentes en el DNA mitocondrial, mientras que en la mayoría de los organismos estudiados a la fecha, estos genes permanecen en el genoma mitocondrial.

En este trabajo se ha demostrado que las proteínas COXII y COXIII se encuentran presentes en la citocromo *c* oxidasa de un miembro de la familia de las Chlamydomonadaceas, *Polytomella* spp. También se demostró que tanto en este organismo como en *C. reinhardtii* los genes que las codifican han sido transferidos al genoma nuclear. Algunas de las características que indican esta localización son:

(i) Uso de codones nuclear, no mitocondrial.
(ii) Adquisición de posibles presecuencias que guían a las proteínas de regreso a la mitocondria.
(iii) Adquisición de señales de poliadenilación "TGTAA", presentes en la gran mayoría de los genes nucleares de las algas Chlamydomonadaceas (Silflow, 1998).
(iv) Presencia de intrones de tipo nuclear.
(v) Es posible en *Polytomella* spp. o *C. reinhardtii* separar electroforéticamente al DNA mitocondrial del nuclear en geles de agarosa. Cuando se hace un análisis tipo southern de estos geles, los genes que codifican para las subunidades II y III de la citocromo *c* oxidasa dan señal exclusivamente con el DNA nuclear.

El gen que codifica para COXIII en *Polytomella* spp. y *C. reinhardtii* predice una proteína madura de 272 residuos, con 7 cruces transmembranales y una estructura similar a la observada para la COXIII de bovino (Tsukihara, 1996) como se muestra en la **figura 2.**

El gen *cox3* predice la existencia de una presecuencia de importación de proteínas a la mitocondria. Adicionalmente, predice una proteína cuyas características hidrofóbicas han disminuido cuando se comparan con otras COXIII codificadas en el DNA mitocondrial. Esta característica, así como la adquisición de una posible presecuencia, sugiere que son modificaciones que los genes sufrieron para poder transferirse exitosamente al núcleo, y para que las proteínas sintetizadas en el citosol pudieran importarse e integrarse correctamente en la membrana interna mitocondrial.

Por otro lado, se encontró que la subunidad II de *Polytomella* spp. y *Chlamydomonas reinhardtii* está codificada por dos genes nucleares. Nuestros resultados sugieren que estos genes se transcriben por separado y se traducen en dos proteínas diferentes. El gen *cox2a* codifica para el extremo amino terminal, los dos cruces transmembranales y la región aromática conservada entre las COXII. Adicionalmente, este gen predice una posible presecuencia para dirigir a la proteína hacia la mitocondria. También predice una pequeña extensión de 20 aminoácidos en el extremo carboxilo terminal. Esta es una región que no se encuentra en otras COXII y que presenta una gran cantidad de residuos ácidos y básicos. Por otra parte, el gen *cox2b* codifica para la región hidrofílica del extremo carboxilo terminal, así como para los residuos que unen al Cu_A. Este gen no predice una presecuencia característica, pero predice una extensión muy polar de 43 residuos en el extremo amino terminal. Es una región que no se ha encontrado en ninguna otra COXII reportada a la fecha, y que no se procesa como una presecuencia, sino que permanece en la proteína madura. La subunidad II puede estar formada por dos proteínas, en que COXIIa se localiza en la membrana interna mitocondrial, mientras que COXIIb se localiza en el espacio intermembranal. Ambas proteínas podrían tener una interacción mediante las extensiones de aminoácidos predichas por cada gen.

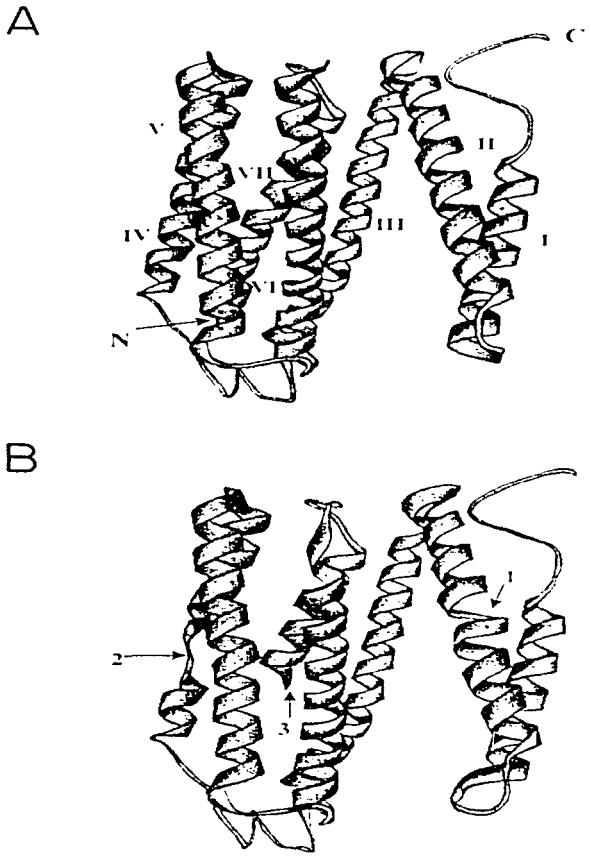

Figura 2. Modelo de la estructura de la subunidad III de la citocromo *c* oxidasa de *Polytomella* spp. (B) comparada con la estructura cristalina de la subunidad III de la citocromo *c* oxidasa de bovino (A). Los cruces transmembranales de la COXIII de *Polytomella* spp. que presentan diferencias importantes con bovino se indican con números. Se empleó el programa Swiss-Model (Guex y Peitsch, 1997) para elaborar este modelo.

La transferencia exitosa de genes mitocondriales al núcleo es un proceso activo que requiere de diversas etapas que se describen a continuación (Brennicke y col., 1993):

Salida de material genético de la mitocondria.

La información genética contenida en la mitocondria puede transferirse en la forma de DNA o RNA. Cuando se identifican en el DNA transferido regiones intergénicas, intrones de tipo mitocondrial o regiones no transcritas, se asume que el intermediario de la transferencia fue DNA (Sun y Callis, 1993; Blanchard y Schmidt, 1995; Blanchard y Schmidt, 1996). Por otro lado, en ciertos casos, se sugiere que la transferencia se llevó a cabo con un intermediario de RNA, cuando el gen nuclear se parece más al RNA mitocondrial editado que a la copia del gen en la mitocondria (Grohmann y col., 1992; Covello y Gray, 1992). En este caso, es necesaria una transcripción reversa que podría llevarse a cabo dentro de la mitocondria o en el citosol. El tamaño de los fragmentos transferidos al núcleo va desde 31 bp (Blanchard y Schmidt, 1996) hasta 270 Kb. Este último se identificó en el cromosoma 2 de *Arabidopsis thaliana*, y representa el 73% de su genoma mitocondrial (Lin y col., 1999; Unseld y col., 1997). No se conocen los mecanismos de escape del RNA o DNA de las mitocondrias, sin embargo, estudios en levadura indican que la frecuencia de escape de DNA depende de las condiciones de crecimiento de las células como temperatura y fuentes de carbono, que pueden afectar la integridad y fluidez de las membranas mitocondriales (Thorsness y Fox, 1990; Shafer y col., 1999). Se han identificado proteínas localizadas en la membrana interna mitocondrial cuya disfunción estimula el escape de DNA de la mitocondria, probablemente al afectar la función e integridad de ésta (Thorsness y Fox, 1993; Campbell y Thorsness, 1998). Un ejemplo es la proteína mitocondrial de 82 kDa Yme1p. Ésta presenta similitud con la proteína FtsH de *Escherichia coli*, la cual participa en la formación del septum durante la división celular bacteriana (Thorness, PE y col., 1993). Esta similitud sugiere que Yme1p podría participar en la fusión y/o en la división mitocondrial.

En el caso de los genes *cox2a*, *cox2b* y *cox3* de *Polytomella* spp. y *C. reinhardtii*, no es posible precisar si fue DNA o RNA el intermediario de la transferencia. No se ha demostrado que el gen mitocondrial que codifica para una posible transcriptasa reversa que únicamente está presente en *C. reinhardtii* sea activo. Esto sugiere que probablemente no participó en la transferencia de estos genes en el ancestro común de las *Chlamydomonas* y *Polytomella* spp. En *Chlamydomonas* no se ha demostrado la existencia de edición mitocondrial de RNA observado comúnmente en plantas , en que se cambia una C por U (Steinhauser y col., 1999). Por otro lado, la fragmentación del gen *cox2* en estas algas pudo haberse llevado a cabo en el DNA de la mitocondria, previamente a su transferencia al núcleo.

Esto se apoya en la siguiente evidencia: se encontró que el genoma mitocondrial del alga clorofita *Scendesmus obliquus* codifica únicamente para el gen *cox2a*, mientras que el gen *cox2b* está ausente (Nedelcu y col., 2000). La proteína predicha por *cox2a* de *S. obliquus* presenta un alto grado de conservación con COXIIa de *Polytomella* spp. y *C. reinhardtii*. Este gen también predice una extensión en el extremo carboxilo terminal, similar a la observada en las COXIIa de *Polytomella* spp. y *C. reinhardtii*, aunque es más pequeña y tiene menos residuos cargados. Estos datos sugieren que esta extensión proviene del gen mitocondrial original. Por otro lado, estos resultados indican que, muy probablemente, primero se transfirió el gen *cox2b* al núcleo y posteriormente se transfirió el gen *cox2a*. Es posible que el gen cox2b se transfiriera con mayor facilidad, ya que la proteína que codifica es altamente hidrofílica, sin ningún cruce transmembranal, mientras que *cox2a* codifica para una proteína hidrofóbica, con dos cruces transmembranales. La fragmentación de un gen dentro de la mitocondria, así como la transcripción independiente de cada fragmento, ha sido reportada previamente en otros organismos, adicionalmente a los genes *cox2a* y *cox2b* de estas algas. En colza se encontró que un análogo del gen bacteriano que codifica para la proteína Ccl1 se encuentra dividido en dos fragmentos separados por 45 Kb que se transcriben independientemente (Handa y col., 1996). Por otro lado, Edqvist y col. (2000) encontraron que la proteína nad1 de *Tetrahymena pyriformis* y *Paramecium aurelia* se encuentra codificada por dos genes que se transcriben independientemente, sin que se detecte un transcrito maduro mediante un procesamiento alternativo que incluya a ambos genes.

Integración del DNA en los cromosomas.

Una vez que el material genético ha salido de la mitocondria e ingresado al núcleo, éste puede ser integrado en algún cromosoma. Estas secuencias se localizan generalmente en regiones no codificantes, como regiones intergénicas, intrones y telómeros. Sun y Callis (1993) proponen que la integración de DNA mitocondrial en el núcleo se lleva a cabo mediante recombinación no homóloga, sin que haya complementariedad entre los extremos del DNA mitocondrial integrado y el cromosomal. Por otro lado, Ricchetti y col. (1999) proponen un mecanismo por el cual los fragmentos de DNA mitocondrial pueden transferirse a los cromosomas de levadura durante la reparación de rupturas de doble hebra mediante recombinación por complementariedad de bases muy limitada (1-4 nt). Esta observación también fue hecha por Blanchard y Schmidt (1996).

Activación del gen transferido.

A continuación es importante que el gen mitocondrial pueda adquirir las características necesarias para su correcta expresión y regulación en su nueva localización. Entre otras cosas, el gen integrado debe cambiar su uso de codones, adquirir regiones promotoras, señales de poliadenilación y sitios de unión a ribosomas.

Adicionalmente, la proteína debe adquirir tanto una presecuencia que la guíe de regreso a la mitocondria, así como las características polares necesarias para que pueda adquirir la conformación adecuada durante su translocación a través de las membranas mitocondriales.

La adquisición de presecuencias mitocondriales pudo ocurrir con la participación conjunta de varios mecanismos. **(i)** Las MTS pueden generarse mediante la acumulación de mutaciones puntuales en la región 5' no traducida del gen transferido, o incluso, en la región que codifica para el extremo amino terminal de la proteína madura. Vassarotti y col. (1987) demostraron que la subunidad β de la F$_1$-ATPasa de levadura puede restaurar su importación a la mitocondria mediante mutaciones puntuales en su extremo amino terminal cuando se ha eliminado artificialmente su presecuencia. **(ii)** El origen de las presecuencias pueden deberse al recambio de exones; se sugiere que en algunas ocasiones los intrones pueden funcionar como sitios de recombinación ilegítima, dando lugar a genes nuevos formados por exones provenientes de otros genes (Stoltzfus y col., 1994). Un ejemplo es el apocitocromo c_1 de papa, en el que se encontró que la parte de la presecuencia que dirige a la proteína a la mitocondria proviene de los tres primeros exones de la gliceraldehido 3 fosfato deshidrogenasa citosólica (Long y col., 1996). Otro ejemplo es la COXII de algunas leguminosas, en la que se observa que la presecuencia adquirida se separa de la proteína madura por un intrón (Nugent y Palmer, 1991; Covello y Gray, 1992). **(iii)** Las presecuencias mitocondriales pueden adquirirse por duplicación o utilización de presecuencias ya existentes. En arroz y maíz, los genes que codifican para la proteína ribosomal RPS14 y SDHB (subunidad B de la succinato deshidrogenasa) se encuentran en un mismo transcrito. Mediante procesamiento alternativo, cada gen usa la misma presecuencia mitocondrial (Kubo y col., 1999; Figueroa y col., 1999). Los autores proponen que primero se transfirió el gen *sdhB* al núcleo, y adquirió las características para poderse expresar, incluyendo una presecuencia y un intrón. Posteriormente el gen *rps14* se transfirió al núcleo y se insertó en el intrón del gen *sdhB*. Finalmente, el gen de *rps14* pudo ser reconocido como un exón durante el procesamiento alternativo. Por otro lado, Kadowaki y col. (1996) encontraron que el gen que codifica para la proteína ribosomal RPS11 de arroz adquirió una presecuencia muy similar a la de la βATPasa de plantas, indicando que ambas presecuencias tienen un origen común. Además, existe una segunda copia del gen *rps11* en el núcleo, pero esta copia inactiva adquirió una presecuencia muy similar a la de la subunidad Vb de la citocromo *c* oxidasa.

Las presecuencias predichas para las proteínas COXIIa y COXIII de *Polytomella* spp. y *C. reinhardtii* pudieron adquirirse de acuerdo al modelo del recambio de exones. Debido a que la frecuencia de mutaciones en los nuevos genes obtenidos mediante este mecanismo es muy alta (Long y Langley, 1993; Ohta, 1994), es difícil deducir la procedencia de los exones que conforman las presecuencias de COXIIa y COXIII.

Cuando se comparan las presecuencias de COXIIa de *Polytomella* spp. y *C. reinhardtii* contra la base de datos de Chlorophyta, se observa que la región de la presecuencia que dirige de la matriz mitocondrial a la membrana interna, posee gran similitud con algunos péptidos de tránsito del cloroplasto. Probablemente, una parte de la presecuencia de COXIIa proviene de la duplicación de péptidos señal del tilacoide o estroma de cloroplasto. Se ha reportado que los péptidos señal que dirigen a las proteínas al estroma en cloroplastos de chícharo pueden fosforilarse *in vitro*, y que este proceso no se observa para proteínas de cloroplasto maduras o presecuencias mitocondriales (Waegemann y Soll, 1996). No se ha observado el fenómeno de fosforilación de péptidos señal en cloroplastos de *Chlamydomonas*, sin embargo esta fosforilación selectiva podría evitar que COXIIa se dirija incorrectamente al cloroplasto a pesar de tener una presecuencia con algunas características compartidas con los péptidos señal de cloroplastos en *Chlamydomonas*. Por otro lado, cuando se compara la presecuencia de COXIII de ambas algas contra la misma base de datos, se observa similitud con la presecuencia de la oxidasa alterna mitocondrial de *Chlamydomonas reinhardtii* (Dinant, M., Laraki, M., Coosemans,N., Joris,B. and Matagne, R. F., sin publicar, No. Acceso AF047832). Es posible que estas presecuencias se hayan adquirido a partir de presecuencias ya existentes, como en los ejemplos reportados por Kadowaki (1996).

Pérdida de la copia mitocondrial

En el proceso de transferencia de genes mitocondriales al núcleo debe existir un momento en que tanto la copia mitocondrial como la nuclear sean activas. El único ejemplo identificado de esta etapa de transferencia ha sido descrito para el gen *cox2* de algunas leguminosas (Adams y col., 1999). En estas plantas han existido eventos de inactivación de una u otra copia con una frecuencia similar. Esto sugiere que, al menos en estos ejemplos, no existe una preferencia aparente por conservar una u otra copia activa después del evento de transferencia.

Tanto en *Polytomella* spp. como en *C. reinhardtii*, se demostró que los genes mitocondriales que codifican para las subunidades II y III de la citocromo *c* oxidasa han sido transferidos exitosamente al núcleo como copia única. Además de contar con la secuencia completa del genoma mitocondrial de *C. reinhardtii*, se conoce la secuencia completa de los genomas mitocondriales de otros miembros de la familia, como son *Chlamydomonas eugametos* (Denovan-Wright y col., 1998), y *Chlorogonium elongatum*

(Kroymann y Zetsche, 1998). Adicionalmente, Turmel y col. (1999) secuenciaron por completo el genoma mitocondrial de otra alga clorofita, *Pedinomonas minor*. Todas estas algas carecen de los genes que codifican para COXII y COXIII. Es de esperar que, al igual que en *Polytomella* spp. y *C. reinhardtii*, los genes se hayan transferido exitosamente al núcleo y la copia mitocondrial se haya desactivado y desaparecido de este organelo.

Disminución de la hidrofobicidad de las proteínas mitocondriales sintetizadas en el citosol.

La mayoría de las proteínas que han permanecido codificadas en la mitocondria son aquellas que presentan varios cruces transmembranales, mientras que las proteínas mitocondriales que se sintetizan en el citosol y se importan a la mitocondria son aquellas solubles o con pocos cruces transmembranales (von Heijne, 1986; Popot y de Vitry, 1990). Adicionalmente, los cruces transmembranales de proteínas sintetizadas en el citosol presentan una menor hidrofobicidad que los cruces transmembranales de proteínas codificadas en la mitocondria. Dos parámetros que nos permiten estimar la dificultad de una proteína para ser importada a la mitocondria desde el citosol son la mesohidrofobicidad (*meso*H) y la hidrofobicidad local <H> (Claros y col., 1995). La mesohidrofobicidad es el promedio de la hidrofobicidad en una región extendida de la proteína, entre 60 y 80 aminoácidos, y <H> es el promedio de la hidrofobicidad local, en una ventana de 13 a 17 aminoácidos. Cuando una proteína tiene bajas *meso*H y <H>, ésta puede ser sintetizada en el citosol y ser importada a la mitocondria. Esto sucede para todas aquellas proteínas cuyos genes son nucleares, incluyendo al acarreador de ATP/ADP. Cuando una proteína tiene valores altos de *meso*H y de <H>, la proteína permanece codificada en el DNA mitocondrial, como es el caso del apocitocromo *b* y COXI. Lo mismo se observa para los genes que codifican para COXII y COXIII en la mayoría de las especies.

Se encontró que las proteínas predichas por los genes *cox3* y *cox2b* de *Polytomella* spp. y *C. reinhardtii* presentan valores de *meso*H y de <H> disminuidos cuando se comparan con los valores para otras COXII y COXIII codificadas en el DNA mitocondrial. Estas adaptaciones pueden contribuir a disminuir los problemas de importación post-traduccional debido al reconocimiento inadecuado de señales de término de transferencia, o a la formación de estructuras hidrofóbicas muy estables que impidan que la proteína adquiera la conformación adecuada durante su importación. La proteína codificada por *cox2a* presenta valores de *meso*H y <H> similares a los observados para algunas COXII codificadas en el DNA mitocondrial, y para COXII de caupí y soya, cuyos genes se localizan en el núcleo (**Figura 3**). Esto indica que la hidrofobicidad general de esta proteína no es determinante para que el gen permanezca retenido en el DNA mitocondrial.

Por otro lado, los genes que codifican para COXIIa y COXIII, predicen presecuencias cuya longitud es mayor a lo esperado para otras presecuencias (Von Heijne y col., 1989). Se ha visto que cuando se duplica una presecuencia mitocondrial, la importación de proteinas hidrofóbicas puede mejorar (Galanis y col., 1991; Claros y col., 1995). Esta longitud puede contribuir a mantener una conformación adecuada de la proteina durante la importación.

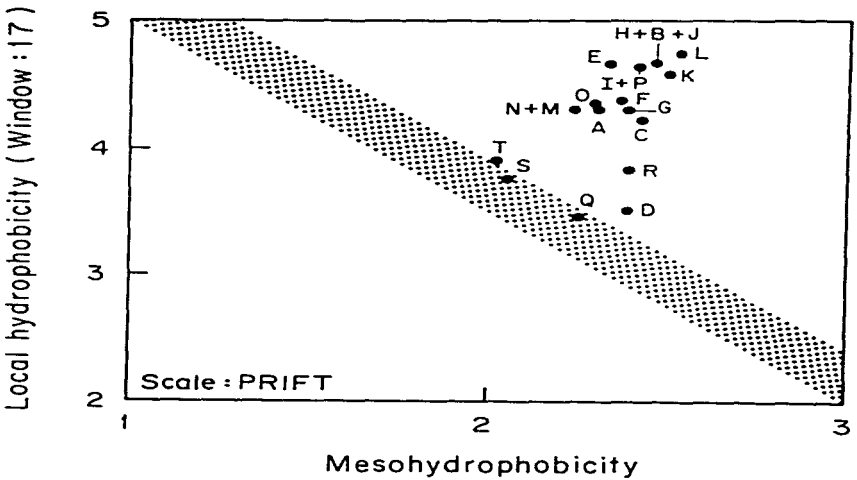

Figura 3. Valores de mesohidrofobicidad contra hidrofobicidad máxima local Para COXIIa de *Polytomella* spp. y *C. reinhardtii*. Comparación con los valores de *Meso*H y <H> de la subunidad II de la citocromo *c* oxidasa de otros organismos. Esta gráfica se elaboró con el programa MitoProtII por Claros y col. (1995). A=*Arabidopsis thaliana*, B= *Brassica napus*, C=*Cyanidium caldarium*, D=*Prototheca wickerhamii*, E=*Raphus sativus*, F= *Triticum aestivum*, G= *Beta vulgaris*, H=*Chondrus crispus*, J=*Zea mays*, K=*Marchantia polymorpha*, L=*Oenothera berteriana*, M=*Orysa sativa*, N=*Pisum sativum*, O=mit *Glycine max*, Q=nuc *Glycine max*, R=*Vigna unguiculata*, S=**C. reinhardtii**, T=***Polytomella*** **spp**

Los genomas mitocondriales de las algas Chlorophyta han evolucionado de maneras diferentes.

Las algas verdes y las plantas tienen un origen monofilético cuya característica es la presencia de cloroplastos rodeados de dos membranas, con tilacoides apilados y clorofilas tipo *a* y *b*. Generalmente se reconocen cinco clases de algas verdes (algas Chlorophyta): Charophyceae, Chlorophyceae, Trebouxiophyceae, Ulvophyceae y Prasinophyceae (Melkonian y col., 1995; Bhattacharya y Medlin, 1998). Actualmente se conoce la secuencia completa del genoma mitocondrial de cuatro algas Chlorophyta de la clase Chlorophyceae: *C. reinhardtii* (Michaelis y col., 1990), *C. eugametos* (Denovan-Wright y col., 1998), *C. elongatum* (Kroymann y Zetsche, 1998) y *S. obliquus* (Nedelcu y col., 2000; Kuck y col., 2000); una de la clase Trebouxiophyceae *Prototheca wickerhamii* (Wolff y col., 1994), una de la clase Prasinophyceae *Nephroselmis olivacea*, y una cuya posición exacta entre las Chlorophyceae, Prasinophyceae o Ulvophyceae se desconoce, *Pedinomonas minor* (Turmel, y col., 1999).

Se ha observado que los genomas mitocondriales de las Chlorophyta difieren en sus estructuras, y se pueden dividir en dos grupos: **(i)** Los genomas mitocondriales de *P. wickerhamii* y *N. olivacea* comparten muchas características con los genomas mitocondriales de las plantas (Oda y col., 1992; Unseld y col., 1997; Kubo y col., 2000) y con sus ancestros procariotes. Son circulares, tienen una gran cantidad de genes que codifican para proteínas de la cadena respiratoria y para proteínas ribosomales, así como para el 5S rRNA. Adicionalmente, presentan una mayor cantidad de RNAs de transferencia y regiones que codifican para las subunidades grande y chica del RNA ribosomal con una estructura convencional. **(ii)** Por otro lado, el resto de las Chlorophyta estudiadas han retenido pocas características de su ancestro. Son genomas circulares o lineales, el DNA mitocondrial se ha reducido de manera importante, tanto en capacidad codificante como en tamaño. Han perdido muchos genes que codifican para proteínas de la cadena respiratoria y carecen de genes para proteínas ribosomales o para el 5S rRNA. Presentan una menor cantidad de tRNAs, y sus RNA ribosomales se encuentran fragmentados y dispersos. Estas Chlorophyta pertenecen en su mayoría a la clase Chlorophyceae. Los representantes con genomas mitocondriales más compactos se encuentran en la familia de las Chlamydomonadaceae, como es el caso de *C. eugametos*, *C. elongatum*, *C. reinhardtii* y *Polytomella* spp.

Se desconocen los factores y mecanismos que dieron lugar a estos dos tipos de genoma mitocondrial entre las Chlorophyta. Nedelcu y Lee (1998) proponen que la reducción del contenido de genes entre las Chlorophyceae se debe a eventos de recombinación entre secuencias directas repetidas pequeñas.

Se observó que en las Chlorophyceae como *C. reinhardtii*, *C. eugametos*, *C. elongatum*, *Polytomella* spp. y *S. obliquus*, existen secuencias repetitivas directas ricas en GC que *P. wickerhamii* no posee.

De igual manera, *N. olivacea* carece de estas secuencias pequeñas, mientras que están presentes en el genoma mitocondrial de *P. minor* (Turmel y col., 1999). Por otro lado, proponen que la fragmentación y dispersión de los RNAs ribosomales observada en estas algas se puede deber a eventos de recombinación entre secuencias pequeñas repetidas e invertidas. Este pudo ser un mecanismo mediante el cual, previamente a la transferencia del gen *cox2* al núcleo, este se fragmentara en dos genes independientes, *cox2a* y *cox2b*, dentro de el genoma mitocondrial de las Chlamydomonadaceas y de *S. obliquus*.

En la **tabla I** se muestra el contenido de genes en el genoma mitocondrial de las algas Chlorophyta conocidos a la fecha. Los tiempos de transferencia de genes mitocondriales entre el grupo con DNA mitocondrial reducido pudo llevarse a cabo de la siguiente manera: Los primeros eventos de reducción del genoma mitocondrial pudieron llevarse a cabo en el ancestro común de *P. minor* y las otras Chloropyceae. Sin embargo, la reducción posterior de los genomas mitocondriales, así como las características que dieron lugar a estos genomas mitocondriales en la actualidad, pudieron ser eventos independientes que se llevaron a cabo una vez que *P. minor* se separó de las Chlorophyceae estudiadas. Los datos que sugieren esto son los siguientes:

(i) El patrón de fragmentacion de los RNA ribosomales entre *P. minor* y las otras Chloropyceae es diferente. La gran mayoría de los sitios de fragmentación de los RNA ribosomales de las Chlamydomonadaceas y *S. obliquus* están conservados, mientras que en *P. minor* el único sitio de fragmentación de la subunidad grande del RNA ribosomal no está conservado (Nedelcu, 1997).
(ii) Si *P. minor* compartiera el evento de transferencia de genes al núcleo con el resto de las Chlorophyceae, sería de esperarse que el gen *atp8* estuviera ausente de su genoma mitocondrial, como sucede en las Chlorophyceae estudiadas. La ausencia del gen *atp8* se observa incluso en el genoma mitocondrial de *S. obliquus*, a pesar de que este genoma es más grande.
(iii) La transferencia de parte de los tRNAs mitocondriales al núcleo pudo ser un evento independiente entre *P. minor* y las Chloropyceae. Si éstas compartieran el ancestro común, sería de esperarse que los tres tRNAs codificados en el genoma mitocondrial de las Chlamydomonadaceas ($tRNA^{Met}_{CAU}$, $tRNA^{Trp}_{CCA}$ y $tRNA^{Met}_{CAU}$) también estuvieran presentes en el genoma mitocondrial de *S. obliquus* y *P. minor*. A pesar de que la primera posee 27 tRNAs en su genoma mitocondrial y la última posee sólo 8, *S. obliquus* comparte con las Chlamydomonadaceas estos 3 tRNAs, mientras que en *P. minor* los tRNA para triptófano (UGG) y metionina (AUG) están ausentes.

Estas observaciones sugieren que *S. obliquus* comparte una mayor cantidad de eventos de reducción de contenido de genes, así como de fragmentación de los RNA ribosomales, con las Chlamydomonadaceas que con *P. minor*. En esta reducción común se incluye la transferencia de los genes *cox2* y *cox3*. En la **figura 4** se muestra un árbol derivado de un análisis filogenético de genes mitocondriales entre las algas Chlorophyta. De acuerdo a esto, en el ancestro común entre *S. obliquus* y las Chlamydomonadaceas, el primer gen de la citocromo *c* oxidasa que se transfirió al núcleo, pudo ser *cox2b*, ya que este gen no está presente en ninguna de estas algas. La transferencia de *cox2a* y *cox3* al núcleo, se llevó a cabo una vez que *S. obliquus* y las Chlamydomonadaceas se separaron, pero antes de que *C. elongatum* se separara de *C. reinhardtii*, *C. eugametos* y *Polytomella* spp. Esto se debe a que los genes *cox2a* y *cox3* aún están codificados en el genoma mitocondrial de *S. obliquus*, pero ya no están presentes en las Chlamydomonadaceas. Para poder establecer con mayor seguridad la historia evolutiva de estos genomas será necesario caracterizar más genomas mitocondriales de algas Chlorophyta, así como a los genes mitocondriales que se han transferido al núcleo.

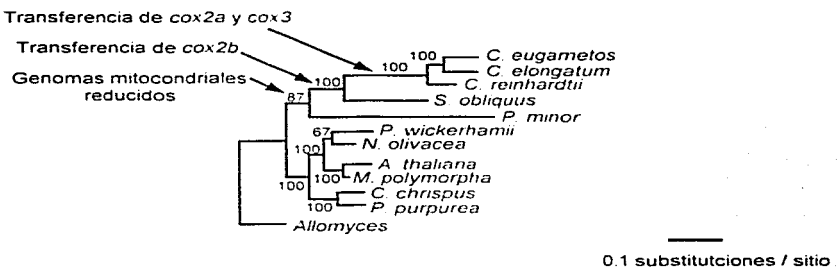

Figura 4. Análisis filogenético empleando 7 secuencias mitocondriales presentes en todas las algas Chlorophyta secuenciadas a la fecha. Basado en: Nedelcu y col. (2000).

Tabla I. Contenido de genes en el genoma mitocondrial de las Chlorophyta estudiadas a la fecha.

Genes	*P. wi*	*N. ol*	*P. mi*	*S. ob*	*C. re*	*C. eu*	*C. el*
COMPLEJO I							
nad1	+	+	+	+	+	+	+
nad2	+	+	+	+	+	+	+
nad3	+	+	+	+	-	-	-
nad4	+	+	+	+	+	+	+
nad4L	+	+	+	+	-	-	-
nad5	+	+	+	+	+	+	+
nad6	+	+	+	+	+	+	+
nad7	+	+	-	-	-	-	-
nad8	-	+	-	-	-	-	-
nad9	+	+	-	-	-	-	-
COMPLEJO III							
Cob	+	+	+	+	+	+	+
COMPLEJO IV							
cox1	+	+	+	+	+	+	+
cox2	+	+	-	+ (cox2a)	-	-	-
cox3	+	+	-	+	-	-	-
COMPLEJO V							
atp1	+	+	-	-	-	-	-
atp6	+	+	+	+	-	-	-
atp8	+	+	+	-	-	-	-
atp9	+	+	-	+	-	-	-
RNA ribosomal							
rnl	+	+	+ (2)	+ (4)	+ (8)	+ (6)	+ (6)
rns	+	+	+	+ (2)	+ (4)	+ (3)	+ (3)
5S	+	+	-	-	-	-	-
TRNA	26	26	8	27	3	3	3
No. de genes	13	19	18	47	21	65	69

P. wi= Prototheca wickerhamii, N. ol=Nephroselmis olivacea, P. mi= Pedinomonas minor, S. Ob= Scenedesmus obliquus, C re= Chlamydomonas reinhardtii, C. eu= Chlamydomonas eugametos, C. el= Chlorogonium elongatum. Los números entre paréntesis indican la cantidad de fragmentos de los RNA ribosomales encontrados.

Por qué unos genes mitocondriales son transferidos al núcleo y otros no?

Una de las características de la evolución de la mitocondria ha sido la reducción de su genoma después del evento endosimbiótico. Esta reducción ocurrió en parte a través de la transferencia de una gran cantidad de genes hacia el núcleo, de hecho, muy pocos genes han quedado retenidos en el DNA mitocondrial. Puede ser un conjunto de factores lo que ha determinado la distribución de genes entre la mitocondria y el núcleo entre las diferentes especies.

La transferencia de genes mitocondriales al núcleo se puede favorecer porque la integridad de éstos en el núcleo se mantiene mejor que en la mitocondria (Blanchard y Lynch, 2000, Wallace y col., 1987). Esto se puede deber a una alta concentración local de radicales libres en la mitocondria que puede dañar al DNA y que favorece que la mayoría de los genes mitocondriales se transfieran al núcleo (Allen y Raven, 1996). También puede deberse a la falta de recombinación sexual en la mitocondria, que repara y mantiene la integridad de los genes nucleares y los protege de las mutaciones dañinas que inevitablemente se acumulan en el DNA (Lynch, 1996, Martin y Herrmann, 1998). Sin embargo, la frecuencia de mutaciones en el DNA mitocondrial de plantas es menor que en el DNA nuclear (Wolfe y col., 1987). Probablemente esto refleja la menor velocidad con que los genes mitocondriales de las plantas se transfieren al núcleo.

Se propone que los pocos genes que aún permanecen en el DNA mitocondrial, son generalmente aquellos que codifican para proteínas muy hidrofóbicas. Su expresión citosólica podría ocasionar una relocalización celular errónea (von Heijne, 1986). Adicionalmente, algunas de estas proteínas pueden ser demasiado hidrofóbicas para ser importadas a través de la doble membrana mitocondrial. Sin embargo, en este trabajo se demostró que los genes *cox2a* y *cox3* que codifican para proteínas muy hidrofóbicas, tienen una localización nuclear. Estos genes predicen una posible presecuencia mitocondrial que podría contribuir a la localización correcta de las proteínas y posteriormente puede facilitar la importación de éstas hasta la membrana interna mitocondrial. Por otro lado, Allen y Raven (1996) proponen que la razón por la que algunos genes han permanecido en el DNA mitocondrial, es que sus productos son en general componentes importantes de la cadena respiratoria y la fosforilación oxidativa. Se requiere un control directo de la expresión de estos genes por cambios en el estado redox de la mitocondria, y de esta manera minimizar la producción de radicales libres de oxígeno que puedan existir en altas concentraciones dentro de la mitocondria. Éste puede ser un factor importante por el cual los genes *cox2* y *cox3* que codifican para las subunidades II y III de la citocromo *c* oxidasa han permanecido en el DNA mitocondrial de la gran mayoría de los eucariotes.

COXII participa directamente en la transferencia de electrones entre el citocromo *c* soluble y la subunidad I de la citocromo *c* oxidasa, mientras que COXIII puede jugar un papel importante, no sólo a nivel estructural, sino también en la canalización del oxigeno hacia el sitio activo de la subunidad I (Riistama y col., 1996). Sin embargo, este modelo no explica la localización nuclear de los genes *cox2* y *cox3* de *Polytomella* spp. y *Chlamydomonas reinhardtii*. Probablemente, en las algas Chlamydomonadaceas la expresión de estos genes no depende directamente del estado redox de la mitocondria, sino que su expresión podría ser constitutiva, de tal manera que la concentración en estado estacionario de estas proteínas compensa la que fuera una regulación redox directa si estos genes hubieran permanecido en la mitocondria. Se ha observado que en células de humano, la regulación de la síntesis de las subunidades nucleares y mitocondriales de la citocromo *c* oxidasa no está íntimamente ligada. Sin embargo, la velocidad de degradación de las subunidades nucleares aumenta cuando se inhibe la síntesis de proteínas mitocondriales (Nijtmans y col., 1995) o cuando se emplean células carentes de DNA mitocondrial, en las cuales la citocromo *c* oxidasa no se ensambla (Taanman y col., 1996).

CONCLUSIONES

1. Las subunidades II y III de la citocromo *c* oxidasa del alga incolora *Polytomella* spp. están presentes en el complejo enzimático. La subunidad II se identificó en geles de acrilamida como una banda con una masa molecular aparente de 18.6 kDa. Esta subunidad se caracterizó mediante una secuencia interna y del extremo amino terminal. La subunidad III se identificó como una proteína de 29.6 KDa mediante una reacción cruzada con anticuerpos contra COXIII de levadura y mediante la secuencia de su extremo amino terminal.

2. El gen que codifica para la subunidad III en *Polytomella* spp. y *Chlamydomonas reinhardtii* es nuclear. Este gen presenta un uso de codones nuclear y una señal de poliadenilación característica de genes nucleares en las Chlamydomonadaceas. Adicionalmente, presenta intrones de tipo nuclear y predice una posible presecuencia mitocondrial.

3. La subunidad II de la citocromo *c* oxidasa está codificada por dos genes nucleares. El gen *cox2a* codifica para la región amino terminal y los dos cruces transmembranales. Además, codifica para una posible presecuencia mitocondrial y para una extensión de aminoácidos no conservada, presente en el extremo carboxilo terminal. El gen *cox2b* codifica para la región carboxilo hidrofílica de la subunidad II, e incluye al sitio de unión de cobre (Cu_A). Además, codifica para una extensión amino terminal no conservada en otras COXII, que no presenta características de presecuencia y que permanece en la proteína madura.

4. Los genes *cox2a* y *cox2b* en *Polytomella* spp. se transcriben y traducen por separado para conformar a la subunidad II como un heterodímero. En este trabajo no fue posible detectar un transcrito maduro que incluyera los mRNA provenientes de *cox2a* y *cox2b*, ni por análisis tipo northern, ni por RT-PCR. La masa molecular disminuida que se observó para COXII de *Polytomella* spp. puede deberse a que ambas proteínas, COXIIa y COXIIb presentan una masa molecular deducida muy similar, y en geles de acrilamida migran como una banda única. Posiblemente sucede algo similar en *C. reinhardtii*.

5. Los genes que codifican para las subunidades II y III de la citocromo *c* oxidasa de *Polytomella* spp. y *C. reinhardtii* predicen proteínas con propiedades físicas que facilitarían su integración en el complejo IV activo. Los genes *cox3* y *cox2b* predicen una proteínas con una hidrofobicidad general disminuida respecto a sus equivalentes sintetizadas en la mitocondria. Además, los genes predicen presecuencias mitocondriales para COXIIa y COXIII. Ambas características podrían ser importantes para facilitar la importación correcta de estas proteínas con 2 y 7 cruces transmembranales respectivamente.

PERSPECTIVAS

1. Tratamiento de enfermedades mitocondriales mediante terapia génica.

Existen muchas enfermedades mitocondriales debidas a mutaciones en el DNA mitocondrial. La mitocondria tiene un papel muy importante en la viabilidad de la célula, por lo cual no es sorprendente que las mutaciones patogénicas en este genoma tengan consecuencias devastadoras. Los tipos de mutaciones que puede sufrir el DNA mitocondrial se clasifican en tres grupos: (1) rearreglos del mtDNA, como duplicaciones y ablaciones; (2) mutaciones puntuales en genes involucrados en la traducción, como rRNA y tRNA; (3) mutaciones puntuales en genes que codifican para proteínas de la cadena respiratoria (Grossman y Shoubridge 1996, Schon y col., 1997, Moraes, 1998; Wallace, 1999).

Algunos defectos parciales en la citocromo *c* oxidasa se han asociado a mutaciones puntuales en tRNAs y a ablaciones en el DNA mitocondrial. Normalmente, estos defectos se acompañan de anormalidades en otros componentes de la fosforilación oxidativa (revisado en Moraes, 1998). Sin embargo, existen algunos casos reportados en que una enfermedad mitocondrial se debe a mutaciones puntuales en el gen *cox2* (Rahman y col., 1999, Clark y col., 1999) o *cox3* (Manfredi y col., 1995, Hanna y col., 1998). Se ha reportado un caso de enfermedad mitocondrial debido a una pequeña ablación de 15 nucleótidos en el gen *cox3* (Keightley y col., 1996). En este caso ha sido posible crear líneas celulares transmitocondriales en que se fusionan células humanas que carecen de DNA mitocondrial (células ρ° King y Attardi, 1989) con fracciones celulares del paciente. El efecto de esta ablación en la función de la citocromo *c* oxidasa en estas líneas celulares se caracterizó por Hoffbuhr y col. (2000).

A pesar de los avances en los diagnósticos y la caracterización de las enfermedades mitocondriales, actualmente no existe un tratamiento satisfactorio para la gran mayoría de estos desórdenes. Se ha visto que el tratamiento de pacientes con cofactores de la fosforilación oxidativa y sustratos oxidables, puede mejorar las condiciones del paciente, sin embrago, las enfermedades mitocondriales comúnmente progresan con efectos devastadores. Las terapias génicas surgen como una estrategia alternativa con efectos más permanentes en el tratamiento de enfermedades mitocondriales.

Una estrategia consiste en dirigir directamente material genético a la mitocondria (Vestweber y Schatz, 1989, Seibel y col., 1995). Otra estrategia consiste en introducir al núcleo una copia correcta del gen mitocondrial afectado. Este gen se traducirá en el citoplasma de la célula, y la proteína se translocará a la mitocondria para remplazar a la proteína mitocondrial defectuosa.

Para ello, es necesario realizar modificaciones en los genes mitocondriales que incluyen la adaptación del uso de codones y la fusión artificial de una presecuencia mitocondrial que dirija a la proteína hacia este organelo. Este tipo de expresión alotópica se ha llevado a cabo con éxito en *S cerevisiae* para reemplazar la deficiencia debida a una mutación en el gen *atp8* (Gray y col., 1998). A diferencia de lo que ocurre en humano, los genes que codifican para COXII y COXIII en *Polytomella* spp. y *C reinhardtii* son de origen nuclear. Esto abre la posibilidad de la expresión alotópica de estos genes en células de humano que contengan mutaciones en el DNA mitocondrial.

2. Estudio de los mecanismos de importación de las subunidades II y III de la citocromo *c* oxidasa de las algas Chlamydomonadaceas.

En este trabajo se ha demostrado que los genes que codifican para las subunidades II y III de la citocromo *c* oxidasa de *Polytomella* spp. y *C reinhardtii* se han transferido exitosamente al núcleo. Ahora continúa el estudio de los mecanismos de importación de las proteínas codificadas por estos genes a la mitocondria, y algunas de las preguntas a resolver son:

- Las presecuencias mitocondriales predichas por los genes *cox2a* y *cox3* ¿son funcionales en realidad?

- ¿Qué parte de las secuencias de *cox3* y de *cox2a* que predicen presecuencias mitocondriales son importantes para la importación de las proteínas?

- ¿Qué parte de la extensión amino terminal no conservada que predice el gen *cox2b* es importante para la importacion de la proteína COXIIb a la mitocondria?

- ¿Qué parte de las extensiones no conservadas del extremo carboxilo terminal predicho por el gen cox2a y del extremo amino terminal predicho por *cox2b* son importantes para el funcionamiento adecuado de la subunidad II?

- ¿Utilizan las proteínas COXIIa, COXIIb y COXIII el mecanismo de importación a la mitocondria que emplean la mayoría de las proteínas sintetizadas en el citosol?

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Apéndice I:

Otros artículos publicados durante mis estudios de Doctorado.

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An Atypical Cytochrome *b* in the Colorless Alga *Polytomella* spp.: The High Potential b_H Heme Exhibits a Double Transition in the α-Peak of Its Absorption Spectrum

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Polytomella **spp. is a colorless alga of the family Chlamydomonadaceae that lacks chloroplasts and cell wall. A highly active ubiquinol-cytochrome *c* oxidoreductase (*bc*$_1$ complex), sensitive to antimycin and myxothiazol, has been purified and characterized from this alga (Gutiérrez-Cirlos *et al.*, 1994, *J. Biol. Chem.* 269, 9147–9154). Both in mitochondrial membranes and in the isolated complex, the visible spectrum of cytochrome *b* from *Polytomella* spp. exhibits an atypical α-band with a maximum at 567 nm. This maximum is shifted 3–4 nm to the red when compared with *b*-type cytochromes from other organisms. Analysis of the *b* hemes of the *bc*$_1$ complex by high performance liquid chromatography revealed no differences in the retention time and in the absorption spectra of the *b*-type hemes from *Polytomella* spp. and hemin, indicating that the prosthetic group in this alga is protoheme and thus ruling out the possibility that the red-shift could be due to different chemical substitutions in the porphyrin rings of the b_L or b_H hemes. The two *b* hemes were characterized by electrochemical redox titration; at pH 7.8–8.0, the midpoint potential for b_L was −143 mV and for b_H +25 mV. The spectra of the two *b*-type hemes were recorded in the presence of different reductants, at selected electrochemical potentials, and in the presence of antimycin A, to distinguish between the contribution of b_L and b_H to the visible spectrum. Both hemes b_L and b_H of the algal cytochrome *b* contribute to the observed bathochromic absorption maximum in the α-band of the spectrum. The data also show that the low potential b_L heme from *Polytomella* spp. is spectroscopically similar to that of other organisms, with two transitions in the α-peak at 558.7 and 568.4 nm. The high-potential heme b_H also exhibits a spectrum with two transitions at 557.2 and 568.9 nm, which surprisingly differs from the spectra of cytochrome b_H of mammals, plants, yeasts, and bacteria, which all exhibit a single transition centered around 560 nm.** © 1998 Academic Press

Key Words: ***Polytomella; Chlamydomonas;*** **mitochondria;** ***bc*$_1$** **complex; cytochrome *b*; Chlorophyceae.**

Ubiquinol–cytochrome *c* oxidoreductase, or *bc*$_1$ complex (EC 1.10.2.2), is an oligomeric membrane enzyme with three redox polypeptides, namely a bihemic cytochrome *b*, a cytochrome c_1, and a Rieske-type iron–sulfur protein. It also contains a variable set of subunits which do not bind prosthetic groups (1). The ubiquinol–cytochrome *c* oxidoreductase activity occurs through a Q-cycle mechanism (2–4) that involves two redox catalytic units. One unit is formed by the Q_o site, the low potential heme (b_L), and the Rieske iron sulfur protein; the other unit is composed of the Q_i site and the high potential heme (b_H) (5). Cytochrome *b* is the most hydrophobic protein of the bc_1 complex (6, 7). Based on sequencing data, inhibitor sensitivities, mutagenesis, and topological studies, models for the disposition of eight putative transmembrane α-helices (A to H) of cytochrome *b* have been proposed (8, 9). Four conserved histidines, two in helix B and two in helix D, at positions 82, 96, 183, and 197 (bovine numbering) coordinate the b_H and b_L hemes (10–12). X-ray data of the bovine *bc*$_1$ complex have confirmed the eight-helices structure of cytochrome *b* and shown that the two *b* hemes are 21 Å apart from each other (13, 14).

All known mitochondrial cytochromes *b* exhibit an α-peak close to 560 nm when fully reduced with dithio-

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nite (8). Electrochemical and high resolution spectral analysis of the mouse bc_1 complex indicated that the b_H heme is characterized by a single α-band peak, while the b_L heme exhibited an overlapped, double α-band absorption peak (5).

A highly active, antimycin A-sensitive, ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex) has been previously purified from *Polytomella* spp., a colorless alga closely related to the photosynthetic *Chlamydomonas* lineage (15). The cytochrome *b* from *Polytomella* spp., both in mitochondrial membranes and in the purified bc_1 complex, shows a maximum in the α-band shifted toward the red, at 567 nm. In this work the *b*-type hemes from the algal bc_1 complex were characterized spectroscopically, electrochemically, and by HPLC analysis. Our results indicate that the b_L and b_H hemes of cytochrome *b* from *Polytomella* spp. are protohemes, that both contribute to the observed red-shifted α-band, and that the high potential heme b_H of this colorless alga has a split α-band different from the one obtained with mammalian, plant, yeast, or bacterial bc_1 complexes.

MATERIALS AND METHODS

Strains. The colorless alga *Polytomella* spp (198.80, E.G. Pringsheim), originally obtained from the algae collection at the University of Gottingen (Germany), was maintained at the Microbiological Collection of the Department of Biotechnology (CINVESTAV del IPN, Mexico) under the register number CDBB-951. The algae were grown and harvested as previously described (15).

Purification of bc_1 complexes and activity measurements. The bc_1 complex from *Polytomella* spp. was purified as described by Gutierrez-Cirlos *et al.* (15). Beef heart mitochondrial bc_1 complex was prepared as described by Ljungdahl *et al.* (16). All complexes were kept at −70°C until used. Cytochrome *b*, cytochrome c_1, and horse heart cytochrome *c* were quantified spectrophotometrically (6). Ubiquinol–cytochrome *c* oxidoreductase activity was measured as described previously (17). Protein concentrations were determined according to Lowry *et al.* (18) with the modifications of Markwell *et al.* (19).

High performance liquid chromatography (HPLC).[2] Runs were performed using two Beckman pumps and an automated gradient controller. Prior to HPLC, samples were centrifuged in an Eppendorf table-top centrifuge and the insoluble material discarded. Proteins were loaded on a Supelco C-4 column (250 × 4.6 mm) with a Rheodyne injector and a 500-μl loop, and eluted at a constant flow rate of 1.5 ml/min. Two buffer systems were prepared: buffer A contained 0.1% TFA in water, while buffer B contained 0.1% TFA in a 2:1 acetonitrile:isopropanol mixture. The column was equilibrated in the presence of 65% buffer A and 35% buffer B, and the samples were eluted with the following profile: an initial wash with 65% buffer A and 35% buffer B (4 min), an upward linear gradient to 35% buffer A and 65% buffer B (40 min) followed by a second upward linear gradient to 100% buffer B (6 min), a downward linear gradient to 65% buffer A and 35% buffer B (7 min), and a final wash with 65% buffer A and 35% buffer B (2 min). Spectra were recorded from 250 to 700 nm at 4-s intervals with a Waters 966 photodiode array detector. Data were processed in a 486 PC (NEC Power Mate 433) with the chromatographic software Millenium 2010.

[2] Abbreviations used: DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid.

Difference spectra of the bc_1 complex. Spectra were recorded using a bc_1 complex concentration of 2.0–5.0 μM of cytochrome *b*. Spectra taken in the presence of inhibitor were carried out as follows: the baseline was made by reducing bc_1 complex with dithionite in both reference and sample cuvets; then, 0.6 μM antimycin A was added to the sample cuvet. Other difference spectra were made using a few grains of dithionite, or 20 mM of ascorbate, pH 7.0, or 55 μM DBH added from an ethanolic stock solution. Deconvolution of the difference spectra was carried out with the Microcal Origin program, version 3.73 (Microcal Software, Inc.). Spectra were obtained with a DW/2a UV/Vis SLM-Aminco spectrophotometer modified with the OLIS DW2 Conversion and OLIS software (On-line Instrument System Inc.). Bilateral curved slits with a spectral bandpass of 3 nm were used and a scanning speed of 5.0 nm/s. The medium response of the photomultipher was of 185 ms. Wavelength calibration was made with a holmium oxide standard.

Redox titrations. The bc_1 complex was present in a concentration range from 2.0 to 4.0 μM (based on cytochrome c_1 concentration). Redox mediators and buffer conditions were as described by Howell and Robertson (5). Electrochemical titrations were carried out in a buffer containing 50 mM Tris-Cl (pH 8.0), 30 mM NaCl, 1 mM $MgCl_2$, 0.1 mg/ml of lauryl maltoside, and 10% glycerol in the presence of the following mixture of redox-mediator dyes: 15 μM 2-hydroxy-1,4-napthoquinone, 75 μM duroquinone, 50 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine, 40 μM 1,2-napthoquinone, 25 μM *N*-methyldibenzopyrazine methosulfate, 40 μM 1,4-benzoquinone, and 40 μM 1,4-naphtoquinone, and an anaerobe atmosphere was maintained under a stream of argon. These mediators were added from stock solutions made in dimethyl sulfoxide. The titration was started by adding 0.5 mM ascorbate, followed by increasing concentrations up to 4.5 mM, and then fresh dithionite solution in increasing concentrations. At each E_h value, absorbance data were acquired between 500 and 600 nm and analyzed using the software package developed by OLIS. After each addition of reducing agent, a 10-min incubation time was allowed for electrochemical equilibration. Titrations were carried out in both the reductive and the oxidative directions. Samples were placed in a bottom-stirred redox cuvet and redox potentials were measured with a Radiometer Model P101 platinum electrode and a Ag/AgCl reference electrode. The electrodes were standarized with commercial horse heart cytochrome *c* (Sigma), considering an E_m of +220 mV. The ratios of absorbance units of the cytochromes were plotted against redox potential, and best-fitting Nernstian curves were drawn through the resulting points.

RESULTS

Spectral characterization of the Polytomella spp. bc_1 complex. The reduced minus oxidized difference spectra of the bc_1 complex of *Polytomella* spp., both in mitochondrial membranes (Fig. 1A) or in its purified form (15), exhibited absorption maxima in their α-peaks at 553 nm for cytochrome c_1 and at 567 nm for cytochrome *b*. This gave rise to a double peak spectrum in the α-band region of the bc_1 complex, where the absorption maximum of cytochrome c_1 is completely resolved from that of cytochrome *b* at room temperature, a spectroscopic feature not seen in bc_1 complexes from other organisms, where the absorption spectra of cytochrome c_1 appears as a shoulder of the cytochrome *b* spectrum. The atypical spectrum of the dithionite-reduced bc_1 complex frozen with liquid nitrogen in a Dewar flask, and in contact with a cold finger with liquid nitrogen, is shown in Fig. 1B. This spectrum exhibits α-bands with absorption maxima at 553 nm for cytochrome c_1 and at 566 nm for cytochrome *b*.

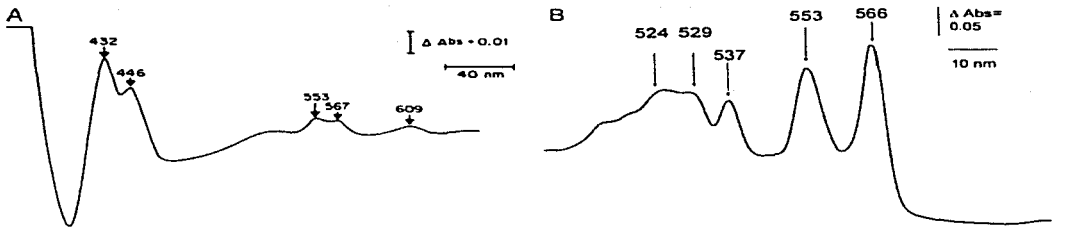

FIG. 1. Visible spectra of the bc_1 complex from *Polytomella* spp. and *C. reinhardtii*. (A) Difference spectrum of *Polytomella* mitochondrial fraction (reduced with dithionite minus oxidized). The absorption peaks shown are all of cytochromes: 432 and 446 nm gamma peaks of cytochrome *b* and cytochrome *c* oxidase, respectively; 553 nm, α-peak of cytochrome c_1; 567 nm α-peak of cytochrome *b* and 609 nm α-peak of cytochrome *c* oxidase. (B) Difference spectrum of the bc_1 complex from *Polytomella* spp. (reduced with dithionite minus oxidized) frozen in liquid nitrogen.

Thus, the *b*-type hemes from *Polytomella* spp. uniquely exhibit a red-shift of 3–4 nm in the α-band, with respect to other bc_1 complexes.

Characterization of the hemes from the bc_1 complex of Polytomella spp. The possibility that the *b*-type hemes from *Polytomella* spp. could be structurally different from the ones found in the bc_1 complexes of other organisms was explored. The bc_1 complex from the alga was denatured by urea and subjected to HPLC in a C_4 column; the resulting fractions were identified by comparing their retention times and spectra with those obtained from purified components. The reverse-phase HPLC profile of the bc_1 complex from *Polytomella* spp. exhibited three peaks absorbing at 400 nm (Fig. 2A). Peak 1, with a retention time of 18.2 min (Fig. 2A), had a maximum absorption peak at 398 nm, and two secondary broad peaks at 499 and 623 nm, but no defined peak at 280 nm (Fig. 3A). This peak exhibited the same retention time of hemin (chloroprotoporphyrin IX iron (III), Aldrich Chemical Co.) (Fig. 2B). The spectrum of pure hemin showed maxima at 398, 499, and 623 nm (data not shown), identical to those of the *b*-type heme fraction shown in Fig. 3A. When a mixture of *Polytomella* spp. bc_1 complex with pure hemin was loaded on the HPLC column, a single peak with a retention time of 18.2 min was obtained (Fig. 2C). The HPLC profiles confirmed the comigration of peak 1 with hemin and allowed its unambigous identification as a *b*-type heme. Peak 2 (retention time of 33.1 min) (Fig. 2A), was assigned to the covalently bound heme c_1 on the basis of its spectra, which exhibits an absorption maximum at 278 with three additional absorbance peaks at 398, 459, and 622 nm (Fig. 3B). When horse heart cytochrome *c* was applied to the column and run under the same HPLC conditions, it exhibited a peak with a retention time of 12 min, and its visible spectrum was identical to the one obtained for cytochrome c_1 (data not shown). Differences were observed in the absorbance ratios A_{400}/A_{275}, which had a value of 8.4 for horse heart cytochrome *c* and 1.9 for cytochrome c_1. These values are in agreement with the molecular mass of the polypeptides covalently bound to the *c*-type hemes. Peak 3, a minor signal with a retention time of 38.3 min (Fig. 2A) had an absorption maximum at 407 nm with an α-peak at 547 nm. This component exhibited the same retention time as hemes of purified bovine cytochrome *c* oxidase (Fig. 3C). Due to its longer retention time, this last peak was identified as heme *a* of *Polytomella* spp., and attributed to the residual presence of cytochrome *c* oxidase aa_3 in some bc_1 complex preparations. The results of HPLC analysis thus confirm that cytochrome *b* of *Polytomella* spp. bears protohemes identical to those of other bc_1 complexes. Therefore, the observed red-shift in the dithionite-reduced spectrum of *Polytomella* spp. bc_1 complex could not be attributed to the presence of different chemical substituents in the tetrapyrrole rings of the *b*-type hemes.

Difference spectra of the two b-type hemes from Polytomella spp. The reduced minus oxidized difference spectra of the *Polytomella* spp. *b* hemes were compared to the corresponding spectra of the bovine enzyme. The optical absorption bands of both *b*-type hemes (b_{11} plus $b_{1.}$) from *Polytomella* spp. were observed in the dithionite-reduced minus ascorbate-reduced spectrum (Fig. 4A). This spectrum shows a maximum at 568.8 nm and a broad shoulder at 558.8 nm with an A_{568}/A_{558} ratio of 2.0, the highest absorbance ratio so far obtained for a cytochrome *b*. In contrast, the same differential spectrum obtained for the beef-heart bc_1 complex exhibited a maximum at 565 nm, with an A_{565}/A_{558} ratio of only 1.4 (Fig. 4B). The fourth derivative analysis of the dithionite minus ascorbate differential absorption spectrum of *Polytomella* spp. exhibited four compo-

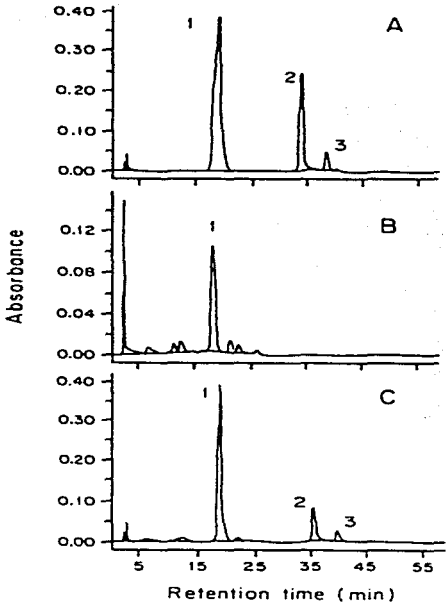

FIG. 2. High performance liquid chromatography of the urea-denatured bc_1 complex from *Polytomella* spp. (A) HPLC profile obtained for the bc_1 complex preparation of *Polytomella* spp. The peaks recorded at 400 nm are numbered for heme *b* (1), cytochrome c_1 (2), and heme *a* (3). (B) HPLC profile of commercial hemin run under the same conditions. (C) HPLC of a mixture of the urea-denatured bc_1 complex from *Polytomella* spp. and the commercial hemin.

nents: the first two, at 531 and 539 nm, are characteristic of the β-band of *b*-type cytochromes, and the last two, at 558 and 568 nm, were ascribed to the α-band. There was no peak at 553 nm which could have indicated a possible contribution of cytochrome c_1 to the absorption (or distortion) of the *b*-type cytochromes spectra (data not shown). The difference spectrum (reduced with dithionite minus reduced with DBH) of the bc_1 complex from *Polytomella* spp. showed two defined peaks at 568.8 and 558.8 nm; these two transitions were assigned mainly to the b_L heme (Fig. 4C). In contrast, the same difference spectrum obtained with the bovine bc_1 complex exhibited a maximum at 565 nm with a broad shoulder at 558 nm (Fig. 4D). The difference spectrum (reduced with DBH minus reduced with ascorbate), representing the b_{H} heme of *Polytomella* spp. (Fig. 4E), shows a split α-band with a small band at 559 nm and a dominant band at 569 nm. The spectrum of the b_{H} heme from this alga clearly differs

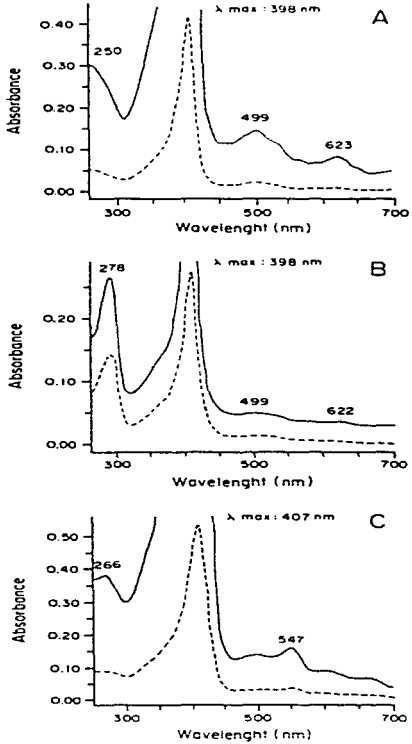

FIG. 3. Absorption spectra of the HPLC fractions. Dashed lines show the absorption spectra obtained. Solid lines show amplifications of the same spectra, showing the minor peak components. (A) Absorption spectra of peak 1 from HPLC, identified as heme *b*. (B) Absorption spectra of peak 2 from HPLC, identified as cytochrome c_1. (C) Absorption spectra of heme *a* of bovine cytochrome *c* oxidase, obtained under the same HPLC running conditions.

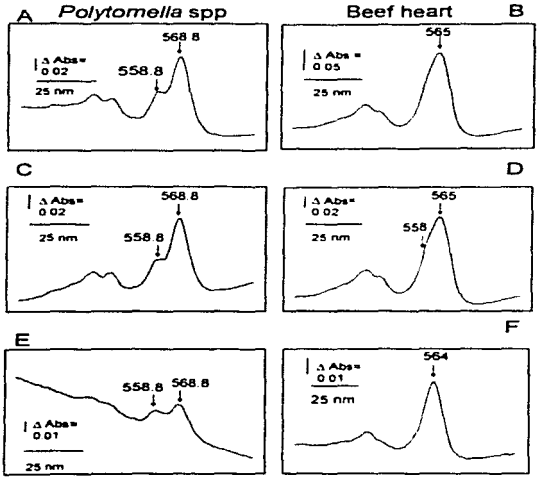

FIG. 4. Comparison of difference spectra obtained with different reductants for the bc_1 complexes from *Polytomella* spp. and from beef heart. (A) Dithionite–ascorbate difference spectrum of the bc_1 complex from *Polytomella* spp. (hemes b_L + b_H). (B) Dithionite–ascorbate difference spectrum of the beef heart bc_1 complex (hemes b_L + b_H). (C) Dithionite minus DBH difference spectrum of the bc_1 complex from *Polytomella* spp. (mainly heme b_L). (D) Dithionite minus DBH difference spectrum of the beef heart bc_1 complex (mainly heme b_L). (E) DBH minus ascorbate difference spectrum of the bc_1 complex from *Polytomella* spp. (heme b_H). (F) DBH minus ascorbate difference spectrum of the beef heart bc_1 complex (heme b_H).

from that of the beef heart bc_1 complex, which exhibits an almost symetric band with a maximum at 564 nm (Fig. 4F). This set of spectra clearly shows that the *b*-type cytochrome from *Polytomella* spp. differ from those of the beef-heart bc_1 complex, namely for the 3- to 4-nm red-shift and for the split α-band of heme b_H.

Midpoint potentials of the b-type hemes of the bc_1 complex from Polytomella spp. and spectra taken at selected redox potentials. The electrochemical titration of the cytochrome *b* hemes in the bc_1 complex from *Polytomella* spp. is shown in Fig. 5. The data indicated the presence of three species of *b*-type hemes. The two major components, representing 39 and 34% of the population, corresponded to the b_L and b_H hemes, respectively. At pH 7.8 (final measurement after titration), the midpoint potentials of b_L was −143 mV and of b_H +25 mV. A third fraction, which represented 27% of the population, exhibited a very high mid-point potential of +146 mV. This component was identified as the b_{150} species observed by other investigators (5, 20, 21), and that probably comprises the fraction of b_H-hemes with a quinone bound at the Q_i center (21). An electrochemical titration of the beef-heart bc_1 complex was carried out in a parallel experiment at pH 6.8 (final measurement after titration), yielding midpoint potentials of −115 mV for b_L and +75 mV for b_H. Noteworthy, the b_{150} species was not detected in this set of experiments.

The spectra of the b_H and b_L hemes were then recorded at selected redox potentials, where the absorption contribution of other component was negligible. The spectra of the individual hemes, optimized for maximal resolution at appropiate redox potentials are shown in Fig. 6. Since redox potentials were chosen to avoid spectral overlap, the intensities of the illustrated spectra do not represent the actual concentrations of the individual components. Heme b_L from *Polytomella* spp. shows a split α-band with a maximum at 568.4 nm and a shoulder at 558.7 nm, and heme b_H also exhibits a dual α-band with maxima at 568.9 and 557.2 nm. However, the ratio 568/558 was found to significantly differ between these two hemes. Heme b_{150} also shows dual α-bands with maxima at 558.8 and 568.8 nm.

The deconvoluted reduced minus oxidized spectra of the two cytochrome *b* hemes are shown in Fig. 7. The optical spectra were fitted with Lorentzian functions; the reported values are the means of five different measurements. For the α-peak of the b_L heme two Lorentzian components were obtained with maxima at

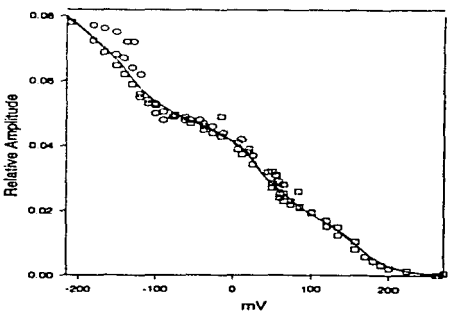

FIG. 5. Electrochemical titration of the cytochrome *b* hemes of the bc_1 complex of *Polytomella* spp. Data points obtained during titration in the reductive direction (□) data points obtained during titration in the oxidative direction (○). The curve was fitted for considering one-electron acceptors. Three components with midpoint potentials of −143, +25, and +146 mV were identified, comprising 39, 34, and 27% of the total cytochrome *b*.

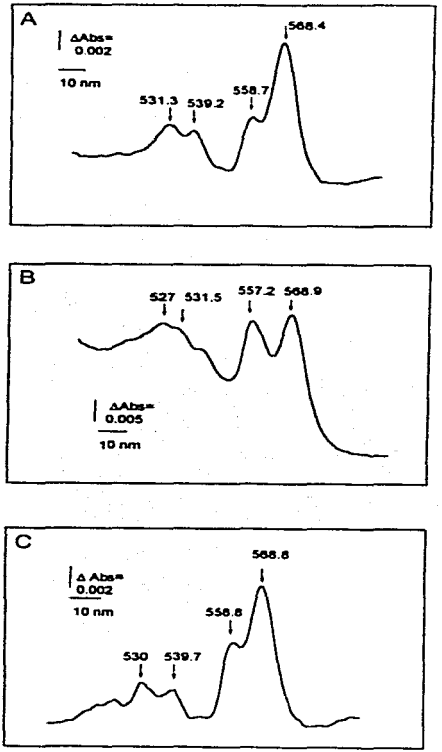

FIG. 6. Difference spectra of the three species of *b* hemes identified by electrochemical titration. The difference spectra were recorded at pH 7.0 and at the following redox potential spans: −367/−75 mV for heme b_L (A), −38/+54 mV for heme b_H (B), and +101/+157 mV for b_{150} (C).

558.7 and 568.4, with relative peak areas of 0.15 and 0.85, respectively. The full-width at half-height (FWHH) of the peaks were 5.96 and 7.82 nm, respectively. Thus, in the b_L heme the lower energy transition is more intense than the higher energy transition. Relative peak areas were about 1:5.5, which contrast with the data obtained for the b_L heme from mouse, with relative peak areas of 4:1 (5). Two Lorentzian functions could also be fitted in the α-peak from the b_H heme, exhibiting maxima at 557.2 and 568.9 nm, with relative peak areas of 0.33 and 0.67, respectively. The FWHH of the peaks were 6.63 and 7.80 nm, respectively. Thus, two spectral components of the b_H heme from *Polytomella* spp. were clearly defined, with relative peak areas of about 1:2, which strongly contrasts with the single transition for the b_H heme in mammalian preparations (5).

The spectral features described above conform to those obtained in the presence of reductants (Fig. 4), which indicates that the b_L and b_H hemes from *Polytomella* spp. exhibit different midpoint potentials, but share similar spectroscopic properties, with two transitions in their α-bands.

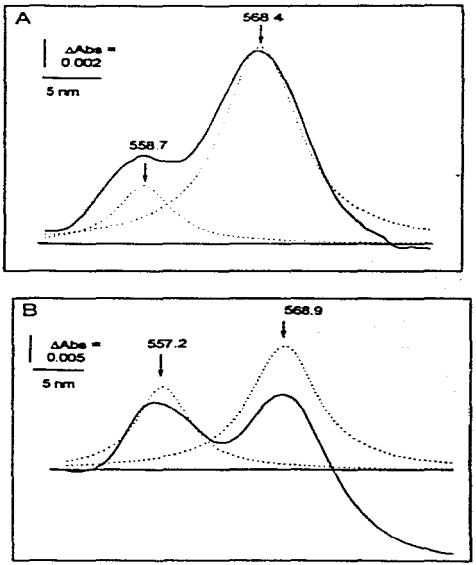

FIG. 7 Difference spectra of the *Polytomella* spp. b_L (A) and b_H (B) hemes. The difference spectra were recorded at pH 7.0 and at the following redox potential spans: −367/−75 mV for heme b_L and −38/+54 mV for heme b_H. The solid lines represent the original spectra, and the dotted lines the Lorentzian components obtained after base line correction and deconvolution.

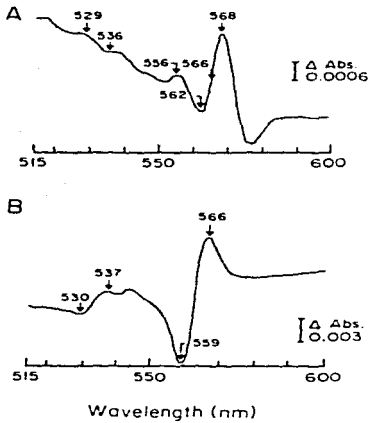

FIG. 8. Comparison between the red-shift caused by antimycin A in the spectra of the *Polytomella* spp. bc_1 complex (A) and the beef bc_1 complex (B). Difference spectra were recorded for the two purified bc_1 complexes (dithionite reduced in the presence of antimycin A minus dithionite reduced). The principal peaks and valleys obtained in the difference spectra for the two complexes are indicated.

The red-shift induced by antimycin A. Antimycin A is a potent inhibitor of the ubiquinol–cytochrome *c* oxidoreductase activity of the bc_1 complexes (22), including the one from *Polytomella* spp. (15). This inhibitor has been shown to affect the spectroscopic properties of heme b_{H} from different organisms (5, 23, 24). The effect of this inhibitor on the spectroscopic features of the *b*-type hemes from *Polytomella* spp. was therefore investigated. Fig. 8 compares the red-shift induced by antimycin A on *Polytomella* spp. and beef heart bc_1 complexes. The *Polytomella* spp. bc_1 complex difference spectrum (dithionite-reduced in the presence of antimycin A minus dithionite-reduced) exhibited a red-shift, with a maximum at 568 nm and a smaller transition at 556 nm. In contrast, the difference spectrum of the bovine bc_1 complex exhibited a single transition with a maximum at 566 nm, consistent with the previously described red-shifts (23, 24). It could thus be concluded that while antimycin A induces a red-shift on the single α-band transition of the b_{H} absorption spectrum of the bovine enzyme, the effect of this inhibitor is evident on the two α-band transitions of the b_{H} heme from *Polytomella* spp., in accordance with the presence of well-defined split α-band of this heme moiety.

DISCUSSION

The mitochondrial cytochrome b from Polytomella spp. exhibits a red-shifted α-band. When the difference spectrum of the reduced minus oxidized mitochondrial fraction from *Polytomella* spp. was recorded, two clearly resolved α-band peaks, with maxima at 553 and 567 nm, corresponding to cytochromes c_1 and *b*, respectively, were observed. These two absorption maxima were also observed in the spectrum of the purified, fully reduced bc_1 complex from *Polytomella* spp., with maxima at 553 and 567 nm at room temperature (15). A similar two-peak spectrum of the bc_1 complex was previously described in mitochondria of *Polytomella caeca* (25), with maxima at 549 nm for cytochrome c_1 and at 562 nm for cytochrome *b* at liquid nitrogen temperature. The corresponding spectra of other bc_1 complexes show overlapping bands, in which the absorption band of cytochrome c_1 appears as a shoulder at 553 nm of the dominant band at 563 nm of the *b* cytochrome (5, 26, 27). Cytochrome c_1 from *Polytomella* spp. shows a maximum in the α-band at 553 nm and contains the classical heme-binding consensus sequence CXXCH (15); therefore, cytochrome c_1 is similar to the corresponding cytochromes in bacteria, animals, higher plants, and fungi. In contrast, the red-shifted α-band maximum of cytochrome *b* from *Polytomella* spp. differs from the one found in all other organisms.

The cytochrome b of the bc_1 complex of Polytomella spp. contains protohemes as prosthetic groups. Structural homologs of *b*-type cytochromes with classical cytochrome *b* functions that bind *a*-type hemes have been identified in archaebacteria (28). The possibility that the atypical absorption spectrum of *Polytomella* spp. *b*-type cytochrome was due to differences in the prosthetic group has been ruled out by HPLC analysis. Therefore, the unique spectroscopic properties of the cytochrome *b* from *Polytomella* spp. must be due to the amino acid environment that surrounds the heme moieties.

The b_{L} heme from Polytomella spp. exhibits two spectroscopic transitions in the α-band. With specific reductants the individual spectra of the *b*-type hemes of the purified bc_1 complex from *Polytomella* spp., showed that the b_{L} heme is spectroscopically similar to those of the corresponding cytochrome from animals, plants, yeast, or bacteria, exhibiting two transitions in the α-region. Nevertheless, the low-energy contribution is much more important in *Polytomella* spp. than in the murine enzyme (5). However, it is the b_{H} heme that exhibits the most striking spectral properties. The high resolution spectral analysis of the b_{L} and b_{H} hemes from the bc_1 complex from mouse (5) showed that heme b_{L} exhibits a split α-band with maxima at 558.1 and 565.2 nm, while heme b_{H} has a single band with maximum at 561.6 nm. Similar results were obtained in the spectroscopic characterization of the b_{L} (b_{566}) and the

b_H (b_{560}) hemes from potato tuber mitochondria (27). In addition, the difference spectra reported for the *b*-type hemes of the bc_1 complex from bacteria, also show a b_L heme with a split α-band, while the b_H heme moiety exhibits a single transition (16, 26, 29). The optical properties of the bc_1 complexes from diverse species have been reported, and generally they show consistent features for the split α-band of the b_L heme; the limited variation of the b_L spectra has been related to the more conserved protein environment predicted to surround this heme within the cytochrome *b* structure (30). However, the spectral differences of *Polytomella* spp. appear to be unique, particularly because in no other species a split and red-shifted high potential b_H heme has ever been observed.

The redox titration of both *b*-type hemes from *Polytomella* spp., yielded values of +25 mV for heme b_H and of −143 mV for heme b_L, giving a redox span of 168 mV. This span is higher than 123 mV obtained by Howell and Robertson (5) for mouse bc_1 complex, i.e., E_h of −31 mV for the heme b_L and E_h of +92 mV for heme b_H, and for the redox span of 128 mV in potato bc_1 complex, with E_h of −77 mV for the heme b_L and E_h of +51 mV for heme b_H (27). These values indicate that redox spans between 120 and 168 mV are compatible with the presence of fully active bc_1 complexes with turnover rates of 300 s^{-1} or more. Further analysis of the residues that surround the hemes remains to be done, to understand how the protein environment modulates the midpoint potentials of these prosthetic groups.

The electrochemical titration also revealed the presence of a third *b*-type component, identified as b_{150}, a fraction of b_H hemes with bound quinone at the Q_i center (21). Surprisingly, the concentration of b_{150} was relatively high, considering that titrations were carried out at pH 7.8, a condition that apparently does not favor its formation (5, 21). In contrast, the b_{150} species was not observed in titrations carried out with the beef-heart enzyme in the same conditions. In addition, a relatively high concentration of lauryl maltoside (2 mg/mg of protein) was required to solubilize the bc_1 complex from *Polytomella* spp. mitochondria (15). This high number of detergent micelles should not favor the binding of the quinone to the Q_i center; an association that appears to be particularly strong in the bc_1 complex from this alga.

The spectra obtained at different redox spans confirmed that the b_H and b_L hemes from *Polytomella* spp. exhibit split α-bands with peaks close to 558 and 568 nm. Small differences were found when comparing the α-peak maxima obtained at the midpoint potentials with those obtained with single reductants. The most striking result obtained in this study is that the b_H heme shows an α-band splitting at room temperature. The deconvolution analysis demonstrated the presence of an important component at 557.2 nm, that contributes with 33% of the total absorption band of the α-peaks of this heme, and that indicates a strong decrease in the *x*–*y* degeneracy of the tetrapyrrole ring.

The effect of antimycin on the absorption spectra of the b-type cytochromes from Polytomella spp. The specific bc_1 complex inhibitors, myxothiazol and antimycin, are known to cause distortions in the absorbance spectra of the bc_1 complex. Myxothiazol binds in the vicinity of the heme b_L center, while antimycin A binds in the vicinity of the b_H center (5, 31).

Antimycin A caused a red-shift on the bc_1 complex spectrum from *Polytomella* spp. Two transitions at 556 and 568 nm were observed. This inhibitor also caused a red-shift on the gamma peak of the cytochrome (data not shown). When compared with the beef bc_1 complex, the shift in *Polytomella* spp. was 2 nm more toward the red, and it exhibited the two transitions not seen in the beef bc_1 complex. Although long-range effects on the b_L heme have been described (5, 21), antimycin A exerts its main effect on the heme b_H moiety of cytochrome *b*. We conclude that the red-shift induced by antimycin A on the b_H heme is consistent with the presence of two spectroscopic transitions in this prosthetic group.

From the data discussed above, we conclude that heme b_H from *Polytomella* spp. is different from the corresponding one from mammals, plants, and bacteria and that this difference makes an important contribution, along with heme b_L, to the observed red-shift of cytochrome *b*. Link *et al.* (32), have modeled a three-dimensional structure of cytochrome *b*. Differences were obtained in the distribution of aromatic residues in the vicinity of the hemes: two phenylalanines and one tyrosine were found to be perpendicular to the b_L porphyrin ring, which allows attractive electrostatic interactions. In contrast, the b_H heme is surrounded by three tryptophan residues that form a densely packed hydrophobic environment. The data obtained from the crystal structure of cytochrome *b* from beef heart mitochondria (14) allows us to pinpoint specific residues that surround the tetrapyrrole rings and that may contribute to the spectroscopic properties of the hemes. The b_H site is relatively loosely surrounded by a section of random coil before the beginning of helix A, helix A, helix B, the BC loop, helix C, helix D, and a portion of the DE loop. When comparing differences in the primary structures of these regions of the bovine enzyme (33), with the one of *Chlamydomonas reinhardtii* (34) a close relative of *Polytomella* spp. (35), the following differences were found (numbered is in accordance with the bovine sequence, followed by the substitution in *C. reinhardtii* in parenthesis): W_{30} (S) and F_{33} (G) in the random coil before helix A; L_{37} (A) in helix A; C_{93} (V) in helix B; Y_{107} (G) and F_{109} (R) in the BC loop, T_{112} (V) in helix C; A_{193} (S) and F_{199} (A) in helix D; and E_{202} (Q), T_{203} (Y), and T_{209} (L) in the DE loop. In contrast, the b_L heme pocket is tightly surrounded by helix A,

the AB loop, helix B, helix C, two portions of the CD loop, and helix D (14). When the same analysis was performed, minor but significant differences between the beef-heart and *C. reinhardtii* structures were found: W_{77} (M) in the AB loop; K_{172} (N) in the CD loop; and F_{189} (Y) in helix D. Based on these structural features, we propose that in the case of the b_H heme from *Polytomella* spp., a set of residues surrounding this prosthetic group alters the x–y degeneracy of the porphyrin ring and that these changes in protein conformation affect the structural anisotropy of the electronic environment around this heme, giving rise to a split and red-shifted α-band. Differences in residues that surround heme b_L may also contribute to the presence of a red-shifted α-maximum in its absorption spectrum.

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Apéndice II:

Métodos.

1. Crecimiento de *Polytomella* spp. y *C. reinhardtii*.

Se empleó una cepa de *Polytomella* spp. (198 80, E. G. Pringsheim) obtenida de la colección de algas de la Universidad de Göttingen, Alemania (Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universitat Göttingen). Actualmente es mantenida en la colección microbiológica del Departamento de Biotecnología del CINVESTAV, I.P.N. con el número de registro CDBB-951. *Polytomella* spp. se creció a temperatura ambiente y sin agitación en matraces de fondo ancho en un medio con 0.4 % de acetato de sodio, 0.2 % de extracto de levadura y 0.2 % de bactotriptona (Wise, 1959). Después de esterilizar el medio, se adicionó vitamina B1, 0.057 µg/ml y vitamina B12, 0.025 µg/ml esterilizadas por filtración. El cultivo se cosechó después de aproximadamente 48 horas de crecimiento mediante centrifugación a 5000 rpm en un rotor GS-3 por 7 minutos. Estas células pueden emplearse inmediatamente o guardarse a -70°C si se resuspenden en un medio con sacarosa 0.32 M, EDTA-K 4 mM y Tris (pH 7.4) 20 mM.

La cepa de *C. reinhardtii* empleada fue obtenida en el *Chlamydomonas* Genetics Center en la Universidad de Duke. Se creció en medio TAP que se prepara de acuerdo a Rochaix y col. (1988):

Solución de Beijerinck 20 X	50 ml
Ácido acético glacial	1 ml
Tris	2.42 g
Solución de fosfatos (K)PO_4 1M pH 7.0	1 ml
Elementos traza	1 ml
Agua hasta 1 litro.	

Solución de Beijerinck 20 X

NH_4Cl	8 g
$CaCl_2 \cdot 2H_2O$	1 g
$MgSO_4 \cdot 7H_2O$	2 g
Agua hasta 1 litro.	

Solución de fosfatos 1M pH 7.0

K_2HPO_4 1M	250 ml
KH_2PO_4 1M	~170 ml
(Agregar hasta tener pH 7.0)	

Elementos traza

EDTA-Na_2	50.0 g
$ZnSO_4 \cdot 7H_2O$	22.0 g
H_3BO_3	11.4 g

$FeSO_4 \cdot 7H_2O$	4.99 g
$MnCl_2 \cdot 4H_2O$	5.06 g
$CoCl_2 \cdot 6H_2O$	1.61 g
$CuSO_4 \cdot 5H_2O$	1.57 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10 g

Se disuelven los siguientes compuestos por separado:
H_3BO_3 en 200 ml
$ZnSO_4$ en 100 ml
EDTA en 250 ml
Todos los demás compuestos se disolvieron en 50 ml cada uno.

Se mezclaron todas las soluciones excepto la de EDTA. Apareció un color violeta. Se hirvió varios minutos hasta que la solución se observó verde translúcido y entonces se adicionó la solución de EDTA
Se enfrió ligeramente pero sin bajar de 70 C y se ajustó el pH de la solución a 6.5-6.8 con KOH al 20% (requiere menos de 100 ml)
La solución se diluyó a 1 litro y se incubó a temperatura ambiente con un tapón de algodón por aproximadamente 2 semanas hasta que se tornó violeta. Se filtró el precipitado rojo que se formó y se conservó a temperatura ambiente

Las células se crecieron por 3 o 4 días con agitación constante y en presencia de luz. Posteriormente se cosecharon mediante centrifugación a 100xg por 10 minutos.
La cepa se puede crecer en medio sólido si se agrega 1 5% de agar al medio TAP.

2. Obtención y solubilización de mitocondrias de *Polytomella* spp.

Las mitocondrias se obuvieron de acuerdo a Gutiérrez-Cirlos y col. (1994). Las células resuspendidas con 4 volúmenes de medio con sacarosa 0 32 M, EDTA-K 4 mM y Tris (pH 7.4) 20 mM. Se rompieron en un homogenizador de vidrio con vástago de teflón haciéndolas pasar por éste 5 veces (Lloyd y Chance, 1968). El homogenizado se centrifugó a 1 000xg 8 minutos y el sobrenadante se centrifugó de igual manera. Para obtener las mitocondrias, este sobrenadante se centrifugó a 10 000 · g 15 minutos (12 000 r.p.m. en rotor SS34) y el botón de mitocondrias obtenido se lavó con sacarosa0.32 M, EDTA-K 4 mM y Tris (pH 7 4) 20 mM. Las mitocondrias se resuspendieron en el mismo amortiguador.

Las mitocondrias se diluyeron con aproximadamente 80% del volumen necesario de amortiguador TM (Tris-HCl 50 mM pH 8.0, $MgSO_4$ 1 mM, PMSF 1 mM, TLCK 50 µg/ml en presencia de NaCl 100 mM para tener una concentración de 10 mg de proteína por ml.

Por otro lado se adicionó gota a gota a las mitocondrias en hielo y con agitación suave el resto de amortiguador TM-NaCl 100 mM con lauril maltósido disuelto en una proporción de 2g de detergente por 1 g de proteína mitocondrial. Las mitocondrias solubilizadas se incubaron media hora en hielo con agitación suave y posteriormente se centrifugaron a 80 000·g por 15 minutos (30 000 r p m. en un rotor 50 Ti) para separar el material no solubilizado. El sobrenadante se dializó contra 10 volúmenes de amortiguador TM sin NaCl y sin detergente durante 2 horas a 4 C.

3. Purificación de la citocromo *c* oxidasa de *Polytomella* spp.

Las mitocondrias solubilizadas y dializadas se incubaron 10 minutos a 4°C y con agitación constante en presencia de colato de sodio al 1.6% y sulfato de amonio a una saturación del 40%. Se centrifugó a 10 000xg 15 minutos y el botón verde, rico en citocromo *c* oxidasa se resuspendió en amortiguador TM con NaCl 100 mM y lauril maltósido al 1.2%. La muestra se centrifugó a 80 000xg 15 minutos y el sobrenadante se dializó contra 10 volúmenes de amortiguador TM. Esta preparación se cargó en una columna de intercambio iónico de DEAE-Biogel A de BioRad previamente equilibrada con amortiguador TM en presencia de 0.1 mg/ml de lauril maltósido. La columna se lavó con 3 volúmenes de este amortiguador. Posteriormente se lavó con 2 volúmenes del mismo amortiguador pero con un gradiente de 0 a 100 mM de NaCl.

La fracción rica en citocromo *c* oxidasa se obtuvo al lavar la columna con el amortiguador de equilibrio en presencia de 200 mM de NaCl. Se colectaron fracciones de 100 gotas por tubo con un colector marca Gilson y se leyo su absorbancia a 280 nm para detectar proteína y a 415 nm para detectar citocromos en un espectrofotómetro Shimadzu. Las fracciones verdes ricas en citocromo *c* oxidasa se concentraron en un ultrafiltro de Amicón de 50 ml con una membrana YM100 de 43 mm. Se determinó la cantidad de proteína en esta preparación, y se guardó en alicuotas a -70°C hasta su uso.

4. Purificación de la citocromo *c* oxidasa de corazón de bovino.

Esta enzima se purificó de acuerdo al método de Capaldi y Hayashi (1972) y se guardó en alicuotas a -70°C.

5. Cuantificación de proteína.

La concentración de proteína se determinó mediante el método de Lowry y col. (1951) con la modificación de Markwell y col. (1978).

6. Actividad de la citocromo *c* oxidasa.

La actividad de la citocromo *c* oxidasa se midió espectroscópicamente de acuerdo al método de Errede y col. (1978). Se midió la actividad en un volumen final de 3ml de amortiguador TM con agitación constante en presencia de lauril maltósido 0.1 mg/ml , antimicina 20μM y citocromo *c* de caballo reducido (marca Sigma) 30 μM. Para reducir al citocromo *c*, se preparó una solución concentrada de éste en un amortiguador de fosfato de potasio 10 mM pH 7.0 y se redujo con unos granitos de ascorbato hasta que la solución adquirió una coloración rosa. Posteriormente se pasó por una columna de 10 mm × 70 mm de Sefadex G25 equilibrada con el mismo amortiguador, con la finalidad de separar al citocromo *c* reducido del exceso de ascorbato. La concentración de citocromo *c* se determinó de acuerdo a González- Halphen y col. (1991), tomando como coeficiente de extinción molar para el citocromo *c* el valor de 18.5 $mM^{-1}cm^{-1}$ a 550 nm. La reacción comienza al adicionar al medio 20-50 μg de oxidasa y se registra la velocidad de disminución de citocromo *c* reducido al medir el cambio de absorbancia a 550 nm con el tiempo. Si se desea observar sensibilidad de la citocromo *c* oxidasa a cianuro, se adiciona con microjeringa KCN preparado el mismo dia a una concentración final de 0.5 a 1 mM. Tanto las mediciones de actividad de la oxidasa como la determinación de su espectro se llevaron a cabo en un espectrofotómetro de longitud de onda dual DW-2a Aminco modificado por ON-Line Instrument System Co. Este equipo emplea una paquetería de Outstanding Laboratory Instrument and Software-Rapid Scanning System (OLIS-RSM).

7. Espectro UV-visible de la citocromo c oxidasa.

El espectro UV-visible se obtuvo a temperatura ambiente a partir de la citocromo *c* oxidasa como se obtiene en la preparación y de la citocromo *c* oxidasa reducida con unos granitos de ditionita ($Na_2S_2O_4$). El amortiguador empleado fue TM con 0.1 mg/ml de lauril maltósido y la concentración de oxidasa fue de aproximadamente 1.5 mg/ml. La concentración de hemos tipo *a* se determinó mediante el coeficiente de extinción molar $\Delta\varepsilon_{(630-605\ nm)}$ = 16.5 $mM^{-1}cm^{-1}$ (Griffiths and Wharton, 1961. Ozawa, T., y col., 1975).

8. Gel desnaturalizante de poliacrilamida

Se hicieron geles de placa de 1.2 mm de espesor al 16% de acrilamida en una cámara BRL (Vertical Gel Electrophoresis System) de acuerdo a la técnica de Schägger y col. (1986).

9. Estimación de masas moleculares de proteínas.

En dos carriles contiguos de un gel de poliacrilamida se cargaron 30 µg de citocromo *c* oxidasa de *Polytomella* spp. y de bovino. Posteriormente el gel se tiñó con azul de Coomassie. Se empleó el equipo UltroScan XL de LKB que realizó barridos densitométricos del gel de poliacrilamida con un haz de láser de helio-neón. A través del barrido densitométrico se determinó la distancia de migración de cada subunidad en el gel denaturalizante. Como marcador de pesos moleculares se empleó a la citocromo *c* oxidasa de bovino, ya que las masas moleculares de todas sus subunidades se conocen (Capaldi y col., 1988). Las masas moleculares aparentes de las subunidades de la citocromo *c* oxidasa de *Polytomella* spp. se calcularon a partir la masa molecular de las subunidades de la citocromo *c* oxidasa de bovino.

10. Inmunorréplicas tipo Western.

Se empleó la técnica descrita por González-Halphen y col. (1988). Los anticuerpos contra COXI, COXII y COXIII de levadura que se usaron se obtuvieron de Molecular Probes (Eugene, Oregon).

11. Secuenciación de proteínas.

11.1. Secuencia del extremo amino terminal por degradación de Edman.

Se prepararon las proteínas de interés de acuerdo al método reportado por Gutiérrez-Cirlos y col., 1994 y la secuenciación del extremo amino terminal de estas se llevó a cabo en un secuenciador Applied Biosystems en el Laboratoire de Microséquençage des Protéines del Instituto Pasteur en París, Francia.

11.2. Secuencia interna de proteínas mediante digestiones proteolíticas.

Se corrió un gel de acrilamida desnaturalizante que contenía aproximadamente 100 µg de oxidasa por carril. Se recomienda que las soluciones para preparar y correr el gel sean frescas. El gel se fijó 2 veces durante 30 minutos con metanol al 50% y ácido acético al 10%. Posteriormente se tiñó por varias horas con amido negro 0.003% en metanol 45% y ácido acético al 10%. Cuando la banda de interés fue visible, ésta se cortó y se lavó con agua desionizada para eliminar el exceso de metanol, ácido acético y colorante. Finalmente se secó ligeramente por centrifugación al vacío sin que la banda se deshidratara por completo.

Las digestiones de la proteína y la secuenciación de cada fragmento se llevaron a cabo por J. D'Alayer en el Laboratoire de Microséquençage des Protéines del Instituto Pasteur en París, Francia. Se llevaron a cabo incubaciones de la muestra con tripsina y endolisina. Posteriormente se separaron los productos mediante columnas de HPLC o mediante geles desnaturalizantes y se analizó la secuencia de sus extremos amino terminales mediante degradación de Edman.

12. Purificación de DNA total de *Polytomella* spp. y *C. reinhardtii*.

El DNA de *Polytomella* spp. y *C. reinhardtii* se obtuvo de igual manera, pero las células se rompieron por métodos diferentes. En el caso de *Polytomella* spp., se empleó un cultivo de 48 horas de 2 litros de medio. Las células se colectaron mediante centrifugación a 5000 rpm en un rotor GS-3 por 7 minutos. Las células se rompieron al resuspender en 25 ml de amortiguador QTP (Tris-HCl 10 mM pH 8.0, NaCl 100 mM, EDTA-Na 10 mM, y SDS 1%.) a 4 °C.

En el caso de *C. reinhardtii*, se cosecharon 500 ml o 1 litro de cultivo crecido por 3 o 4 días en agitación constante y con luz. Las células se lavaron con 100 ml de Tris 10 mM pH 8.0, EDTA-Na 1mM. Posteriormente, las células se resuspendieron en 5 ml de citrato de sodio 100 mM y se congelaron en nitrógeno líquido. Se adicionó un volumen de SDS 2% y se incubaron a 60 °C 15 minutos.

Las células rotas se extrajeron 2 veces con un volumen de fenol-cloroformo (1:1) y una vez con un volumen de cloroformo. La fase acuosa se precipitó con 0.1 volúmenes de acetato de sodio 3M pH 5.0 y 3 volúmenes de etanol y se incubó 15 minutos a -70 °C. Posteriormente se centrifugó a 10000 x g 10 minutos y el DNA precipitado se lavó con etanol al 70%. Se resuspendió en 4 o 5 ml de agua y el DNA se incubó con 2.5 µg de RNasa de Boehringuer por 3 horas a 37°C. Posteriormente se llevaron a cabo dos extracciones con un volumen de fenol-cloroformo (1:1) y finalmente con un volumen de cloroformo. El DNA se precipitó y lavó de igual manera que anteriormente. Se resuspendió en 1 o 2 ml de agua y se guardó a 4°C toda la noche para completar su disolución. Posteriormente se guardó a -20°C.

13. Purificación de RNA total de *Polytomella* spp. y *C. reinhardtii*.

El RNA total de estas algas se obtuvo de 80 ml de cultivo de *Polytomella* spp. o 50 ml de cultivo de *C. reinhardtii* y se empleó el sistema de RNeasy de QIAGEN de acuerdo a las instrucciones del distribuidor.

La concentración de RNA se determinó midiendo la absorbancia a 280 nm en un espectrofotómetro. La pureza y calidad del RNA se determinó mediante la relación A_{260nm}/A_{280nm} así como con la forma del espectro obtenido en esta región. El RNA obtenido puede emplearse para correr geles de agarosa o para preparar primera hebra de cDNA.

Cuando se prepara RNA para obtener primera hebra de cDNA a veces es conveniente hacerle un tratamiento con la DNasa libre de RNasas de QIAGEN durante la purificación del RNA, de acuerdo a las instrucciones del proveedor.

14. Geles de formamida-formaldehído para RNA.

El RNA se analizó en geles de agarosa 1 o 1.2% con formamida-formaldehído 0.66 M. Se cargaron entre 15 y 20 µg de RNA por carril. El tratamiento del material de vidrio y plástico, la preparación de soluciones, así como la preparación de la muestra se hicieron de acuerdo al método de Sambrook y col. (1989).

15. Purificación de DNA de geles de agarosa.

La banda de DNA que se deseaba purificar de un gel se corrió lo suficiente para separarla de otras bandas no deseadas. Esta se cortó del gel, se colocó en un tubo de 1.5 ml y se pesó. Para purificar la banda del gel se emplearon dos sistemas diferentes, uno de Boehringer Mannheim y el otro conocido como QIAEX II Agarose Gel Extraction Protocol, de QIAGEN. Las instrucciones de uso de cada sistema se proporcionan por el proveedor.

16. Marcaje radioactivo de DNA para sondas.

Se empleó el sistema de cebadores al azar de Gibco BRL para marcar aproximadamente 30 ng de DNA usado como sonda con ^{32}P-dCTP 10 mCi/ml.

17. Análisis tipo Southern.

Se cargaron 30 µg de DNA por carril de un gel de agarosa al 0.8% o 1%. La transferencia de DNA de geles de agarosa a membranas de nylon (Hybond de Amersham) por capilaridad se llevó a cabo de acuerdo a Sambrook y col. (1989). Ésta se lleva a cabo toda la noche, y al día siguiente la membrana se entrecruzó con luz UV en un entrecruzador de Stratagene.

18. Análisis tipo Northern.

La transferencia de RNA de geles de formamida-formaldehído a membranas de nylon por capilaridad se llevó a cabo de acuerdo a Sambrook y col. (1989). Antes de la transferencia, el gel se incubó por dos horas en agua tratada con DEPC (dietil pirocarbonato) con un cambio de agua después de la primera hora. El dispositivo de transferencia fue igual al de las transferencias tipo Southern, pero se empleó como amortiguador de transferencia SSC 10X tratado con DEPC. La membrana se sometió a luz UV como en el caso anterior para que el RNA quede protegido de degradación y fijo a la membrana.

19. Prehibridación e hibridación de membranas de Nylon.

Las membranas se prehibridan durante 2 o 3 horas a la temperatura deseada con el siguiente amortiguador:

SSC 6×
Reactivo de Denhardts 5×
Tris-HCl 20 mM pH 8.0
EDTA-Na 2mM pH 8.0
SDS 0.2%
DNA de esperma de arenque 0.1 mg/ml.

El DNA de esperma de arenque se incubó en agua en ebullición 10 minutos e inmediatamente después se incubó en hielo 10 minutos antes de agregarse al medio de prehibridación. Para hibridar la membrana se empleó el mismo amortiguador pero solamente se adicionó la sonda marcada radioactivamente y se dejó incubar en esta solución toda la noche a la temperatura deseada.

20. Reacción en cadena de la polimerasa (PCR).

En general, se preparó la siguiente mezcla de reactivos para un volumen final de 50 µl y por cada tubo de reacción:

- 1 µl de oligodesoxinucleótidos (dATP, dCTP, dGTP, dTTP) 10 mM cada uno.
- 5 µl de amortiguador de polimerasa Taq 10× marca Gibco BRL.
- 2 µl de $MgCl_2$ 50 mM para una concentración final de Mg^{2+} de 2 mM.
- 0.5 µl de polimerasa Taq recombinante (5 unidades/µl) de Gibco BRL.

- (opcional) Cuando se emplea DNA de *C. reinhardtii*, con un alto contenido de G y C, se recomienda agregar 10 µl de amortiguador Q (QIAGEN).
- Agua para 39 µl.

En cada tubo se adicionaron 200 ng o 20 pmoles de cada oligodesoxinucleótido en 5 µl y entre 200 ng a 1 µg de DNA, normalmente contenido en 1µl. Si el templado es DNA total, es necesario romperlo un poco pipeteando varias veces. Los ciclos empleados en general fueron:

5'	94°C	
45"	94°C	35 ciclos
1'	45°C	
2'	72°C	
7'	72°C	

Para analizar los productos de PCR, se corrieron entre 10 y 15 µl de cada uno en un gel de agarosa al 1% o 1.2%. Las secuencias de todos los desoxioligonucleótidos empleados en este trabajo se encuentran en el apéndice III.

21. Preparación de la primera hebra de cDNA.

Las reacciones se llevaron a cabo en un termociclador que usa tubos de paredes delgadas de 200 µl. El cDNA obtenido puede ser guardado a -20°C. Se emplearon 4 diferentes sistemas de transcriptasa reversa. Thermo RT™ (Display Systems Biotech), M-MuLV RT (Promega), Superscript™II (Gibco BRL) y Omniscript (QIAGEN). En todos los casos se emplearon 1 o 2 µg de RNA total y 25 unidades de inhibidor de Rnasas (Boehringer). Para preparar primera hebra de cDNA de *C. reinhardtii*, es recomendable incubar al RNA en agua a 65°C 5 minutos y posteriormente en hielo. Adicionalmente, se recomienda agregar amortiguador Q 1x de QIAGEN.

Como cebadores de la transcriptasa reversa pueden emplearse oligodesoxinucleótidos especificos, oligo d(T)/adaptador u oligo Q_T (para consultar las secuencias de estos oligodesoxinucleótidos, dirigirse al apéndice III)

22. Adición de colas poli A al extremo 5' del cDNA.

La finalidad de esta reacción es adicionar al extremo 5' del cDNA una cola de poli (A) que nos permita amplificar completa esta región del gen de interés. Se empleó el sistema de transferasa terminal de Boehringer Mannheim. La mezcla de reacción fue la siguiente:

2 o 4µl de primera hebra de cDNA.
4 µl de amortiguador para transferasa terminal 5×.
1 µl dATP 4 mM.
6 µl de $CoCl_2$ 2 5 mM.
1 µl transferasa terminal (25 U).
Agua hasta 20 µl.

Se Incubó 8 minutos a 37°C.

Posteriormente se incubó 5 minutos a 65°C para desactivar a la transferasa terminal.

El cDNA se guardó a -20°C.

23. Amplificación rápida del extremo 5' de cDNA.

Como templado para esta reacción de PCR se usó 1µl de cDNA con una cola de poli (A) en el extremo 5'. Cuando se emplea esta técnica con cDNA de *C. reinhardtii* se recomienda agregar amortiguador Q (QIAGEN). Este PCR se llevó a cabo en dos etapas:

Primer PCR.

Sin Taq, con sólo 2 pmol de 1) oligo d(T)/adaptador ó 2) QT.

5' 95°C
6' 75°C ◄— Se adicionaron 2.5 unidades de polimerasa Taq por tubo.
5' 48°C
15' 72°C ◄— Se adicionaron 20 pmol de adaptador + 20 pmol de oligodesoxinucleótido especifico.
↓
1' 95°C
1' 55°C } 40 ciclos
2' 72°C
↓
7' 72°C

Segundo PCR.

Es un PCR anidado en el que se emplea 1 µl del primer PCR diluído 1:20. Las condiciones de reacción fueron las siguientes:

Sin Taq, con 20 pmol de adaptador y 20 pmol de cebador especifico anidado.

5' 95°C
6' 75°C ◄— Se adicionaron 2.5 unidades de polimerasa Taq por tubo.

1' 95°C
1' 55°C } 30 ciclos
2' 72°C
↓
7' 72°C

24. Clonación de productos de PCR.

24.1. Ligación y transformación.

Los productos de PCR purificados del gel de agarosa se ligaron con el sistema de pMos-blunt Ended Cloning de Amersham™ ó con el sistema de pGem®–T Easy Vector System de Promega. Las instrucciones para preparar la mezcla de ligación así como las condiciones de transformación de células competentes de *E. coli* se proporcionan por el proveedor. Las células transformadas se sembraron en cajas de LB con ampicilina 100 µg/ml agregando directamente a las bacterias transformadas 10 µl de IPTG 800 mM y 40 µl de x-Gal 2%. Se seleccionaron las colonias con inserto por su resistencia a ampicilina y por la falta de actividad de la β galactosidasa (colonias blancas).

24.2. Minipreparación de plásmidos.

Se empleó la técnica propuesta por Zhou y col. (1990) para minipreparaciones rápidas. Aquellas colonias que presentaron el inserto esperado se crecieron en LB liquido con ampicilina 100 µg/ml para llevar a cabo una preparación del plásmido más limpia y apropiada para secuenciar.

25. Preparación de plásmido. Esta se llevó a cabo mediante midi columnas marca QIAGEN ó con el sistema Wizard® Plus *SV* para minipreparaciones de Promega.

26. Tamizaje de la biblioteca de cDNA en λgt10 de *C. reinhardtii*.

Para conocer la secuencia de los genes *cox2a*, *cox2b* y *cox3* de *C.reinhardtii* se empleó una biblioteca de cDNA preparada en λgt10 proporcionada por Franzén y Falk (1992). La búsqueda de los genes en esta biblioteca, así como las soluciones empleadas se hicieron de acuerdo a Sambrook y col. (1989). La cepa de *E. coli* empleada como receptora del fago fue la *C600hfz*

26.1. Titulación de la biblioteca. La finalidad es conocer el número de placas líticas por ml de solución de fagos (pfu/ml). El procedimiento fue el siguiente:

- Se sembró la cepa bacteriana C600 h*fl* en una caja de LB toda la noche a 37°C (esta caja se llamó "caja primaria" y se guardó a 4°C). A la mañana siguiente se tomó una colonia y se sembró nuevamente en una caja de LB toda la noche a 37°C. Esta caja fue empleada para tomar de ahí las bacterias para ser infectadas a lo largo del tamizaje siempre y cuando no tuvieran más de 3 semanas a 4°C. Si este era el caso se resembró una colonia de la caja primaria para obtener colonias frescas cuyo crecimiento fuera el óptimo.

- Se tomó una colonia de bacterias de la caja de LB y se creció en 50 ml de LB con **maltosa 0.2%** y $MgSO_4$ 0.1 mM toda la noche a 37°C y con buena aereación. Se recomienda emplear un matraz de 250 o 500 ml con agitación de 250 r.p.m.

- Al día siguiente se cosecharon las células y se resuspendieron en 1/5 o 1/10 de su volumen original con amortiguador SM. Las bacterias deben ser guardadas a **4°C**, y pueden permanecer en condiciones óptimas hasta tres días.

- Se preparó una serie de diluciones de la biblioteca original de la siguiente manera: se tomaron 5 µl de la biblioteca original y se diluyeron en 500 µl de amortiguador SM (esta dilución fue de 10^{-2}). De esta dilución se tomaron 100 µl y se llevaron a 1ml con SM (dilución 10^{-3}) . Se preparó 1 ml de una serie de diluciones hasta 10^{-7}, todas en 1 ml de SM.

- En un tubo de ensayo o tubo Falcon tapado y estéril se agregaron 100 µl de bacteria concentrada, 100 µl de SM y 100 µl de una de las diluciones entre 10^{-3} y 10^{-7}. Se mezcló suavemente. Se recomienda preparar un par de controles para verificar que las condiciones son las adecuadas, uno que no contenga fagos, sólo el SM y las bacterias, y otro control con fagos y SM pero sin bacterias.

- Se incubó 30 minutos a 37°C para que el fago infectara a las bacterias.

- Se agregaron al tubo 7 ml de agar superior **completamente fundido** y equilibrado a 45°C en un baño de temperatura y se mezclaron por inversión.

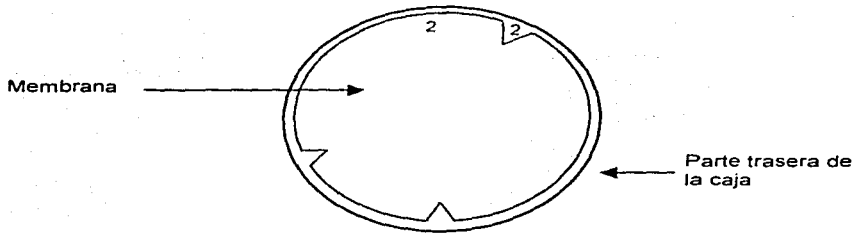
- Inmediatamente se vació la mezcla sobre un plato grande (150 mm) de LB con agar 1.4% y se distribuyó completamente en toda la superficie del plato evitando que el agar superior se fundiera antes de concluir. Se repitió este paso para cada una de las diluciones y los dos controles. Se permitió que el agar superior se endureciera a temperatura ambiente durante aproximadamente 10 minutos y posteriormente se incubó entre 8 y 12 horas a 37 C. Es importante que las cajas de LB estén previamente secas y equilibradas a 37°C. Después de varias horas de incubación comienzaron a aparecer plaquitas translúcidas sobre la cama opaca de bacterias que indicaron las posiciones donde se estaba efectuando la lisis bacteriana por parte de los fagos.

- Cuando las placas líticas alcanzaron un tamaño de aproximadamente 1.5 mm de diámetro, se contaron en aquellas cajas en que fueron perfectamente distinguibles unas de otras y se calcularon los pfu que hay en 1 ml de biblioteca original.

26.2. Plaqueo y búsqueda de clonas positivas.

- Se plaquearon 5 cajas de LB agar 1.4% con la dilución que contenía aproximadamente 10^5 pfu. Para ello se siguieron los mismos pasos que para llevar a cabo la titulación, pero sólo se empleó aquella dilución que fue la adecuada. Las cajas sembradas se incubaron a 37 C entre 8 y 12 horas.

- Una vez que las placas líticas crecieron hasta 1.5 mm aproximadamente, éstas se transfirieron a una membrana de nylon del mismo tamaño que las cajas. Para una orientación posterior de aquellas placas líticas positivas, fue necesario hacer 3 recortes triangulares en posiciones asimétricas en el borde de las membranas y numerarlas. Cuando la membrana se colocó sobre una de las cajas numeradas, fue necesario marcar con un plumón de color la forma de los cortes de la membrana en el reverso de la caja de LB.



- Se emplearon membranas de nylon Hybond-N de Amersham. Se colocó cuidadosamente la membrana sobre el LB agar de una de las cajas. Es importante comenzar a colocar desde el centro la membrana y permitir que ésta se vaya adhiriendo a la superficie de agar sin que queden burbujas atrapadas. Para la primera réplica se incubó de esta manera 5 minutos y para la segunda 7. Si se desean hacer más réplicas es necesario incubar 2 minutos más cada vez, es decir, 9, 11 minutos, etc.

- La membrana se separó de la caja con pinzas, cuidando de no romper el agar superior. La membrana se colocó con las placas líticas hacia arriba, sobre un papel Whatman saturado con una solución desnaturalizante de NaOH 0.5N y NaCl 1.5M. Se incubó por 5 minutos.

- Después se colocó la membrana sobre un papel absorbente para eliminar el exceso de solución y con pinzas se colocó con las placas líticas hacia arriba sobre un papel Whatman saturado con solución neutralizadora de Tris 0.1M pH 7.5 y SSC 2× durante 5 minutos.

- Finalmente, la membrana se transfirió por 5 minutos a un papel Whatmann saturado con SSC 2× para lavar los restos celulares.

- Este proceso se repitió para todas las membranas. Si las soluciones de los papeles Whatman se llenan de restos celulares, es necesario cambiar estos papeles y saturarlos con soluciones frescas.

- El DNA del fago se fijó a la membrana mediante incubación a 80°C por 2 horas en un horno de vacío.

- La solución para prehibridar e hibridar las membranas fue la siguiente:

5× SSC
5× Denhardts
0.5% SDS

- Las membranas se mojaron con un poco de solución para hibridar y se colocaron en una bolsa de plástico perfectamente sellada. Se agregaron 10 ml de solución para la primera membrana y 2 ml más por cada membrana adicionada a la bolsa. Se eliminaron las burbujas de aire y la bolsa se selló por completo. Se prehibridó a 65°C 1 hora en un hibridizador rotatorio.

- Para colocar la sonda marcada radioactivamente se cortó una de las esquinas de la bolsa y se agregó la sonda. Al mismo tiempo se agregó DNA de esperma de salmón desnaturalizado, a una concentración final de 0.1 mg/ml. Posteriormente la bolsa se selló completamente. Por seguridad es necesario envolverla en una segunda bolsa de plástico perfectamente sellada. Las membranas se hibridaron a 65 C toda la noche.

- Al día siguiente las membranas se extrajeron de la bolsa de plástico y se colocaron en un recipiente con 200 ml (para 10 membranas) de una solución que contenía 2xSSC y 0.2% de SDS a temperatura ambiente. En una nueva bolsa de plástico se hicieron los siguientes lavados:

2x SSC + 0.2% SDS 15 minutos a 65 C	dos lavados
0.2x SSC + 0.2% SDS 30 minutos a 65 C	un lavado

Finalmente las membranas se incubaron en una solución 0.1x SSC + 0.1% SDS a temperatura ambiente para evitar que se secaran mientras éstas se colocaban en bolsas de plástico perfectamente selladas para impedir que se secaran durante su exposición en placa fotográfica.

26.3. Tamizaje secundario.

De acuerdo a las placas reveladas, a los cortes hechos en el borde de las membranas y al dibujo de estos trazado en la parte posterior de cada caja, se localizó la posición de aquellas placas positivas.

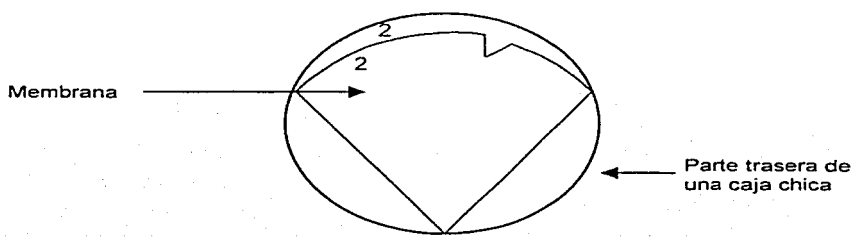
Es importante verificar que las placas seleccionadas sean positivas en ambas réplicas, es decir, que su posición en ambas réplicas sea exactamente la misma.

Debido a que la población de placas líticas en estas cajas es muy alta, hay una gran cantidad de placas no positivas que están estrechamente cercanas a la placa lítica de interés. Por ello es necesario hacer un segundo tamizaje con menos placas líticas por caja para poder aislar colonias únicas en este paso

- Se cortó con navaja la punta de una micropipeta de 1000 µl y se colocó justo donde se localiza la placa lítica positiva La punta se introdujo en el LB-agar y se atravezó hasta el fondo de la caja Después se succioó el LB y se incubó en 1 ml de SM a 4 °C toda la noche para permitir que el fago difundiera hacia fuera del LB-agar

- Al día siguiente se tituló la preparación de fago, para ello se hicieron nuevas diluciones del fago desde una dilución de 10^{-1} hasta una 10^{-5} en cajas de LB-agar 1.4% de **100 mm** de diámetro Se seleccionó aquella dilución en que se contaban aproximadamente 150 o 200 placas líticas bien separadas Para el caso de cajas de Petri chicas fue necesario agregar 3 5 ml de agar superior fundido en vez de los 7 ml empleados para cajas grandes

- Se transfirieron las placas líticas a membranas de nylon de acuerdo a lo indicado con anterioridad, pero en esta ocasión una membrana grande (para cajas de 150 mm) se cortó en 4 partes iguales y una de estas partes es la que se empleó para la transferencia. Es importante hacer un corte triangular asimétrico en el borde de la membrana y marcar su contorno sobre la parte posterior de la caja de petri



- Una vez que las placas líticas se transfirieron y fijaron a la membrana, éstas se hibridaron con **sonda marcada nueva**. Las membranas se hibridaron y lavaron en las mismas condiciones que durante el primer tamizaje

- Una vez que se identificó una colonia positiva completamente aislada, se succionó el LB-agar con la punta de micropipeta de 200 µl cortada con una navaja y se incubó en 1 ml de SM toda la noche a 4°C para permitir que el fago difundiera hacia fuera del LB-agar.

26.4. Purificación del DNA de fago. Para purificar el DNA del fago es necesario sembrar una caja chica de LB- **agarosa** (en esta etapa, el agar podría inhibir reacciones enzimáticas, como la ligación o digestión con enzimas de restricción). Se emplearon 50 µl de la solución de fago (aproximadamente 10^5 pfu) en el SM **sin diluir.** Después de incubar la caja toda la noche a 37°C se deben ver sólo placas confluentes.

- El lisado de lambda en placas se preparó de acuerdo al protocolo II de Sambrook y col. (1989). La agarosa superior se raspó suavemente de la caja de Petri con una pipeta Pasteur doblada en L y se colocó en un tubo Falcon estéril.

- Se agregaron 10 ml de amortiguador SM a la caja y se enjuagaron los restos de agarosa superior. La caja se incubó con el SM 15 minutos a temperatura ambiente.

- Se agregaron los 10 ml de SM junto con los restos de agarosa al tubo Falcon que contenía la agarosa superior que se raspó de la caja. Se agregaron 100 µl de cloroformo y se agitó suavemente por inversión. Se incubó 2 horas a temperatura ambiente para permitir que el fago difundiera hacia fuera de la agarosa.

- Se centrifugó a 10,000 · g 10 minutos para separar la agarosa del sobrenadante. Este se colocó en un tubo Falcon estéril nuevamente y se completó su volumen a 10 ml con SM. Si la muestra se va a guardar es importante agregar 100 µl de cloroformo y agitar varias veces por inversión. En este caso la muestra se incuba a 4°C.

Para preparar el DNA del fago se emplean columnas de QIAGEN Lambda Midi Kit especiales para este tipo de purificación. Es importante no permitir que el precipitado de DNA obtenido al final se seque más de 5 minutos, porque de ser así, resulta imposible solubilizarlo posteriormente.

26.5. Amplificación por PCR del cDNA de *C. reinhardtii* aislado de la biblioteca de λgt10.

Una manera rapida de saber si el inserto contenido en el DNA de fago aislado es el gen de interés, consiste en amplificar por PCR el inserto mediante el uso de oligodesoxinucleótidos basados en la secuencia de λgt10 que flanquea al inserto de cDNA de *C. reinhardtii*. Las secuencias de estos oligodesoxinucleótidos son:

Hacia adelante 5'-AGCAAGTTCAGCCTGGTTAAGT-3'
Hacia atrás 5'-CTTATGAGTATTTCTTCCAGGGTA-3'

El producto de PCR obtenido se clonó en el vector pMos-blunt o pGem-T como se mencionó anteriormente o se secuenció directamente con los mismos oligodesoxinucleótidos empleados para amplificar este producto de PCR.

26.6. Subclonación del cDNA de *C. reinhardtii*.

26.6.1. *Preparación del cDNA* de *C. reinhardtii*.

- Se cortó aproximadamente 1 μg de DNA de fago purificado por columnas de QIAGEN con la enzima de restricción *EcoRI* en 200 μl de volumen final y se incubó toda la noche a 37°C.

- El DNA digerido se precipitó con 1/10 de volumen de acetato de sodio 3M pH 5.0 + 3 volúmenes de etanol al 100%. Después se corrió en un gel de agarosa al 1% y se purificó el inserto. Se corrió una décima parte del volumen de inserto puro en un gel de agarosa para estimar su concentración.

26.6.2. *Preparación del vector pBluescript.*

- Se digirieron aproximadamente 15 μg de pBluescript (Stratagene) en 50 μl de volumen final con EcoRI toda la noche a 37°C.

- Se agregaron 6 μl de amortiguador 10× para fosfatasa alcalina (Boehringer) y 1.5 unidades de fosfatasa alcalina de camarón (1.5 μl). Se incubó a 37°C 3 horas. Posteriormente se desactivó la fosfatasa alcalina al incubar 20 minutos a 65°C.

- El plásmido cortado y desfosforilado se corrió en un gel de agarosa al 1% y se purificó. Se corrió una décima parte del plásmido puro en un gel de agarosa para estimar su concentración.

26.6.3. *Ligación, transformación y purificación del plásmido.*

- Para la reacción de ligación se agregaron aproximadamente 150 ng de vector digerido y desfosforilado y 20 ng de inserto liberado con EcoRI. La ligación se llevó a cabo a 16°C toda la noche con la siguiente mezcla de reacción:

x μl de pBluescript
y μl de inserto
0.5 μl ATP 10 mM
0.5 μl DTT 100 mM
2.2 μl amortiguador 5· de Gibco BRL
1 μl de T4 ligasa de Gibco BRL.
Agua para 12 μl

- La transformación se llevó a cabo con células competentes DH5α. Las células transformadas se siembran en cajas de LB con ampicilina 100 μg/ml agregando directamente al tubo 10 μl de IPTG 800 mM y 40 μl de x-Gal 2%. Se incubó toda la noche a 37°C.

- Se hizo una minipreparación de plásmido de 8 colonias blancas, y aquellas que tuvieron un plásmido con inserto se crecieron en un mayor volúmen. Posteriormente se hizo una preparación de plásmido con columnas de QIAGEN o con el sistema Wizard de Promega.

Apéndice III:

Oligodesoxinucleótidos empleados.

1. OLIGODESOXINUCLEÓTIDOS DISEÑADOS SOBRE *cox3*.

1.1 Oligodesoxinucleótidos degenerados diseñados sobre el extremo amino terminal de la proteína y sobre regiones conservadas del extremo carboxilo terminal para *Polytomella* spp.

Residuos DAGHHLSP

Amino 5'-TCT GAT GCT GGT CAT CAT CTT TCT CC-3'
C C C C C C C C

Residuos WH(M/F)VDVVWL

Carboxilo 5'-GG CAC ATG GTC GAT GTC GTC TGG CT-3'
T T C T C T T

1.2 Oligodesoxinucleótidos diseñados sobre la secuencia genómica para la amplificación rápida de los extremos 3' y 5' del cDNA de *cox3* de *Polytomella* spp.

Residuos EHYLVHTA

PC35'F 5'-G CAT TAC CTC GTC CAC ACT GC-3'

Residuos EMGMHTDVN

PC3N1F 5'-AG ATG GGC ATG CAT ACC GAT G-3'

Residuos PYGTTFFM

PC3NR 5'-CAT GAA GAA GGT GGT ACC GTA GG-3'

Residuos CNVFLGL

PC35'NR 5'-GAG GCC AAG GAA GAC GTT GC-3'

Residuos QYFHGVA

PC35'R 5'-CAG CGA CAC CGT GGA AGT ACT G-3'

Residuos PLLFGSVA

PC35'R4 5'-C AAC GGA TCC GAA CAA CAA GG-3'

Residuos EMGMHTDV

PC35'R3 5'-C ATC GGT ATG CAT GCC CAT C-3'

1.3 Oligodesoxinucleótidos diseñados sobre los extremos del cDNA de *cox3* de *Polytomella* spp. para obtener un producto de PCR de la secuencia genómica o de cDNA del gen completo.

CO3P.1F 5'-CGTTTTTGGTCAAGTTGAAA-3'

CO3PF1 5'-GAGGTCTCAGCTTCTTAAGGCTC-3'

CO3PR1 5'-GCTCATGTAACTATGCCACAAGAC-3'

CO3PR2 5'-CGCATAACGCGAAGTCACTAC-3'

1.4 Oligodesoxinucleótidos diseñados para amplificar el extremo 5' del cDNA de *cox3* de *C. reinhardtii*.

cr3g2R 5'-GTGCTCCATGTAGAACTCCTTGG-3'

c3cr207R 5'-GTTGGGCACCTGAGGCTGC-3'

1.5 Oligodesoxinucleótidos diseñados para amplificar la secuencia genómica y de cDNA completa de *cox3* de *C. reinhardtii*.

c3cr10F 5'-AGCGCGACCGGTGAAACCAG-3'

c3cr40F 5'-GGGTCACTTGTCAGGAAGTCTTG-3'

cr3g2F 5'-CCAAGGAGTTCTACATGGAGCAC-3'

cg680F 5'-GTGGCGCTGCAGATGCAGTGGC-3'

Cg350R 5'-TGGAAGGGGTGGCGCTTGCCG-3'

cg785R 5'-CCTTGGCCACCATGGCCACGTTGG-3'

cr3g3R 5'-CTGCCACACACACCCGTCATACG-3'

2. OLIGODESOXINUCLEÓTIDOS DISEÑADOS SOBRE *cox2*.

2.1 Oligodesoxinucleótidos diseñados sobre el gen *cox2b* de *Polytomella* spp.

Residuos L G I K M D A I

F3Pst 5'-CTC GGT ATT AAG ATG GAT GCT ATC-3'

Bint 5'-AGC TGT TTA AGA CCA TGA CTT C-3'

Residuos L G I K M D A I

B1Pst 5'-GAT AGC ATC CAT CTT AAT ACC GAG-3'

Residuos L R M L E V D E D

B2Pst 5'-CTC ATC GAC CTC AAG CAT ACG GAG-3'

Residuos D A K D Q L K E

F4Pst 5'-GGA TGC TAA GGA CCA GCT CAA GG-3'

2.2 Oligodesoxinucleótidos diseñados sobre el extremo amino terminal de la proteina basados en el uso de codones nuclear de *Polytomella* spp

Residuos E A P V A W Q L G

F1p 5'-GAG GCT CCT GTT GCT TGG CAG CTT GG-3'
A C C C C G

Residuos Q D S A T S Q A Q A

F2p 5'-CAG GAT TCT GCT ACT TCT CAG GCT CAG G-3'
C C C C C C

2.3 Oligodesoxinucleótidos diseñados en regiones conservadas cercanas a los 2 cruces transmembranales para encontrar la secuencia del gen *cox2a* de *Polytomella* spp.

Residuos KAIGHQWYW

PCO2R1 5'-CCA ATA CCA CTG ATG ACC AAT AGC C-3'
G G T G G
G

Residuos PSFALLYS

PCO2R2 5'-GA ATA AAG AAG AGC AAA AGA AGG-3'
G G G G G G G

Residuos HGTTIEI

PCO2R3 5'-AT CTC AAT AGT AGT ACC ATG-3'
G G G T G
G

2.4 Oligodesoxinucleótidos diseñados sobre un fragmento del gen *cox2a* de *Polytomella* spp.para amplificar los extremos 3' y 5' del cDNA.

Residuos

PCO2R1-5' 5'-TC AGG GAG AGC TTG CTT AGT GTA G-3

Residuos FLYHIATK

PCO2R2-5' 5'-TT GGT GGC GAT GTG GTA GAG G-3'

Residuos HYTKQALPE

PCO2F1-3' 5'-AC TAC ACT AAG CAA GCT CTC CCT G-3'

Residuos FLYHIAT

PCO2F2-3' 5'-TC CTC TAC CAC ATC GCC ACC-3'

2.5 Oligodesoxinucleótidos diseñados sobre el gen *cox2a* de *Polytomella* spp. para amplificar un producto que contenga la secuencia genómica o de cDNA completa.

PC2AmF1 5'-AAT GCT CGC CCA GCG TAT C-3'

PC2AmR1 5'-AAA CCT TCA CAC ACC CAT AGG C-3'

PC2AmR2 5'-AGA AAG TCT AAA CCA CGA CTC CG-3'

2.6 Oligodesoxinucleótidos para amplificar el extremo 5' del cDNA de *cox2a* de *C. reinhardtii*.

Crnc2-60 5'-TGG TGG TCA GGC CCA GAG C -3'

Crnc2-120 5'-CAA TCA TGG CCT GAG CAG TGG-3'

Crnc2-180 5'-GTG ATC AGG AAG AAG AAG ATG TCG-3'

Crnc2-200 5'-TCT GGA ACA TCA TGT AGA ACA CC-3'

3. OLIGODESOXINUCLEÓTIDOS d(T) EMPLEADOS EN LA SÍNTESIS DE PRIMERA HEBRA DE cDNA Y EN LA AMPLIFICACIÓN RÁPIDA DE EXTREMOS 5' Y 3'

3.1 Oligo d(T)/adaptador

5'- GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT -3'

Adaptador

5'- GACTCGAGTCGACATCGA –3' Tm= 52°C

3.2 QT

5'- CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT 3'

Q_0

5'- CCAGTGAGCAGAGTGACG -3' Tm= 46.2°C

ADO

5'- CCAGTGAGCAGAGTGACGAGG -3' Tm= 54.7°C

cQ_1

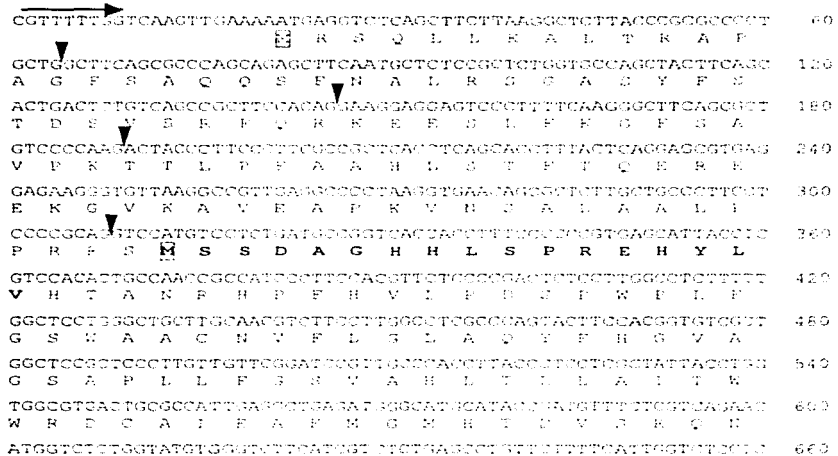
5'- CGAGGACTCGAGCTCAAGC –3' Tm= 52.4°C

$gacQ_1$

5'- GACGAGGACTCGAGCTCAAGC –3' Tm= 55.6°C

Apéndice IV:

Secuencias reportadas.



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Intrón 1=
GTAAGATTATTTCCTTTATTTAGGGCCGTGTTTATAATAACGGAGATGTGAATTTTATCG
AGACCTGTGTTTTAGACAAATTTTGCTGAATCTATATTCCTATAGTTGTTAAAGCTTACT
TCGACAGCTTTTTAATGTTTTTGTATAAATATTTTGAATCTTTGTAG

Intrón 2=
GTAAGAACCAGCTGATTAATGTTCTGGCCTCTTGAATTTATATTAATAAGTACTATTTAT
ATATTCTTTAG

Intrón 3=
GTAATTTGAAATTAATATTTTTGCTTCTTTTTTGTTTAAGATATGCATGCCATTTAACTA
ACGTTTCTGTTTGTGCTTTTTTATAG

Intrón 4=
GTAAGATTATATTGATAGAGAAATTGTGATATATTTTGTCGGTGTGACTGTTTTTATCGT
TGGACAAATTGATCATGTTCATTTTTTAATTTAACGTCAGAAGGAAATAAAGCTGTTCA
GTATTATTCTATGTCTTTTTTTTTGTTACCAATGCCTCATTATTTTTTAAATCATTTTCCT
GGTATTTCGAAAGATCGTTCCGACAGGGGTGTTAGGTCCATTTCTGTATATGTGACGTAC
TTTCGTACCGCAGAAACTAGTATTTATTTTCTTAAAATTTGTTTTAGCTATGAAAGTGCT
ACTGATAGCAATGCCAAAAAGAATTAATGGATAATATTTGGTTCTTGTGGTCAAAAATTT
CTTAACTTCAGGCCCTTCTCAGGGTAGCTATGACATAAATGTGCTTTTAGTCACTTACCC
CCTTTTTTCCTATGGGTCTTATAG

Figura A. Secuencia completa del gen *cox3* de *Polytomella* spp. Las letras negritas indican la región amino terminal de la proteína determinada por degradación de Edman. Las metioninas de la presecuencia están encerradas en un recuadro. Los triángulos negros indican la posición de los 4 intrones. Las flechas indican la posición donde se diseñaron oligodesoxinucleótidos para amplificar al gen completo, tanto en su secuencia de cDNA como en la genómica. La señal de poliadenilación TGTAA está subrayada. Número de acceso en la base de datos de DDBJ/EMBL/GenBank: AF233514 para la secuencia de cDNA y AF286057 para la secuencia genómica

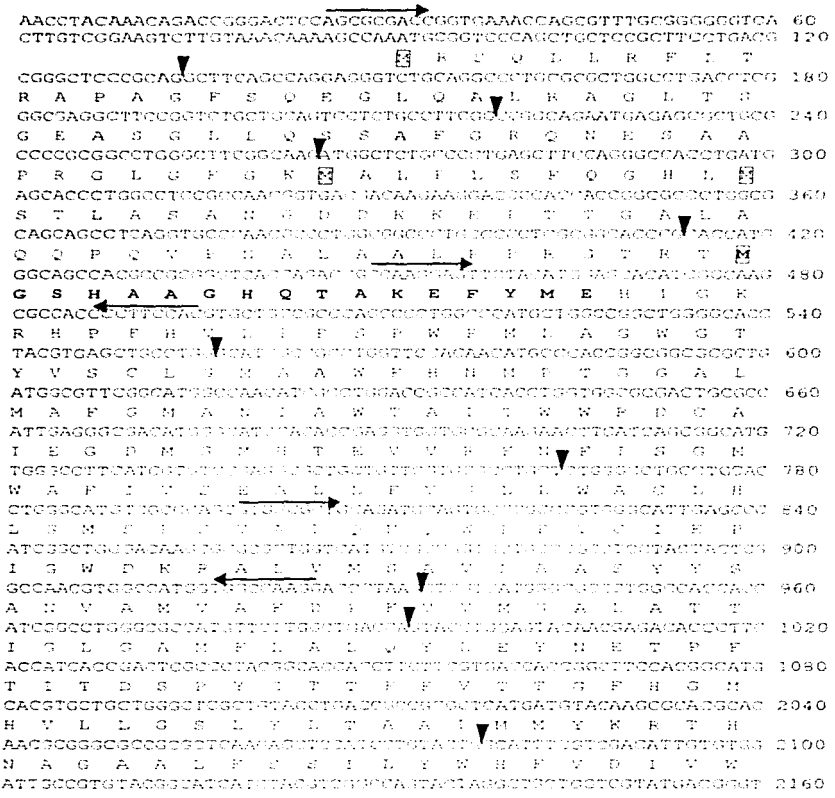
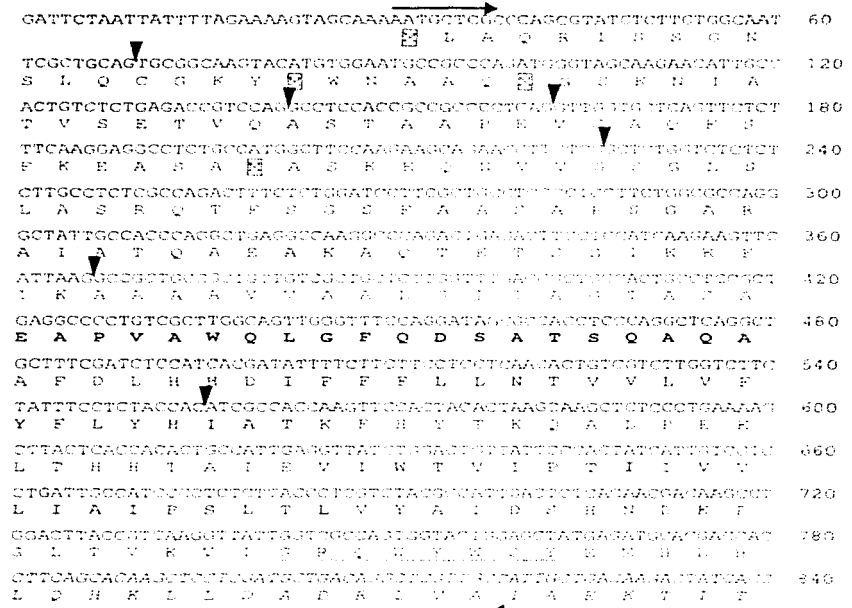


Figura B. Secuencia completa del gen *cox3* de *Chlamydomonas reinhardtii*. Las letras negritas indican la región que corresponde a la secuencia amino terminal de la proteína determinada para *Polytomella* spp. Las metioninas de la presecuencia están encerradas en un recuadro. Los triángulos negros indican la posición de los 9 intrones. Las flechas indican la posición donde se diseñaron oligodesoxinucleotidos para amplificar la secuencia genómica. La señal de poliadenilación TGTAA está subrayada. Número de acceso en la base de datos de DDBJ/EMBL/GenBank: AF233515 para la secuencia de cDNA y AF286058 para la secuencia genómica.



Intrón 1.
GTTAATAATATTTATTTATACTATAGACAATTTTAAATATCTTATTAGACTTGTTTTCTA
ACATTTTTGTTGGCTTTTTTATAG 84

Intrón 2.
GTAATTGTTTACGAATATATTTTCAATGGAACATGATGCCCTATATGTTTAACTACATAA
CGGAATTTCTTATTATAA[illegible]
TTTTTTTTCTTAAAG 104

Intrón 3.
GTGCTGGTTGACTATTTATACTATAGCACTGCATTGTATCTCTTTTTTAAATTTTGTCTT
AAACTTATTATCAAAATATGTAAAG 86

Intrón 4.
GTAAGTACCACTATTTCTATAGCGTAGTTGTGTGCAAATAATAATTTTTTATTTTTTTAC
ACATAG 66

Intrón 5.
GTAACGCTGAATATTTGTCTTGTTTATAGCTGTAAACAGCCTTTTTGGCTCTTATCTTTG
TGCTTGGTATGTTTGAAAATTTTGTTTTATTGTAAGTACTAATTTAATCCAAGATGTTT
CGCTATTTTCTTTCAAGTAATTTTTTGTTTGATTCACTTTATGAAACAATCGTATGGAG
CTTCTAATCTTATATGTTAATAAATTTCTAATTATTATTTTTCCAATAG 229

Intrón 6.
GTAAGTAGCTCTCATAATAAGCCAACGGTTAATGTTTCTGCGTTTGAGCTGTATTGTATA
TTTAATTTAGTAATGGTGTTTTAACATTCAAGTAATTATCACTTTTTGACATTTTCCCAT
TCGTAG 126

Figura C. Secuencia completa del gen *cox2a* de *Polytomella* spp. Las letras negritas indican la región amino terminal de la proteína determinada por degradación de Edman. Las metioninas de la presecuencia están encerradas en un recuadro. Los triángulos negros indican la posición de los 6 intrones. Las flechas indican la posición donde se diseñaron oligodesoxinucleótidos para amplificar al gen completo, tanto en su secuencia de cDNA como en la genómica. Los posibles cruces transmembranales se encuentran sombreados. La región aromática conservada se encuentra subrayada, al igual que la señal de poliadenilación TGTAA. La región carboxilo terminal no conservada se encuentra en letras itálicas. Número de acceso en la base de datos de DDBJ/EMBL/GenBank: AF305078 para la secuencia de cDNA y AF305541 para la secuencia genómica.

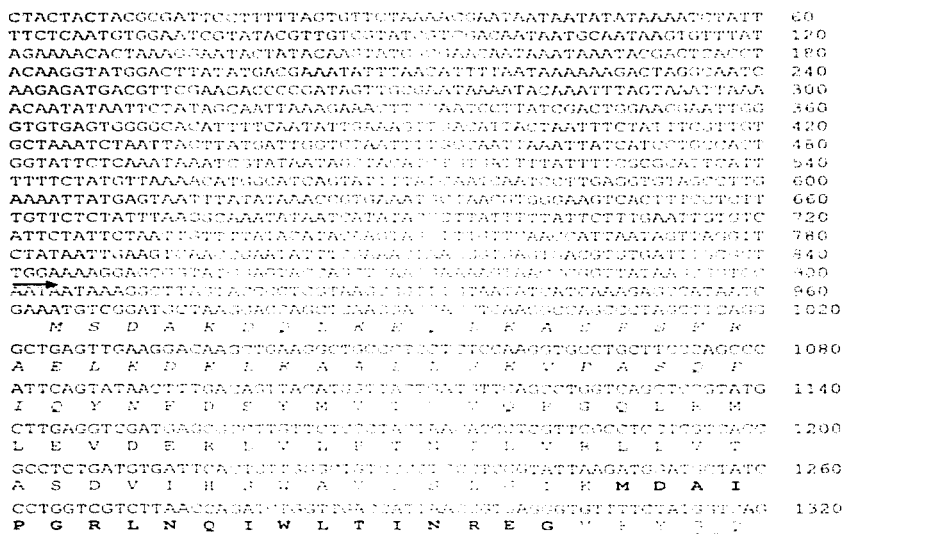


Figura D. Secuencia genómica del gen *co×2b* de *Polytomella* spp. obtenido por Antaramián, A. (tesis de doctorado, 1998) Las letras negritas indican la región interna de la proteína determinada por degradación de Edman. Las flechas indican la secuencia incluida en el cDNA obtenida en este trabajo. El sitio de unión a cobre se encuentra subrayado, al igual que las 3 señales de poliadenilación TGTAA. La región amino terminal no conservada se encuentra en letras itálicas. Número de acceso en la base de datos de DDBJ/EMBL/GenBank: AF305079 para la secuencia de cDNA y AF305542 para la secuencia genómica

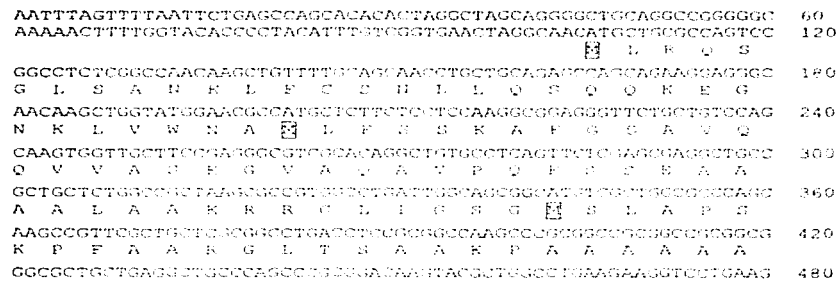
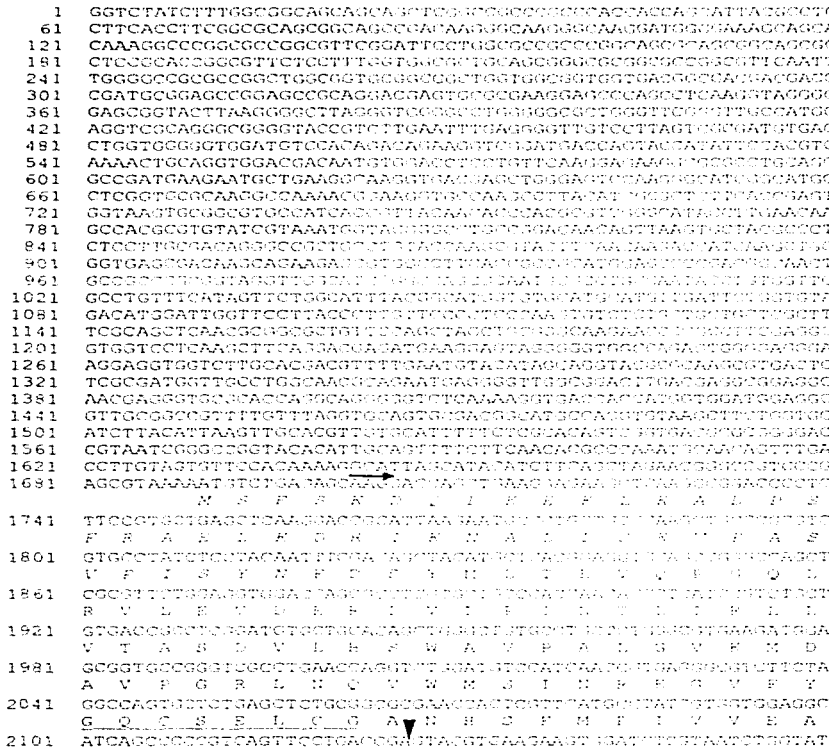


Figura E. Secuencia completa de cDNA del gen *cox2a* de *Chlamydomonas reinhardtii*. Las letras negritas indican la región que corresponde a la secuencia amino terminal de la proteína determinada para *Polytomella* spp. Las metioninas de la presecuencia están encerradas en un recuadro. Los posibles cruces transmembranales se encuentran sombreados. La región aromática conservada se encuentra subrayada, al igual que la señal de poliadenilación TGTAA. La región carboxilo terminal no conservada se encuentra en letras itálicas. Número de acceso en la base de datos de DDBJ/EMBL/GenBank: AF305080.



Intrón.

GTGAGTTGCGCATGGGGCCGCGAAAGCGCGCGCTGGGAAGCACTGATTAATGCGCTAGCG
AATGTTGGGGTTTTCGGGTTGCTGGGCTGTGGCCGCTGTGCCGCCACGATGACTGTCGAG
GTGTCGGTGATGGCCAACACAACTAGTTTTCTTGCATGCTAACCCCGTCGGTTCTGCTCT
ATTGCAG 187.

Figura F. Secuencia genómica del gen *cox2b* de *Chlamydomonas reinhardtii*. Las flechas indican la secuencia incluida en el cDNA obtenida en este trabajo. El sitio de unión a cobre se encuentra subrayado, al igual que la señal de poliadenilación TGTAA. La región amino terminal no conservada se encuentra en letras italicas. La posición del intrón se indica con un triángulo negro. Número de acceso en la base de datos de DDBJ/EMBL/GenBank: AF305540 para la secuencia de cDNA y AF305543 para la secuencia genómica.