THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 8, Issue of February 22, pp. 6051–6058, 2002

Printed in U.S.A.

The Typically Mitochondrial DNA-encoded ATP6 Subunit of the F_1F_0-ATPase Is Encoded by a Nuclear Gene in *Chlamydomonas reinhardtii**

Received for publication, October 16, 2001, and in revised form, December 11, 2001
Published, JBC Papers in Press, December 14, 2001, DOI 10.1074/jbc.M109993200

Soledad Funes‡§, Edgar Davidson¶, M. Gonzalo Claros‖, Robert van Lis‡, Xochitl Pérez-Martínez‡ ‡‡, Miriam Vázquez-Acevedo‡, Michael P. King¶, and Diego González-Halphen‡§§**

From the ‡Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, 04510 México D.F., Mexico, the ¶Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, and the ‖Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, Málaga E-29071, Spain

The *atp6* gene, encoding the ATP6 subunit of F_1F_0-ATP synthase, has thus far been found only as an mtDNA-encoded gene. However, *atp6* is absent from mtDNAs of some species, including that of *Chlamydomonas reinhardtii*. Analysis of *C. reinhardtii* expressed sequence tags revealed three overlapping sequences that encoded a protein with similarity to ATP6 proteins. PCR and 5′- and 3′-RACE were used to obtain the complete cDNA and genomic sequences of *C. reinhardtii atp6*. The *atp6* gene exhibited characteristics of a nucleus-encoded gene: Southern hybridization signals consistent with nuclear localization, the presence of introns, and a codon usage and a polyadenylation signal typical of nuclear genes. The corresponding ATP6 protein was confirmed as a subunit of the mitochondrial F_1F_0-ATP synthase from *C. reinhardtii* by N-terminal sequencing. The predicted ATP6 polypeptide has a 107-amino acid cleavable mitochondrial targeting sequence. The mean hydrophobicity of the protein is decreased in those transmembrane regions that are predicted not to participate directly in proton translocation or in intersubunit contacts with the multimeric ring of *c* subunits. This is the first example of a mitochondrial protein with more than two transmembrane stretches, directly involved in proton translocation, that is nucleus-encoded.

The F_1F_0-ATP synthase/ATPase (EC 3.6.1.3) is present in the three domains of life: archea, prokarya, and eukarya. This membrane-bound complex catalyzes ATP synthesis using the electrochemical gradient generated by light-driven or redox-driven electron transfer chains (1). Two main structural domains constitute this oligomeric protein, the membrane-bound sector F_0 involved in proton translocation, and the extrinsic domain F_1 that catalyzes the synthesis of ATP. The F_1 domain contains five subunits in a $3\alpha/3\beta/1\gamma/1\delta/1\epsilon$ stoichiometry (2). The structure of the F_1 sector of the bovine enzyme has been determined crystallographically (3). The F_0 portion of the ATP synthase is less well characterized, due to its highly hydrophobic nature. The structure of a subcomplex of the *Saccharomyces cerevisiae* ATP synthase shows that 10 *c* subunits are arranged around a central stalk (4). In addition, one *a* subunit, also known as ATP6, is thought to interact with the multimeric ring of *c* subunits, translocating protons from the intermembrane space to the mitochondrial matrix. Subunit *a* is predicted to contain two hemichannels that are an obligate route for protons during ATP synthesis driven by the chemiosmotic gradient (5, 6).

In eukaryotic organisms, all of the subunits of the F_1 and a subset of the F_0 portion of the ATP synthase are nucleus-encoded and cytoplasmically synthesized. In most organisms, only two or three of the hydrophobic components of the F_0 sector are mtDNA[1]-encoded. The genes of the F_0 sector that are usually found in the mitochondrial genome are *atp6*, *atp8*, and *atp9* (encoding ATP6 or subunit *a*, ATP8 or A6L, and ATP9 or subunit *c*, respectively). These genes encode highly hydrophobic polypeptides with multiple, putative transmembrane regions (five for ATP6, two for ATP8, and two for ATP9).

The 15.8-kb linear mitochondrial genome of the green alga *C. reinhardtii* lacks several genes that are usually present in mitochondrial genomes, including *nad3*, *nad4L*, *cox2*, *cox3*, *atp6*, *atp8*, and *atp9* (7, 8). These seven genes encode essential components of oxidative phosphorylation complexes and are also absent from the mitochondrial genomes of the related green algae *Chlamydomonas eugametos* (9) and *Chlorogonium elongatum* (10). The genes *cox2a*, *cox2b,* and *cox3*, which encode subunits IIA, IIB, and III of cytochrome *c* oxidase, have been transferred to the nucleus in at least two members of the family

* This work was supported by the Fogarty International Center at the National Institutes of Health (NIH) Grant TW01176; NHLBI, NIH, Grant HL59646; Consejo Nacional de Ciencia y Tecnologia (CONACyT) Grant 27754N; and Dirección General de Estudios de Personal Académico, Universidad Nacional Autónoma de México (UNAM), Grant IN202598. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AF411119 (atp6 cDNA sequence from C. reinhardtii) and AF411921 (atp6 genomic sequence from C. reinhardtii).

§ Recipient of a fellowship from CONACyT and Dirección General de Posgrado (UNAM) and a graduate student grant from Progama de Apoyo a los Estudios de Posgrado (UNAM).

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§§ To whom correspondence should be addressed: Dept. de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, 04510 México D.F., Mexico. Tel.: 52-55-5622-5620; Fax: 52-55-5622-5611; E-mail: dhalphen@ifisiol.unam.mx.

[1] The abbreviations used are: mtDNA, mitochondrial DNA; Cr-ATP6, *C. reinhardtii* subunit ATP6 of mitochondrial F_1F_0-ATP synthase; EST, expressed sequence tag; <H>, local hydrophobicity; MTS, mitochondrial targeting sequence; nt, nucleotide(s); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Chlamydomonadaceae (11, 12). This gene transfer from the mitochondria to the nucleus was accompanied by several structural changes in these genes. The proteins contain mitochondrial targeting sequences (MTS) and diminished overall mesohydrophobicity that allow the import and assembly of these proteins in the inner mitochondrial membrane.

In this work, the cDNA of the *atp6* gene from *C. reinhardtii* was cloned and sequenced, and the corresponding genomic sequence was obtained. We show that *atp6* is localized in the nuclear genome. The ATP6 polypeptide encoded by this *atp6*, named Cr-ATP6, is homologous to all known ATP6 proteins and is shown to be a constituent of the mitochondrial ATP synthase of *C. reinhardtii*. This is the first description of an *atp6* gene that resides in the nuclear genome and the first example of a nuclear gene that encodes a mitochondrial protein that exhibits structural features characteristic of a proton channel. We demonstrate that the largest decrease in hydrophobicity of Cr-ATP6 occurs in those transmembrane regions that do not participate directly in proton translocation and that are not predicted to interact with adjacent subunits. This characterization of a nuclear version of a gene that is normally present in mitochondrial genomes will facilitate the allotopic expression of mtDNA-encoded genes and its future application to human mitochondrial gene therapy.

EXPERIMENTAL PROCEDURES

Strain and Culture Conditions—Cell wall-less *C. reinhardtii* strain CW15 was grown in TAP medium (13) with 1% sorbitol (Sigma) under continuous light, with agitation at 100 rpm. The cells were harvested at the late exponential phase of growth.

Nucleic Acid Preparation and Sequencing—Total DNA from *C. reinhardtii* was obtained as previously described (11) or, alternatively, using the DNeasy Plant Mini Kit (Qiagen). Total RNA from *C. reinhardtii* was obtained using the RNeasy Mini Kit (Qiagen). All standard molecular biology techniques were as described (14). Sequencing was performed by the Kimmel Cancer Center DNA Sequencing Facility at Thomas Jefferson University.

Cloning of the cDNA of the Gene atp6 from C. reinhardtii—We identified an EST clone (AV394701) (15) as a fragment of the *atp6* gene from *C. reinhardtii*, because the deduced amino acid sequence at the 3′ end of the EST exhibited high similarity with other ATP6 subunits. The 5′ end showed sequence similarity to two additional clones (AV388269 and AV395475) (15). Based on these EST sequences, two deoxyoligonucleotides were designed (5′-GAGGGTGTTCGGCCTCTTGG-3′ and 5′-CGAAGAACGACAGCGAGAAAGG-3′) and used to amplify a PCR product of 822 nt, containing a portion of the *atp6* cDNA, using a *C. reinhardtii* cDNA library (16) as template. The sequences at the 5′ and 3′ ends of the cDNA were obtained following RACE PCR (17). The primers used were as follows: 5′ RACE, forward (oligo(dT)/adapter) and reverse (5′-CGCCAGAAGCGGTAGATGCC-3′); for nested PCR, forward (oligo adapter) and reverse (5′-GCAGCGAATGGCACCATCG-3′); 3′ RACE, forward (5′-CTACGTTGGCGAGTTCAACAAGC-3′) and reverse (oligo(dT)/adapter); for nested PCR, forward (5′-GTGGTCAAGAAGGCGCTGTAAGC-3′) and reverse (oligo adapter). The sequences of the dT/adapter and adapter deoxyoligonucleotides were described previously (11). The first cDNA strand for these reactions was obtained using the kit Omniscript (Qiagen) with a specific primer (5′-GAG AAG CCC AGC TTG TAC AGA CC-3′) to obtain the 5′ end and oligo(dT) to obtain the 3′ end of the cDNA.

Cloning of the atp6 Gene from C. reinhardtii—Three pairs of deoxyoligonucleotides were designed using the cDNA sequence to amplify the genomic sequence for the *atp6* gene (5′-AGACGAAGAATATAGATTGG-3′ and 5′-CGCCAGAAGCGGTAGATCCC-3′; 5′-CATTCGCTGCCCAGCAGGGC-3′ and 5′-GCGAAGAACGACAGCGAGAAGGG-3′; 5′-CCTCCAACTTGCTGGGTCTGGTG-3′ and 5′-ACGAAGCTTACAGTCTCCTC-3′). The PCRs were performed with the *Pfu* Turbo DNA polymerase (Stratagene). For PCR amplification, samples were denatured for 5 min at 94 °C; subjected to 10 cycles of 10 s of denaturation at 94 °C, 1 min of annealing at 55 °C, and 4 min of extension at 68 °C; and subjected to 25 cycles of 10 s of denaturation at 94 °C, 1 min of 55 °C annealing, and 4 min (plus 10 s each cycle) of 68 °C extension. Three different overlapping PCR products of 959, 1973, and 2030 nt were obtained and cloned into pGEM-T Easy Vector (Promega) after the addition of terminal Adenines.

Mitochondrial Protein Analysis—Mitochondria from *C. reinhardtii* were isolated as described (18). The final mitochondrial pellet was resuspended in 35 ml of Percoll dilution buffer with a protease inhibitor mixture (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 mM ϵ-aminocaproic acid). Mitochondria were diluted to a final concentration of 5 mg of protein/ml, solubilized in 1% dodecylmaltoside, and subjected to blue native electrophoresis using a gradient of 5–12% acrylamide (19). Respiratory chain complexes were separated in the second dimension by Tricine-SDS-PAGE (12% acrylamide) (20). Apparent molecular masses were calculated using prestained molecular weight markers (BenchMark Prestained Protein Ladder; Invitrogen). Protein concentrations were determined according to Markwell *et al.* (21). The isolation of polypeptides for N-terminal sequencing was done as previously described (22). 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.

Sequence Analysis in Silico—ATP6 sequences used in this work for mesohydrophobicity analysis were obtained from the SwissProt and TrEMBL data bases at the European Bioinformatics Institute (available on the World Wide Web at srs.ebi.ac.uk). The accession numbers are as follows: Q31720 (*Brassica napus*); P07925, Q36271 (*Zea mays*); P26853 (*Marchantia polymorpha*); P05500 (*Oenothera bertiana*); P05499 (*Nicotiana tabacum*); Q04654 (*Vicia faba*); P20599 (*Triticum aestivum*); P92547, P93298 (*Arabidopsis thaliana*); O21786 (*Oryza sativa*); Q36513 (*Platymonas subcordiformis*); Q35748 (*Raphanus sativus*); Q35781 (*Sorghum bicolor*); Q36376, Q36379 (*Helianthus annuus*); Q37624 (*Prototheca wickerhamii*); Q36730 (*Petunia parodii*); Q34008, Q34004, Q9XPH3, Q9XPH4, Q9TGM2 (*Beta vulgaris*); O79682 (*Glycine max*); Q9ZY27 (*Pedinomonas minor*); Q9XL97 (*Solanum tuberosum*); Q9TC92 (*Nephroselmis olivacea*); Q9MGK0 (*Scenedesmus obliquus*). Alignments were carried out with the ClustalX program (23) with default parameters. Protein weight matrix was Gonnet, although results obtained with Blosum and Pam were similar. The hydrophilic gap penalty was enabled.

Mitochondrial targeting sequence analysis utilized the program MitoProt II (24). The same program was used to calculate the segments with high local hydrophobicity (<H>) in a distance comprising 13–17 amino acids. The mesohydrophobicity was estimated by scanning each sequence for a maximum average hydrophobicity measured in windows from 60 to 80 amino acids and averaging the values. We used several hydrophobicity scales to reduce the possibility of bias.

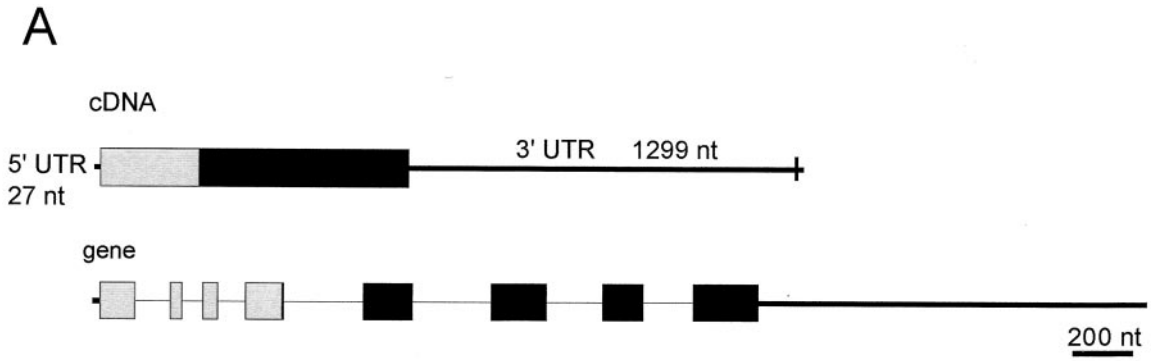
Protein transmembrane regions and secondary structure were predicted using the program TodPred II (25), the PredictProtein Web server (www.es.embnet.org/Services/MolBio/PredictProtein/) (26), and the ExPASy Molecular Biology Server (www.expasy.ch).

Data used to analyze the *atp6* gene codon usage were obtained from the Codon Usage Database (www.kazusa.or.jp/codon/) with accession numbers [gbpln]:237 for nuclear genes and [gbpln]:21 for mitochondrial genes of *C. reinhardtii*.

RESULTS

Characterization of the atp6 cDNA from C. reinhardtii—We identified a *C. reinhardtii* EST sequence as a partial fragment of an *atp6* cDNA by the similarity of the predicted translation product with known plant ATP6 proteins. This sequence was used to identify two additional, partially overlapping, EST sequences as potential components of a full-length *atp6* cDNA, although portions of the predicted polypeptide lacked similarity with known ATP6 proteins. Based on these EST sequences, deoxyoligonucleotides were designed and used for PCR amplification. A PCR product of 822 nt was obtained using a *C. reinhardtii* cDNA library as template. The sequence of the amplified product confirmed the co-linearity of the EST sequence fragments. The 5′- and 3′-ends of the cDNA were obtained by RACE PCR (17) using cDNA made from *C. reinhardtii* total RNA. The full-length cDNA was PCR-amplified as two overlapping fragments and sequenced, and a length of 2349 nt was obtained for the *atp6* cDNA.

The *C. reinhardtii atp6* cDNA contains a 5′-untranslated region of 27 nt followed by an open reading frame of 1014 nt (Fig. 1*A*). The sequence flanking the proposed open reading frame initiating methionine codon, AACCATGG, is a consensus translation initiation site (A/C)A(A/C)(A/C)ATG(G/C) for *C. reinhardtii* (27). The TAA stop codon corresponds to the one



B

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Cr                MALQQAA PRVFGLLGRA PVALGQSGIL TGSSGFKNQG FNGSLQSVEN   -61
So                ------- ---------- ---------- ---------- ----------

Cr     HVYAQAFSTS SQEEQAAPSI QGASGMKLPG MAGSMLLGKS RSGLRTGSMV PFAAQQAMNM    -1
So     ---------- ---------- ---------- ---------- ---------- ----------

Cr     ---------- -----STAAQ VQSGATVNSL LLGIY----- -RFWRSQAPM DKPHAPVDDR    39
So     MILNYFLFNY SSRIIASAAE AWQSVPLVSL HLPFMGAVFS LNIWAVTVAI AMLVLVVNHS
                     ::**:       : **  * :         :*     :        *:

Cr     MLPAIVDASD ---------- ---------- ---------- ------NRAA LGT-------    56
So     LNHSSAQAGN SALSGFRKSV SGFWNTQTLE RVHLKWRQLS ARSAQTDRVS LSTNRLPSAW
       :  :  :* :                                          :* : * *

Cr     ---------- ---WATALFC TILASNLLGL VPTNEAPTSG LGFATGLGVS VWATATTLGL   103
So     RALSDIQGKT TVVWSSVLFF ILLASNSFGL VPLCSAITGQ AGFTLGMSIR LLSGITYLGV
                    *:: **   :**** :** **   * *    **: *: :  : :  * **:

Cr     YKLGFSFPGH FIPGGTPWPM AFIFVPLETI SYTFRAVSLG VRLWVNMLAG HTLLHILTGM   163
So     KRQGMRFVRL FLPSGPSWPM APLFILLESI SYGFRAISLG TRLYCNMFAG HLLLHLFTSL
        : *: *    *:* *  *** * :*: **:* ** ***:***  **: **:** * ***::* :

Cr     ALALPFSLSF FAMVPATFAV ACLLSALVGL EYLVAVLQSG VFSILSTVYV GEFNSVKLAG   223
So     SLVPVLCTPV LLGAPFTVIA ASILMALSAL ETIVAVLQSG VFCLLSGFYV TEVLRRKDE-
       :*   :     :   * *    * :* **  * * :******* ** :**  **  *    *

Cr     PLAKVVKKAL   233
So     -LASAL----
        **  :
```

FIG. 1. **Organization of the *atp6* gene of *C. reinhardtii* and sequence comparison of *C. reinhardtii* and *S. obliquus* ATP6 subunits.** *A*, diagram of the organization of the *C. reinhardtii atp6* gene. The coding regions of the *atp6* cDNA and *atp6* gene are shown as *boxes*, the nontranslated regions are shown as *thick black lines*, and introns are indicated by a *thin black line*. The putative mitochondrial targeting sequence is *gray* and the polyadenylation signal is indicated as a *vertical black bar*. *B*, the amino acid sequence alignment of the ATP6 proteins of the F_1F_0-ATP synthase from *C. reinhardtii* (*Cr*) and *S. obliquus* (*So*) (28) are shown. *Black triangles* indicate the positions of introns in the corresponding gene sequence of *C. reinhardtii*. The *C. reinhardtii* sequence is *numbered*, using 1 as the first amino acid of the mature protein. The amino acids of the MTS have negative numbers. The MTS of the *C. reinhardtii* protein is in *boldface type*. The amino-terminal sequence of mature Cr-ATP6 obtained by Edman degradation is *boxed*. *, identical amino acids; :, similar amino acids. The sequence of the mature Cr-ATP6 is 39% identical and 46% similar to that of the *S. obliquus* ATP6. *UTR*, untranslated region.

present in the majority of nuclear genes (70% of the reported genes use this stop codon), and the flanking nucleotides GTAAG are identical to the consensus sequence (G/C)TAA(G/A), characteristic of *C. reinhardtii* nuclear genes (27). The deduced sequence predicts a Cr-ATP6 preprotein of 340 amino acids. A BLAST search of the SwissProt nonredundant protein data base using the Cr-ATP6 sequence produced the highest similarity with the mtDNA-encoded ATP6 subunit from the green alga *S. obliquus* (28, 29), also a chlorophyte alga. The two proteins shared 39% identity and 46% similarity (Fig. 1*B*). The similarity is highest at the carboxyl-terminal region of the protein (59% similarity over amino acids 114–233).

The *atp6* cDNA contains a 3′-untranslated region of 1299 nt (Fig. 1*A*). Two different putative polyadenylation signals were found in the Cr-ATP6 cDNA: TGTAA, the typical signal of nuclear genes of this alga, located 15 nt before the end of the cDNA sequence, and TGTAG, a variation of the most common signal (27), located 1048 nt upstream from the first polyadenylation site. When total RNA was isolated and hybridized with the *atp6* probe in a Northern blot analysis, a single band of 2.4 kb was observed. This result suggested that only the orthodox TGTAA polyadenylation site is functional in the *atp6* RNA sequence (Fig. 2*C*).

Genomic Sequence of atp6 from C. reinhardtii—The complete genomic sequence of the *atp6* gene was obtained following PCR amplification of three overlapping genomic regions using prim-

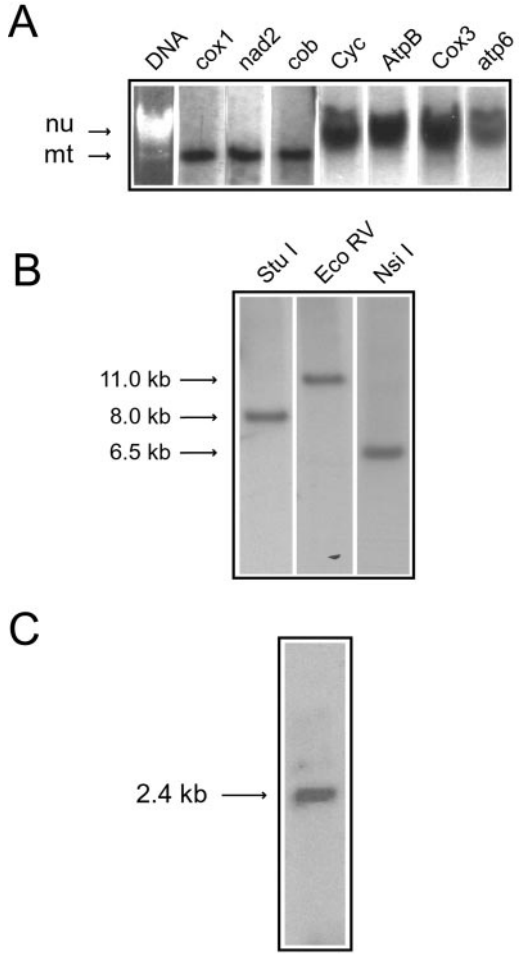

FIG. 2. **The *atp6* gene is nucleus-localized, present in a single copy, and expressed in *C. reinhardtii*.** *A*, nuclear localization of the *atp6* gene. Thirty micrograms of total DNA from *C. reinhardtii* was electrophoresed through a 0.7% agarose gel. The *first lane* (*DNA*) shows the ethidium bromide-stained gel. The *subsequent lanes* show Southern blot analyses of the same gel hybridized with different mtDNA-encoded (*cox1*, *nad2*, and *cob*) and nucleus-encoded (*Cyc*, *AtpB*, and *Cox3*) gene probes. The *atp6* probe hybridized with the nuclear DNA and not with the mtDNA. *B*, *atp6* is a single copy gene. Total DNA from *C. reinhardtii* was digested with the restriction enzymes *Stu*I, *Eco*RV, and *Nsi*I and subjected to Southern blot analysis with a labeled probe for the *atp6* gene. Single hybridizing fragments were detected, suggesting the presence of a single *atp6* gene. *C*, Northern blot analysis of total RNA from *C. reinhardtii*. Twenty micrograms of total RNA from *C. reinhardtii* was electrophoresed through 1% agarose, 0.66 M formaldehyde gels and subjected to Northern blot analysis with a labeled probe for the *atp6* gene. A single hybridizing band of 2.4 kb was detected.

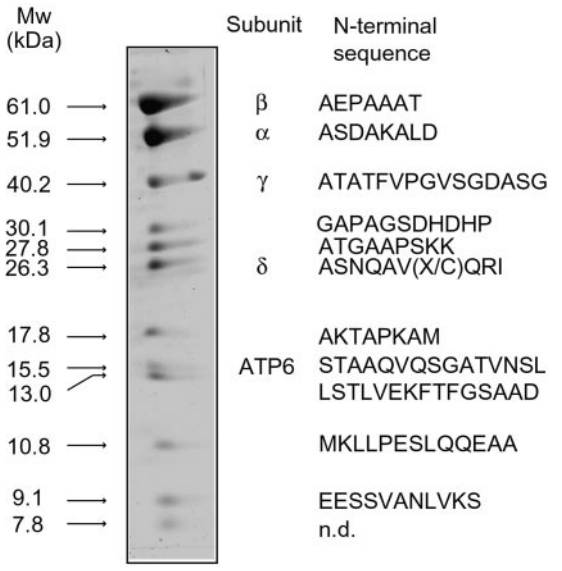

FIG. 3. **Subunit composition of the F_1F_0-ATP synthase complex from *C. reinhardtii*.** The mitochondrial respiratory chain complexes were separated by blue native electrophoresis and in the second dimension by Tricine-SDS-PAGE. Shown is the ATP synthase complex after electrophoresis on a Tricine-SDS-polyacrylamide gel. The amino-terminal sequences of the polypeptides were determined, and the identified subunits are indicated. *n.d.*, not determined.

ers derived from the cDNA sequence. The 3577-nt gene contained seven introns, ranging from 72 to 263 nt in length. These introns were present in the coding regions for both the mitochondrial targeting sequence and the mature portion of the protein (Fig. 1, *A* and *B*).

The atp6 Gene Is Located in the Nucleus in C. reinhardtii—When total DNA isolated from *C. reinhardtii* was electrophoresed on agarose, the mtDNA separated as a discrete band running below the major band representing nuclear and chloroplastic DNA (11). Southern blot analysis was carried out to ascertain if the *atp6* gene was present in the nuclear genome. The lower band hybridized with three different mtDNA probes from *C. reinhardtii*, *cox1*, *nad2*, and *cob* (7). In contrast, nuclear DNA hybridized with the *atp6* gene, obtained in this study, and three probes derived from *C. reinhardtii* nuclear genes: *Cyc* encoding cytochrome *c* (30), *AtpB* encoding the β subunit of the ATP synthase (16), and *Cox3* encoding subunit III of cytochrome *c* oxidase (11) (Fig. 2*A*). A second Southern blot analysis was carried out to determine whether the *atp6* gene was present as a single copy gene in the genome of *C. reinhardtii*. Total DNA was digested with three different restriction enzymes. Southern analysis utilizing an 822-nt PCR product of the coding region of the *atp6* gene as a probe, resolved a single band in each of the restriction digests, suggesting that this gene is present in a single copy in the *C. reinhardtii* genome (Fig. 2*B*).

The pattern of codon utilization of the *atp6* gene was compared with the pattern of codon usage of other known nuclear, chloroplast, and mitochondrial genes of *C. reinhardtii*. Each codon family of the *atp6* gene favored C or G in the third position and is therefore typically nuclear and different from mitochondrial and chloroplast genes (27) (data not shown).

ATP6 Is a Constituent of F_1F_0-ATP Synthase of C. reinhardtii—If the nuclear localized *atp6* gene of *C. reinhardtii* is expressed, the corresponding Cr-ATP6 subunit should be present in the F_1F_0-ATP synthase complex. To identify the Cr-ATP6 subunit, isolated mitochondria from *C. reinhardtii* were solubilized and subjected to blue native electrophoresis followed by second dimension analysis by denaturing Tricine-SDS-PAGE. The F_1F_0-ATP synthase was identified by its characteristic electrophoretic pattern on the second dimension gels. Twelve different polypeptides were present in this complex (Fig. 3). The three major subunits were identified as subunits α, β, and γ, since their apparent molecular masses are similar to those reported previously (31). Their identities were confirmed by N-terminal sequence analysis of their first five amino acids. The additional eight polypeptides were subjected to more extensive N-terminal sequencing (Fig. 3). The 26.3-kDa subunit was identified as subunit δ of F_1F_0-ATP synthase because of its sequence similarity with other δ subunits. The 15.5-kDa polypeptide was identified as subunit ATP6, the protein product encoded by *atp6*, since its initial 15 amino acids matched exactly amino acids 108–122 of the deduced protein sequence encoded by the *atp6* cDNA. This confirms that the *atp6* gene product is a constituent of the F_1F_0-ATPase complex of *C. reinhardtii*. The N-terminal sequences of the eight remaining

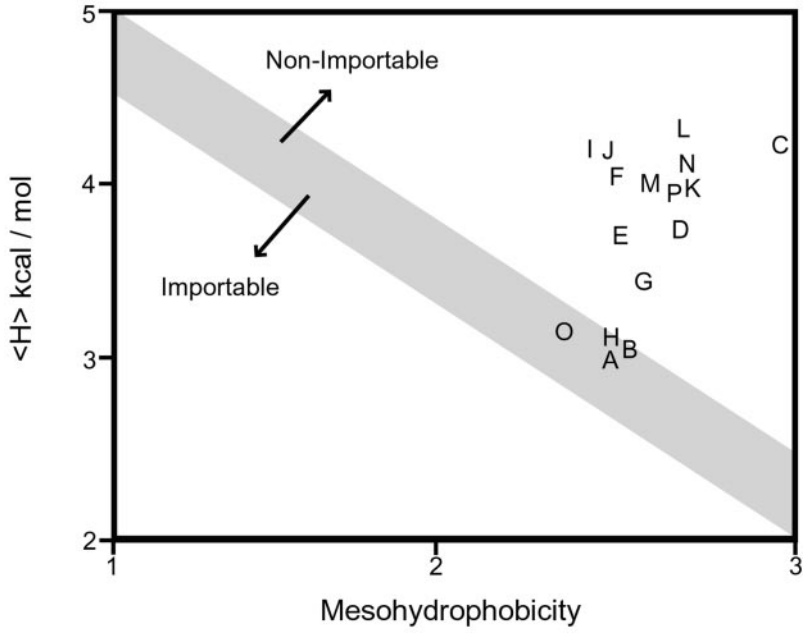

FIG. 4. **Plot of mesohydrophobicity *versus* maximal local hydrophobicity for ATP6 proteins.** The MitoProtII program was used to calculate local hydrophobicity values (<H>) and mesohydrophobicity values for ATP6 proteins, using the PRIFT scale. Proteins are distributed on the *abscissa* according to their mesohydrophobicity value and on the *ordinate* according to the hydrophobicity of the most hydrophobic 17-residue segment. The hypothetical boundary between importable and nonimportable proteins, indicated by a *broad*, *gray diagonal*, was derived from Claros *et al.* (39) and Pérez-Martínez *et al*. (11). The following proteins with their symbols were analyzed: *C. reinhardtii* (*A*), *S. obliquus* (*B*), *P. minor* (*C*), *H. annuus* (*D*), *P. subcordiformis* (*E*), *Z. mays* (*F*), *P. wickerhamii* (*G*), *O. sativa* (*H*), *S. tuberosum* (*I*), *N. tabacum* (*J*), *B. napus* (*K*), *T. aestivum* (*L*), *M. polymorpha* (*M*), *B. vulgaris* (*N*), *G. max* (*O*), *A. thaliana (P)*.

polypeptides showed no evident similarity with other ATPase subunits, and their identities remain to be established.

Our data indicate that the mature Cr-ATP6 subunit is a protein of 233 amino acids with an expected molecular mass of 24,577 Da and predicts that Cr-ATP6 has an MTS of 107 amino acids, which is cleaved upon import of this polypeptide into mitochondria. For numbering purposes, the mature protein Cr-ATP6 is considered to start at amino acid number 1, and the amino acids that belong to the putative MTS are numbered accordingly (Fig. 1*B*).

Hydrophobicity and Importability of the Nucleus-encoded Subunit ATP6 from C. reinhardtii—Mitochondrial protein import studies suggest that the highest average hydrophobicity over 60–80 amino acids of a polypeptide chain (mesohydrophobicity), along with the maximum hydrophobicity of the putative transmembrane segments, are useful indicators of the likelihood that a protein could be imported into mitochondria (32). We have previously shown that the transfer of genes from the mtDNA to the nucleus in chlamydomonad algae is accompanied by a decrease of these parameters for the COX IIA, COX IIB, and COX III subunits of cytochrome *c* oxidase (11, 12). The physical characteristics of the predicted Cr-ATP6 polypeptide were examined *in silico* and compared with those of ATP6 subunits encoded by other complete plant and algal mitochondrial *atp6* genes. Fig. 4 shows the plot of mesohydrophobicity *versus* maximal local hydrophobicity (<H>) for Cr-ATP6 and for different mtDNA-encoded ATP6 sequences. When compared with several of its mitochondrial counterparts, Cr-ATP6 displays both decreased <H> and mesohydrophobicity. However, other mtDNA encoded ATP6 subunits, like those from *S. obliquus*, *O. sativa*, and *G. max*, also exhibit reduced mesohydrophobicity and <H>.

The reduction of mesohydrophobicity and <H> depends on two factors: the length of the hydrophilic loops between transmembrane domains and the mean hydrophobicity of each transmembrane segment. The alignment of all available ATP6 sequences reveals that the distances between transmembrane domains have not changed (results not shown). Hence, the mean hydrophobicity of each transmembrane segment ought to be reduced in Cr-ATP6. To determine in which regions the hydrophobicity was diminished, hydropathy plots of Cr-ATP6 were compared with those of ATP6 sequences from plants and algae. Analysis of Cr-ATP6 predicts that it contains at least six hydrophobic regions that could be transmembrane regions (data not shown). However, topological considerations argue against the possibility that all hydrophobic segments span the membrane. The most precise positioning of the putative transmembrane helices was obtained by aligning all reported ATP6 sequences and predicting the secondary structure. This analysis suggested a model in which there are five transmembrane segments, named A–E (Fig. 5). This model is in accordance with the topology of the amino and carboxyl termini and the presence of conserved and functionally required amino acids (33–36).

Establishing the boundaries of the five putative transmembrane regions allowed us to quantitate the mean hydrophobicity for each in *C. reinhardtii* and mtDNA-encoded ATP6 subunits from other algae and plants (Fig. 6). While transmembrane helices D and E exhibit similar <H> values when compared with the mean <H> values in helices D and E of plant and algal mitochondrial sequences, significant differences in <H> values were found in the putative transmembrane regions A, B, and C, which are not believed to participate directly in proton translocation and are not thought to interact with the multimeric ring of *c* subunits. This decrease was more than 50% in helices A and C of Cr-ATP6 in comparison with the <H> values of helices A and C of other mtDNA-encoded ATP6 subunits. We conclude that the Cr-ATP6 subunit exhibits diminished overall hydrophobicity as compared with the majority of mtDNA-encoded homologs and that the main decrease in hydrophobicity occurred in those transmembrane regions that exhibit poor sequence conservation and that seem not to be critical for proton translocation.

DISCUSSION

The Gene atp6 of C. reinhardtii Exhibits Nucleus-encoded Characteristics—The 15.8-kb linear mitochondrial genome from *C. reinhardtii* lacks several genes that are typically mtDNA-encoded, including three genes that encode subunits of the F_1F_0-ATP synthase (7). In this work, we show that the *atp6* gene was transferred from the mitochondrial genome to the nucleus in *C. reinhardtii* and demonstrate that the ATP6 protein is present in the mitochondrial F_1F_0-ATP synthase. The *atp6* gene has been previously found only in mitochondrial genomes. This is the first biochemical and genetic evidence for the nuclear localization of *atp6*. We show that the *atp6* gene is

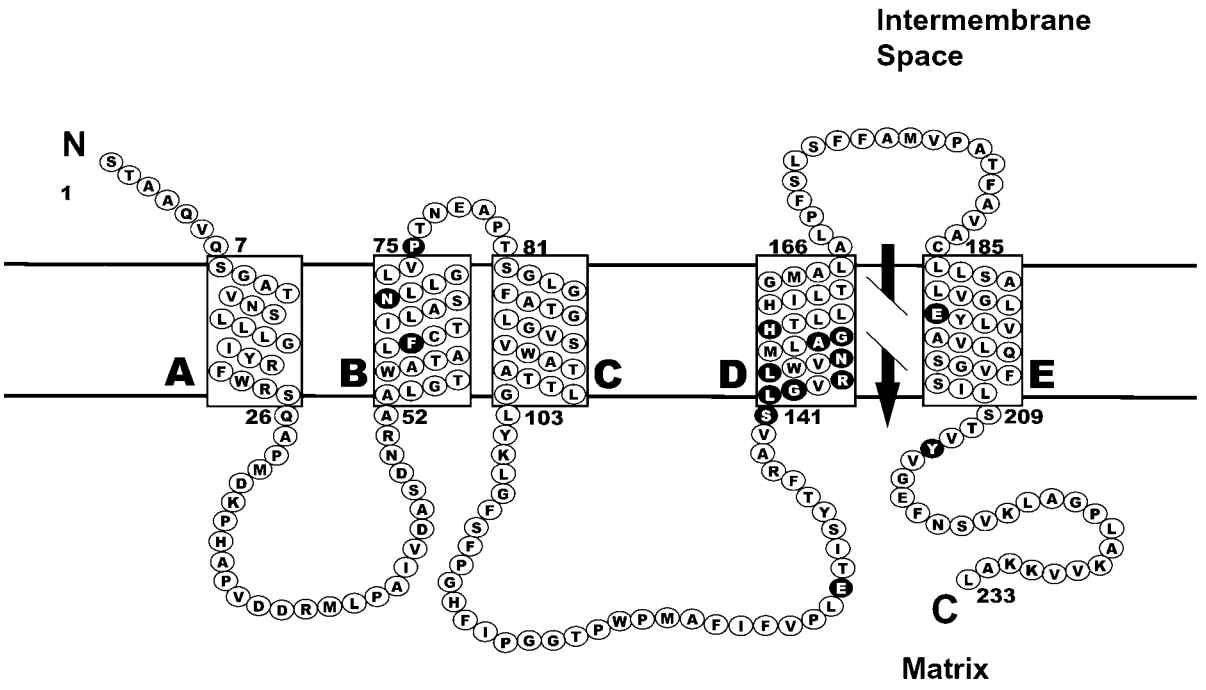

FIG. 5. **Model for the transmembrane helices of the ATP6 subunit of the F_1F_0-ATP synthase from *C. reinhardtii*.** Shown is a model for the location of the transmembrane helices of Cr-ATP6. Conserved amino acids are shown as *black circles*. Transmembrane regions D and E participate in proton translocation. Proton translocation is depicted as a *broken arrow* to illustrate the participation of two hemichannels in subunit ATP6. For simplicity, the multimeric ring of *c* subunits is not depicted.

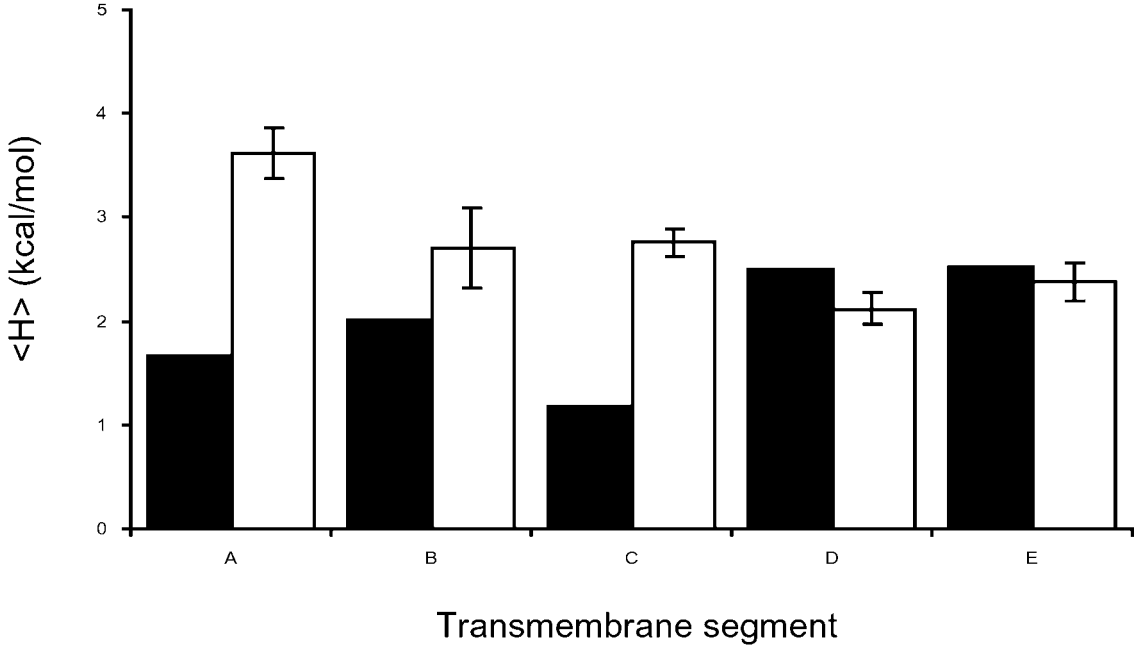

FIG. 6. **Analysis of the mean hydrophobicity values of the five transmembrane regions.** Hydrophobicity values for the five putative transmembrane helices of the Cr-ATP6 (*black bars*) were compared with the mean value of hydrophobicity for 21 mtDNA-encoded ATP6 proteins from plants (*white bars*). In Cr-ATP6, mean hydrophobicity is reduced in transmembrane regions A, B, and C (54, 25, and 57% diminished) as compared with the mean of the mean hydrophobicity values of algal and plant transmembrane regions.

nucleus-localized in *C. reinhardtii* by Southern blot hybridization (Fig. 2*A*). The *atp6* gene exhibits all of the characteristics of a mitochondrial gene that was functionally transferred to the nucleus (37): a nuclear codon usage, a typical nuclear polyadenylation signal, the presence of introns, acquisition of a DNA sequence encoding an amino-terminal MTS, and diminished hydrophobicity relative to many equivalent mtDNA-encoded proteins.

The deduced amino acid sequence of the ATP6 cDNA and the N-terminal sequence of the mature protein predict the existence of a 107-amino acid MTS. This MTS is likely to have been acquired after the original mitochondrial gene was transferred to the nuclear genome. This MTS has a low number of acidic residues, and amino acids 2–24 are predicted to form the amphipathic domain necessary for import of the protein into mitochondria. The methionine (Met^1) adjacent to the MTS cleavage site of the full-length Cr-ATP6 may have been the initiating methionine of the ancestral mitochondrial protein before its corresponding gene was transferred to the nucleus. The MTS is unusually long when compared with other *C.*

reinhardtii MTSs, which exhibit a mean length of 30 amino acids (38). However, we previously found that the nuclear genes encoding COX IIA and COX III of *C. reinhardtii* and *Polytomella* sp., proteins that are typically encoded in the mtDNA, are also predicted to have long MTSs, of 98 and 130 amino acids (11, 12). This suggests that the mitochondrial import of the more hydrophobic mitochondrial proteins, such as COX IIA, COX III, and ATP6, may benefit from an extended MTS.

The duplication of the MTS was found to improve the *in vitro* and *in vivo* import of hydrophobic proteins into yeast mitochondria (32, 39). It was suggested that a long MTS can improve the interaction of the precursor with the mitochondrial import machinery. Alternatively, a long MTS could facilitate the folding of the protein to increase its importability and assembly in the membrane.

The vast majority of the plant mitochondrial ATP6 sequences exhibit physical constraints that are expected to hinder their import into mitochondria (Fig. 4), consistent with their presence in the mitochondrial genome. In contrast, Cr-ATP6 has a lower overall hydrophobicity that allows its import into mitochondria, most probably through the TOM-TIM machinery (40). In addition, some mtDNA-encoded ATP6 sequences also exhibit low <H> and mesohydrophobicity values (*S. obliquus*, *O. sativa*, and *G. max*). These genes seem to be "prepared," from the hydrophobicity point of view, for transfer to the nucleus. We hypothesize that the ancestral Cr-ATP6 protein had a low overall hydrophobicity that enabled the *atp6* gene to be functionally transferred to the nucleus.

Transfer of atp6 and Other mtDNA-encoded ATP Synthase Subunits to the Nucleus—Mitochondrial genomes range widely in size and gene content. Only two genes encoding components of the mitochondrial respiratory chain are invariably present in all mitochondrial genomes known to date: *cob* and *cox1.* Their polytopic protein products are central components of proton-translocating complexes: cytochrome *b* of the bc_1 complex and subunit I of cytochrome *c* oxidase. Cr-ATP6 is the first example of a mitochondrial protein with more than two transmembrane helices, directly involved in proton translocation, that is nucleus-encoded.

The highly reduced 6-kb mtDNA of the parasite *Plasmodium falciparum* contains three genes encoding components of the mitochondrial respiratory chain: *cob*, *cox1*, and *cox3* (41). At the other end of the mtDNA size spectrum, the 60-kb mtDNA of the flagellate *Reclinomonas americana* (42) encodes 23 proteins that participate in oxidative phosphorylation. Five of these are constituents of the F_1F_0-ATP synthase complex: the proteins encoded by the genes *atp1*, *atp3*, *atp6*, *atp8*, and *atp9*. In the mitochondrial genomes of fungi and plants, three genes encoding hydrophobic components of the F_0 sector of F_1F_0-ATP synthase are usually found: *atp6*, *atp8*, and *atp9*. These three genes are absent in the mtDNA of *C. reinhardtii*.

The relocation of *C. reinhardtii atp6* to the nucleus occurred relatively late in evolution, after the massive transfer of genes from the protomitochondrion to the nucleus (43). In the evolution of the green algae, the transfer of *atp6* to the nucleus occurred after the separation of the *Scenedesmus* and *Prototheca* lineages, where *atp6* is still mtDNA-encoded, from the *Chlamydomonas* and *Chlorogonium* lineages, where the *atp6* gene is no longer encoded in the mitochondrial genome.

The transfer of the *atp6* gene to the nuclear genome is unlikely to be exclusive to *C. reinhardtii*. Other organisms that lack the *atp6* gene in their mitochondrial genomes and that are likely to have transferred it to the nucleus are the closely related algae *C. eugametos* (9) and *C. elongatum* (10), the apicomplexan organisms *P. falciparum* (41) and *Plasmodium reinchenowi* (44), and the ciliates *Paramecium aurelia* (45) and *Tetrahymena pyriformis* (46). It would be interesting to examine the sequences of ATP6 from more distantly related organisms to determine whether they have reduced physical constraints for import and to determine where reductions in hydrophobicity occur in the protein.

Mean Hydrophobicity Has Strongly Decreased in Those Transmembrane Regions of Cr-ATP6 That Are Not Critical for Function—The transfer of the *atp6* gene from the mtDNA to the nucleus was accompanied by, or preceded by, a strong decrease in the overall hydrophobicity of the encoded protein (Figs. 4 and 5) and probably facilitates the import and assembly of Cr-ATP6 into an active F_1F_0-ATP synthase. Hydrophobicity analyses, in combination with the alignment of all ATP6 sequences available and previous published observations (33–36), allowed us to predict the presence of five well defined transmembrane regions in Cr-ATP6, named A–E (Fig. 5). The decreased hydrophobicity occurs predominantly in transmembrane regions A, B, and C (Fig. 6), which are poorly conserved among other ATP6 proteins and are thought not to participate directly in the proton translocating function of ATP6. In contrast, the well conserved transmembrane regions D and E maintain similar levels of mean hydrophobicity to mtDNA-encoded ATP6 subunits. We previously observed a similar phenomenon in the COX III proteins of *C. reinhardtii* and *Polytomella* sp. COX III is typically mtDNA-encoded but has been transferred to the nucleus in these algae (11). The nucleus-encoded COX III proteins showed greater diminished hydrophobicity in regions of the protein not in contact with the COX I subunit. This suggests that decreases in mean hydrophobicity of mitochondrial proteins whose genes have been relocated to the nucleus are more likely to occur in regions of the protein not involved in subunit-subunit interactions or in protein function.

Cr-ATP6 contains several amino acids that are conserved in all ATP6 sequences. Helix B ends in a highly conserved Pro and contains two additional conserved amino acids, Phe^{62} and Asn^{69}. The presence of Asn is not very common in transmembrane segments, so we believe its presence may have a role in transmembrane helix association through interhelical hydrogen bonding (47, 48). Transmembrane helices D and E contain most of the conserved amino acids; helix D is preceded by a highly conserved Glu^{131} involved in the proton translocation pathway and contains the invariant amino acids Ser^{141}, Leu^{142}, Gly^{143}, Leu^{146}, Asn^{149}, Ala^{152}, Gly^{153}, and His^{154}. There is also an invariant Arg^{145} that has been previously described as a residue involved in the protonation of the *c* subunit oligomer. Helix E contains the conserved Glu^{194}, which is hypothesized to be involved in proton translocation, and Tyr^{212}, which may be structurally important, after the end of this helix (Fig. 5).

Implications for Human Mitochondrial Gene Therapy—A considerable number of human diseases have been associated with point mutations or deletions in the mitochondrial genome (49–51), and strategies to develop mitochondrial gene therapies have been suggested (52, 53). One promising approach for overcoming mutations in mtDNA-encoded proteins is to place a wild-type copy of the affected gene in the nucleus and target the expressed protein to the mitochondrion to replace the defective mtDNA-encoded protein. Such allotopic expression of a normally mtDNA-encoded gene has been successfully performed in *S. cerevisiae* to overcome a mutation in the mtDNA-encoded *atp8* gene (54).

Analysis of nuclear forms of proteins that are normally encoded in the mtDNA may provide insights that will facilitate the allotopic expression of genes harboring mutations in the mtDNA-encoded genes. The observed decreases in hydrophobicity of nuclear expressed COX III and ATP6 of *C. reinhardtii*

suggest that human mitochondrial genes could potentially be engineered for allotopic expression for gene therapy purposes by modifications to decrease the mean hydrophobicity of the protein product, especially in transmembrane regions that are not highly conserved or that are known to be noncritical for function. Such changes would be in addition to the changes in codon usage and the addition of an MTS-coding sequence that are a prerequisite for nuclear expression of a mitochondrial protein. The presence of long MTSs in COX III, COX IIA, and ATP6 from *C. reinhardtii* suggests that a long MTS would facilitate import in allotopic expression systems. A detailed knowledge of the topology and function of the nucleus-encoded hydrophobic mitochondrial proteins may enhance our understanding of the problems involved in allotopic expression in addition to providing insights into the evolutionary forces that have led to the nuclear localization of genes that are typically mtDNA-encoded.

Acknowledgments—We thank Dr. J. d'Alayer (Institut Pasteur) for expert help in sequencing peptides, Dr. L.-G. Franzén (Goteborg University) for the kind gift of the cDNA library of *C. reinhardtii*, Drs. M. Futai and H. Omote (Osaka University) for carrying out initial immunoblot analysis with antibodies against the *E. coli* subunit *a*, Dr. S. B. Vik (Southern Methodist University) for helpful discussions while predicting the Cr-ATP6 model, and several colleagues and researchers who kindly provided DNA probes that were used in this work: Drs. H. Handa (Hokkaido National Agricultural Experiment Station, Japan), K. Kadowaki (National Institute of Agrobiological Resources, Ibaraki, Japan), and F. Kempken (Ruhr-Universitat Bochum, Germany). We are grateful to Drs. A. Atteia (Instituto de Fisiología Celular, Universidad Nacional Autónoma de México), D. W. Krogmann (Purdue University), M. Tuena (Instituto de Fisiología Celular, Universidad Nacional Autónoma de México), and S. B. Vik (Southern Methodist University) for critical comments to the manuscript.

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