An Algal Nucleus-encoded Subunit of Mitochondrial ATP Synthase Rescues a Defect in the Analogous Human Mitochondrial-encoded Subunit

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Unlike most organisms, the mitochondrial DNA (mtDNA) of *Chlamydomonas reinhardtii*, a green alga, does not encode subunit 6 of F_0F_1 -ATP synthase. We hypothesized that *C. reinhardtii* ATPase 6 is nucleus encoded and identified cDNAs and a single-copy nuclear gene specifying this subunit (*CrATP6*, with eight exons, four of which encode a mitochondrial targeting signal). Although the algal and human *ATP6* genes are in different subcellular compartments and the encoded polypeptides are highly diverged, their secondary structures are remarkably similar. When *CrATP6* was expressed in human cells, a significant amount of the precursor polypeptide was targeted to mitochondria, the mitochondrial targeting signal was cleaved within the organelle, and the mature polypeptide was assembled into human ATP synthase. In spite of the evolutionary distance between algae and mammals, *C. reinhardtii* ATPase 6 functioned in human cells, because deficiencies in both cell viability and ATP synthesis in transmitochondrial cell lines harboring a pathogenic mutation in the human mtDNA-encoded *ATP6* gene were overcome by expression of *CrATP6*. The ability to express a nucleus-encoded version of a mammalian mtDNA-encoded protein may provide a way to import other highly hydrophobic proteins into mitochondria and could serve as the basis for a gene therapy approach to treat human mitochondrial diseases.

INTRODUCTION

Complex V of the respiratory chain/oxidative phosphorylation system, or F_0F_1 -ATP synthase (E.C. 3.6.1.14), couples the passage of protons across the intermembrane space to the synthesis of ATP from ADP and P_i (Elston *et al.*, 1998; Noji and Yoshida, 2001). In humans, the complex comprises at least 14 nucleus-encoded subunits (α , β , γ , δ , ϵ , b, c, d, e, f, g, h, IF1, and OSCP) and two mitochondrial DNA (mtDNA)-encoded subunits (ATPase 6 [subunit a in *Escherichia coli*] and ATPase 8). The F_0 portion of the complex, located in the mitochondrial inner membrane, contains a ring of c subunits surrounding a central γ subunit “stalk” that rotates within the F_1 portion of the complex, which is a spherical hexamer

of three α - β dimers that protrudes into the matrix. ATPase 6, which is part of F_0 , forms a channel through which proton flow is coupled to rotation of the c-ring (Rastogi and Girvin, 1999; Hutcheon *et al.*, 2001).

Maternally inherited mutations in the human gene encoding ATPase 6 (*MTATP6*) are responsible for two related mitochondrial encephalomyopathies: neuropathy, ataxia, and retinitis pigmentosa (NARP) (Holt *et al.*, 1990) and maternally inherited Leigh syndrome (MILS) (Tatuch *et al.*, 1992). The most common mutation in these disorders is a T→G transversion at nucleotide (nt) 8993 in human mtDNA (Anderson *et al.*, 1981), converting Leu-156 to Arg. In both disorders, the mutation is heteroplasmic, that is, the patient harbors both wild-type and mutated mtDNAs, with 70–90% mutated mtDNA in NARP patients and 90–95% in MILS patients; asymptomatic or oligosymptomatic mothers of these patients usually have <70% mutation in blood cells (i.e., the mutation behaves in a recessive manner). Importantly, in cells harboring high levels of the mutation, ATP synthesis is decreased by ~50–70% (Tatuch and Robinson, 1993; Vazquez-Memije *et al.*, 1996; Manfredi *et al.*, 1999; Garcia *et al.*, 2000; Schon *et al.*, 2001; Manfredi *et al.*, 2002).

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Abbreviations used: EST, expressed sequence tag; mtDNA, mitochondrial DNA; MILS, maternally inherited Leigh syndrome; NARP, neuropathy, ataxia, and retinitis pigmentosa; nt, nucleotide.

In humans, as in almost all other organisms examined to date, ATPase 6 is encoded by mtDNA. Among the unicellular algae, however, only some species contain an mtDNA-encoded *ATP6* gene, for example, *Prototheca wickerhamii*, *Pedinomonas minor*, and the stramenopile algae *Cafeteria roenbergensis* and *Chrysodidymus synuroideus*. In other algal species, however, including *Chlorogonium elongatum* (Kroymann and Zetsche, 1998), *Chlamydomonas eugametos* (Denovan-Wright *et al.*, 1998), and *Chlamydomonas reinhardtii* (Gray *et al.*, 1988), no gene encoding ATPase 6 can be found in their mtDNA. Because the mitochondrial F_0F_1 -ATP synthase in *C. reinhardtii* is sensitive to oligomycin (Nurani and Franzen, 1996) and because oligomycin sensitivity is conferred by ATPase 6 (Breen *et al.*, 1986; John and Nagley, 1986), we reasoned that a gene specifying this subunit is encoded in the *C. reinhardtii* nuclear genome. In support of this view, it had already been shown that the genes specifying cytochrome *c* oxidase (COX) II and COX III, two subunits of cytochrome *c* oxidase that are typically mtDNA-encoded but that are also absent from the *C. reinhardtii* mitochondrial genome (GenBank U03843), are nuclear encoded instead (Perez-Martinez *et al.*, 2000, 2001, 2002; Watanabe and Ohama, 2001).

We recently showed that expression of the human mtDNA-encoded *MTATP6* gene from a relocated position in the nucleus ("allotopic expression"; Law *et al.*, 1988; Nagley *et al.*, 1988; Claros *et al.*, 1996; Gray *et al.*, 1996; de Gray, 2000; Zullo, 2001) could complement a deficiency in ATP synthesis in transmitochondrial cells harboring the T8993G mutation associated with NARP and MILS (Manfredi *et al.*, 2002). We show herein that the nucleus-encoded *ATP6* gene from *C. reinhardtii* (Funes *et al.*, 2002) can be expressed in human cells and can also rescue the ATP synthesis defect in human cells harboring this mtDNA mutation.

MATERIALS AND METHODS

Isolation of *C. reinhardtii* ATP6

C. reinhardtii strain 21 gr (mt+) (CC-1690) was obtained from the *Chlamydomonas* Genetic Center (Duke University, Durham, NC) and was cultured under continuous light at room temperature (Snell, 1976). Using the *Chlamydomonas* EST Database (<http://www.kazusa.or.jp/en/plant/chlamy/EST/>) (Asamizu *et al.*, 1999, 2000), we identified a number of overlapping expressed sequence tags (ESTs) encoding the putative *ATP6* mRNA. Sets of oligonucleotides were designed based on predicted overlapping ESTs to amplify both the full-length cDNA and the chromosomal gene. Total RNA was extracted using standard methods (Wegener and Beck, 1991). First-strand cDNA was obtained using the SuperScript First Strand Synthesis System for reverse transcription-polymerase chain reaction (PCR) (Invitrogen, Carlsbad, CA). The SMART RACE cDNA Amplification kit (CLONTECH, Palo Alto, CA) was used for determination of the 5'- and 3'-untranslated regions (UTRs). Amplified PCR products were inserted into pCRII-TOPO Vector (Invitrogen) for sequencing. Isolated clones were screened by automated sequencing using the ABI Prism Big Dye kit (PerkinElmer Life Sciences, Boston, MA). For amplification of the genomic sequence, genomic DNA was isolated by standard methods (Pan and Snell, 2000), and 1 μ g of total DNA was used as a template in a PCR reaction using high-fidelity *Taq* Polymerase (Roche Applied Science, Indianapolis, IN). The *C. reinhardtii* *ATP6* (*CrATP6*) cDNA and genomic sequences have been deposited in GenBank (AF388174 and AY063772, respectively).

Southern Blot Hybridization

Ten micrograms of total genomic DNA were digested with appropriate restriction enzymes, separated through a 1% agarose gel, transferred onto nylon membranes (Schleicher & Schuell, Keene, NH), and probed with a random-primer-labeled (Rapid Prime labeling kit; Roche Applied Science) PCR fragment corresponding to the *CrATP6* coding region. Incubation of the probe with the membrane was carried out as described previously (Pan and Snell, 2000).

Expression of *CrATP6*

Because no antibody to *CrATP6* is available, we appended an in-frame sequence encoding a FLAG epitope tag (DYKDDDDK) to the 3' end of the coding region of the full-length *CrATP6* cDNA and inserted the construct into the *Bam*HI and *Eco*RI sites of the mammalian expression vector pCDNA3 (Invitrogen), by using *Bam*HI and *Eco*RI linkers flanking the insert. Positive clones were confirmed by sequencing. A positive plasmid (plasmid pcDNA3/5a-a, termed herein pCrA6F for clarity and brevity) was isolated using the plasmid midi kit (QIAGEN, Valencia, CA). Transient transfections of human embryonic kidney 293T and monkey COS7 kidney cells were carried out with FuGENE 6 (Roche Applied Science), a nonliposomal transfection reagent, according to manufacturer's recommendations. Briefly, the DNA-FuGENE 6 complex was made fresh at a ratio of 3 μ l of FuGENE 6 to 1 μ g of plasmid DNA in serum-free DMEM before overlaying onto preplated cells overnight at 37°C. Cells were cultured to confluence for further analyses. For stable transfections of human cybrids JCP213 (100% wild-type mtDNA; 8993T) and JCP261 (100% mutant mtDNA; 8993G), cells were transfected as described above; upon confluence, the neomycin analog G418 was added to the high-glucose DMEM (Invitrogen) to select for positive cells. Stable transfections were maintained in medium with G418 for 4 wk before growth experiments were carried out. For growth in glucose, cells were grown in DMEM supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 4.5 mg/ml glucose. For growth in galactose, the above-described medium contained 5 mM galactose instead of glucose and the cells were left in this medium for 4 d before changing to glucose medium. To determine the sensitivity to oligomycin (Manfredi *et al.*, 1999), cells were grown in galactose medium containing 0.1 ng/ml oligomycin for 4 d; the medium was then changed to glucose for recovery.

ATP synthesis was measured as described previously (James *et al.*, 1999). Briefly, cells were incubated in 150 mM KCl, 25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM potassium phosphate, 0.1 mM $MgCl_2$, and 0.1% bovine serum albumin, with 50 μ g/ml digitonin. Mitochondria were energized using 1 mM malate and 1 mM pyruvate as substrate in the presence of 1 mM ADP and 0.15 mM of the adenylate kinase inhibitor P^i , P^s -di(adenosine)pentaphosphate, and incubated for 10 min at 37°C. A 50- μ l aliquot was resuspended in 25 mM Tris-HCl, pH 7.4, and boiled for 2 min. Parallel incubations were also carried out in the presence of 2 μ g/ml oligomycin to measure mitochondrial-specific ATP synthesis. The supernatant was assayed for ATP by using a luciferin-luciferase assay (Manfredi *et al.*, 2001) with an ATP bioluminescence assay kit CLSII (Roche Applied Science) in an Optocomp-1 luminometer (MGM Instruments, Hamden, CT).

Immunological Techniques

Colocalization of the expressed protein with mitochondria was assessed by treatment of the transfected cells, grown on coverslips, with 500 nM of the mitochondrial-specific fluorescent dye Mitotracker Red (Molecular Probes, Eugene, OR) and by immunodetection of the FLAG epitope with anti-FLAG M2 primary IgG antibody (Sigma-Aldrich, St. Louis, MO) and a goat anti-mouse IgG secondary antibody conjugated to Oregon Green (Molecular Probes). Briefly, the cells were incubated with Mitotracker, diluted in basic DMEM at 37°C, washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (wt/vol), and permeabilized with cold

acetone. Nonspecific reaction was blocked with nonimmune goat serum in PBS. Coverslips were mounted on slides with an aqueous mounting medium (Biomed, Foster City, CA). Immunofluorescence was visualized using an IX70 microscope (Olympus, Tokyo, Japan) and images were captured using an RT color SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Expression from transfected pCrA6F was detected by Western blotting. Briefly, cells were harvested, washed in PBS (1× PBS; Invitrogen), and homogenized in mitochondrial isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, pH 7.4). Crude mitochondria were isolated as described previously (Ojaimi *et al.*, 1999). Protein samples at a concentration of 10 µg of total homogenate, and isolated mitochondria were electrophoresed through a 15% SDS-PAGE gel and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). For blue native PAGE, 20 µg of crude mitochondria were solubilized by sonication and electrophoresed through a 5–18% continuous gradient gel under nonreducing conditions. Lanes from the first dimension (blue native) were cut out, treated to denature the protein subunits, and inserted horizontally into a denaturing 15% SDS-PAGE gel for analysis in the second dimension. Proteins from the first and second dimensions were electrotransferred onto a PVDF membrane. Expressed proteins were detected using a mouse monoclonal antibody against the FLAG epitope and were visualized using the ECL-Plus chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

RESULTS

Isolation and Characterization of *C. reinhardtii* ATP6

We originally designed degenerate primers corresponding to the most conserved region of ATP6 among various species to amplify subregions of the putative *CrATP6* gene from both *C. reinhardtii* mRNA and genomic DNA, but these attempts were unsuccessful. Using the translation product from the mtDNA-encoded *ATP6* gene from another algal species, *P. wickerhamii* (GenBank U02970) to screen the *Chlamydomonas* EST database, however, we identified three overlapping ESTs (BE121716, AV623443, and AV621415) corresponding to the putative full-length *CrATP6* mRNA. Using these cDNAs as templates, we eventually assembled a 1079-base pair *CrATP6* cDNA containing 31 nt of the 5'-UTR, a 1020-nt open reading frame specifying a 340-amino acid (aa) polypeptide, and at least 28 nt of the 3'-UTR of the *ATP6* mRNA (Figure 1A). Using PCR primers from regions of this cDNA to amplify *C. reinhardtii* total genomic DNA, we obtained a 2222-base pair fragment representing the *ATP6* gene (Figure 1A). During the course of this work, another group (Funes *et al.*, 2002) also reported the isolation of a full-length *CrATP6* cDNA (GenBank AF411119) and gene (GenBank AF411921). That cDNA, which is 2349 base pairs in length, is essentially identical to that reported herein, but has an additional 1.3 kb of 3'-UTR sequence. *CrATP6* is almost certainly a single-copy gene (Funes *et al.*, 2002), because Southern blot hybridization analysis revealed that it is contained on a single *EcoRI* and *SphI* fragment, and, as predicted by the gene sequence, on only two *MboI* fragments (Figure 1B).

The deduced translation product (Figure 1C) is 340 amino acids in length (predicted molecular mass of 35.5 kDa), of which the first 107 amino acids constitute the mitochondrial targeting signal (MTS) (Funes *et al.*, 2002). In the 233 aa mature polypeptide (predicted molecular mass of 24.6 kDa),

the N-terminal 60 amino acids have a very low degree of primary sequence identity with the mitochondrial DNA-encoded ATPase 6 polypeptides from highly diverse organisms. The remaining C-terminal residues are more conserved (Figure 1C), not only in the related algae *P. wickerhamii* (~42% identity) and *P. minor* (33%) but also in *Saccharomyces cerevisiae* (37%), *Homo sapiens* (32%), and even in *E. coli* (25%) and in ATP1, the analogous subunit of the ATP synthase in *C. reinhardtii* chloroplasts (22%). In spite of the low degree of sequence identity overall, the conservation of secondary structure along the entire length of these orthologous polypeptides is remarkably high (Figure 1A), presumably reflecting the evolutionary constraints on the requirement of this subunit to be able to couple proton flow to the rotation of the ring of c subunits with which it makes contact (Rastogi and Girvin, 1999).

Expression of *C. reinhardtii* ATPase 6 in Mammalian Cells

The high conservation of secondary structure encouraged us to determine whether CrATP6 could be expressed and targeted to mitochondria in mammalian cells. Because no antibodies to CrATP6 are available, we appended sequences encoding a FLAG epitope tag to the C terminus of the full-length *CrATP6* cDNA (termed *CrATP6F*) and inserted this construct into pCDNA3, a mammalian expression vector (termed herein plasmid pCrA6F). Transient expression of pCrA6F in both simian virus 40-transformed monkey COS7 and human 293T kidney cells showed that the expressed CrATP6F protein was targeted to mitochondria (Figure 2, A and B).

Western blot analysis of both total cell homogenate and purified mitochondria isolated from 293T cells confirmed that CrATP6F was targeted to mitochondria (Figure 3A). Moreover, the precursor protein (predicted size 36.5 kDa) was imported into the mitochondria and was processed to produce the presumably mature polypeptide, with a size of ~26 kDa. Because it is difficult to deduce the molecular mass of highly hydrophobic proteins, such as CrATP6F, in SDS-PAGE gels (Mariotti *et al.*, 1986), we cannot know with any degree of certainty whether the site of cleavage of the CrATP6F precursor in human mitochondria is the same as that of CrATP6 in algal mitochondria. However, the ~10-kDa difference in the sizes of the precursor and the mature polypeptides (Figure 3A) implies that the cleavage site is, in fact, very close to the expected position at aa 107 (the predicted size of mature CrATP6F is 25.6 kDa), implying that the unusually long and presumably complex MTS (Daley *et al.*, 2002) of CrATP6F is "recognized" by the human importation machinery. The amount of mature polypeptide was ~40% of the total expressed protein, implying that importation of CrATP6F into human mitochondria was relatively inefficient. We believe that, similar to what we observed upon allotopic expression of human ATPase 6 (Manfredi *et al.*, 2002), the unprocessed precursors present at relatively high levels in the isolated mitochondrial fraction were either loosely attached to the mitochondrial outer membrane or were attached but not imported efficiently.

Analysis using a combination of blue native and SDS gels (Schägger *et al.*, 1988; Schägger and Ohm, 1995) strongly implied that the imported and processed CrATP6F was incorporated into human F₀F₁-ATP synthase (Figure 3, B and

Figure 1. Characterization of *C. reinhardtii* ATP6. (A) The gene (top map) and processed mRNA (bottom map) contain eight exons (boxes), of which the first four (gray shading) encode the MTS (Funes *et al.*, 2002) and the last four (black shading) encode the mature protein; the 5'- and 3'-UTRs are unshaded. Below the maps are Kyte-Doolittle hydropathy plots (hydropathy scale at left) for ATPase 6 from the indicated organisms. Dashed lines denote exon-intron boundaries in *CrATP6*. (B) Southern blot hybridization of genomic *C. reinhardtii* DNA digested with the indicated restriction enzymes and probed with the coding region of *CrATP6* cDNA. Markers, in kilobases, are at left; the approximate sizes of the hybridizing bands, in kilobases, are at right. (C) Alignments of ATPase 6 polypeptides from the indicated species; aa numbering is at right. Exon-intron boundaries for *CrATP6* are indicated by the vertical lines; the MTS is underlined. Leu-156 in human ATPase 6, which is mutated in NARP/MILS, is boxed. Residues conserved among all four species are in bold.

C). In particular, use of antibodies to both FLAG and to subunit α of F_1 ATPase to probe a Western blot of proteins from 293T cells transfected transiently with pCrA6F and separated on a nondenaturing blue-native gel revealed an essentially identically sized signal with both antibodies, migrating at ~ 600 kDa, consistent in size with that of complex V (Figure 3B). Moreover, when the lane containing this band was run in the second dimension on a denaturing SDS gel, the anti-FLAG signal was now detected at the appropriate lateral position on the gel and with an estimated molecular mass of 26 kDa (Figure 3C). This result implies that the mature 26-kDa CrATP6F polypeptide was indeed incorporated into human complex V. Two other minor spots, also in the size range of 26–27 kDa, were also observed in the second dimension. The horizontal distance separating the main spot from these two minor spots implied that some anti-FLAG-reactive material had been present in the blue native gel in a complex of ~ 500 kDa. We do not know the origin of these two spots, because their sizes are not consis-

tent with those estimated for either the F_0 (~ 200 kDa) or F_1 (~ 400 kDa) subcomplexes of ATP synthase.

Expression of CrATP6F in Human Cybrids Harboring the T8993G Mutation

Having shown that ATPase 6 from *C. reinhardtii* could assemble into human complex V, we wanted to determine whether such a “chimeric” ATP synthase could function in human mitochondria. We had shown previously that allotopic expression of a genetically modified, nucleus-localized, version of the (normally mtDNA-encoded) human ATP6 gene was able to rescue a deficiency in ATP synthesis in cytoplasmic hybrid (cybrid) cells harboring homoplasmic levels of the T8993G mutation found in NARP and MILS patients (Manfredi *et al.*, 2002). We therefore carried out a similar analysis by using the *Chlamydomonas* construct. To do this, we transfected wild-type (i.e., 100% 8993T) and mutant (i.e., 100% 8993G) cybrids with pCrA6F and used

Figure 2. Targeting of *C. reinhardtii* ATPase 6 to mammalian mitochondria. pCrA6F was expressed transiently in human 293T (A) and monkey COS7 (B) simian virus 40-transformed kidney cells, and stably in human cybrid line JCP261 harboring 100% mutated (i.e., 8993G) mtDNAs from a MILS patient (C). Immunohistochemistry with an anti-FLAG antibody was carried out to detect the FLAG epitope tag. Note the colocalization of the anti-FLAG signal (green fluorescence) with that of Mitotracker Red (red fluorescence) in the merged panels at the right.

selection for resistance to the neomycin analog G418 to isolate stably transfected cells. As with the transiently transfected cells, we confirmed that the expressed CrATP6F was targeted to the mitochondria of stably transfected wild-type (our unpublished data) and mutant cybrids (Figure 2C).

After 1 mo in culture, we monitored the growth rate of the cells. Similar to what had been shown by us previously (Manfredi *et al.*, 1999), we found that the mutant cybrids grew as well as did wild-type cybrids in rich medium containing glucose (Figure 4, top) and were only slightly affected when grown in medium containing galactose instead of glucose (Figure 4, middle). We had also shown previously that when grown in medium containing galactose plus low levels of oligomycin, which binds specifically to ATPase 6 (Breen *et al.*, 1986; John and Nagley, 1986) and inhibits complex V function, the growth of cells containing mutant mtDNA is inhibited compared with that of both wild-type

cells (Manfredi *et al.*, 1999) and of mutant cybrids in which human ATPase 6 was expressed allotopically (Manfredi *et al.*, 2002). We found that in the presence of galactose plus oligomycin, the stably transfected mutant lines that were expressing CrATP6F grew better than did the untransfected mutant cybrids, albeit to a somewhat lesser extent than did the wild-type cybrids (Figure 4, bottom).

We measured mitochondrial (i.e., oligomycin-sensitive) ATP synthesis in triplicate platings of cells from two independent stable transfections of pCrA6F in wild-type and mutant cybrids, as well as from untransfected control cybrids grown in parallel (Figure 5). ATP synthesis in mutant cybrids before transfection was ~35% of the level in wild-type cybrids, values that are consistent with what had been observed previously in patient cells (Tatuch and Robinson, 1993; Vazquez-Memije *et al.*, 1996; Manfredi *et al.*, 1999, 2002; Garcia *et al.*, 2000; Schon *et al.*, 2001). In the wild-type cybrids

Figure 3. Western blot analyses. (A) Western blot of proteins isolated from human 293T cells transfected transiently with pCrA6F or from untransfected cells (UT), by using anti-FLAG antibodies. Sizes of markers, in kilodaltons, are at left. The predicted sizes of the precursor (P) and mature (M) CrA6F polypeptides, in kilodaltons, are at right. (B) Proteins from 293T cells either UT or transfected with pCrA6F were electrophoresed through a 5–18% continuous gradient blue-native gel. The proteins were transferred onto a PVDF membrane, and parallel lanes from the membrane were probed with antibodies to either FLAG or to subunit α of F_1 ATPase, as indicated. Both antibodies revealed a signal of the same size, migrating at ~600 kDa, consistent with the size of complex V. Markers, in kilodaltons, are at right. (C) Proteins from 293T cells were electrophoresed through 5–18% continuous gradient blue-native gels run in parallel. One gel was transferred onto PVDF membranes and was probed with anti-FLAG antibodies, again revealing a signal migrating at ~600 kDa (left). A lane from the parallel gel was excised (dashed box) and run in the second dimension under denaturing conditions through a 15% SDS-PAGE gel, with the top of the gel oriented as shown (right), and probed with anti-FLAG antibodies. Markers, in kilodaltons, are at right in both gels.

transfected with pCrA6F, ATP synthesis remained unchanged in one transfection but, for unknown reasons, increased in the second one, compared with the values in the control wild-type cybrids. It is noteworthy that we did not detect any diminution in ATP synthesis in these cells, implying that the expressed CrATP6F had little, if any, negative effects on the functioning of complex V.

In the mutant cybrids transfected with pCrA6F, mitochondrial ATP synthesis increased above control values by ~75%. Note, however, that the amount of ATP synthesis in the transfected mutant cybrids was still less than that in the wild-type cybrids (Figure 5). In other words, although the expression of CrATP6F in mutant cybrids almost doubled the ability of those cells to generate ATP, this improvement was still well below normal levels. It should be noted, however, that expression of the *C. reinhardtii* ATP6F polypeptide

Figure 4. Growth curves. Shown is an example of a set of growth curves of cybrids containing 100% wild-type (WT) (open symbols) and 100% mutant (M) (filled symbols) mtDNAs, either untransfected (squares) or transfected with pCrA6F (circles) and grown in the indicated media. Arrow denotes confluence.

was able to increase ATP synthesis in the mutant cells despite the fact that endogenously synthesized mutant human mtDNA-encoded ATPase 6 polypeptides were competing

Figure 5. ATP synthesis. Measurement of oligomycin-sensitive (i.e., mitochondrial) ATP synthesis by using malate/pyruvate as the substrate, in wild-type and mutant cybrids transfected with pCrA6F in two independent transfections (1 and 2), compared with values in the respective untransfected (UT) cells. Measurements were performed in triplicate. Asterisks denote values that were statistically significant ($p < 0.05$ in a paired Student's *t* test) compared with those from the respective untransfected cells.

with the algal polypeptides for assembly into complex V holoproteins. Importantly, this degree of improvement in oxidative energy metabolism was clearly sufficient to allow the cells to grow in the galactose-oligomycin medium (Figure 4).

DISCUSSION

The endosymbiont hypothesis for the origin of mitochondria (Lang *et al.*, 1999) implies that >99% of the ~4000 genes originally present in the protomitochondrion are not present in the organelle today. Many of these genes were presumably nonessential to the viability of the eukaryotic host and were lost during the course of evolution, whereas the remainder were transferred to the nucleus and are the ancestors of many of the "housekeeping" genes present in modern eukaryotes (Lang *et al.*, 1999; Gray *et al.*, 2001). Most of the proteins in this latter group have evolved to be retargeted back to mitochondria, where they comprise the majority of the estimated 850 gene products present in the modern mammalian organelle. The evolution of organellar genetic codes that differ from the universal code cemented this division between nuclear DNA- and mitochondrial DNA-encoded polypeptides. However, a tiny subset of pro-

teins, all components of the mitochondrial respiratory chain/oxidative phosphorylation system, escaped this massive gene transfer process and remained in the mitochondrial genome. With but few exceptions, a "canonical" set of mtDNA-encoded proteins, typically six subunits of NADH dehydrogenase ubiquinone oxidoreductase, the cytochrome *b* subunit of ubiquinone-cytochrome *c* oxidoreductase, three subunits of cytochrome *c* oxidase, and two subunits of ATP synthase, is remarkably conserved among all species examined, ranging from the protist *Reclinomonas americana* to mammals. Although the reason for this conservation has been the subject of speculation, the most widely held view is that these proteins are so hydrophobic that they are unable to be imported from the cytoplasm, and therefore this set was constrained by evolutionary pressure to remain in the mitochondrial genome (Claros *et al.*, 1995, 1996; Perez-Martinez *et al.*, 2000, 2001).

However, the rules determining which hydrophobic protein genes are retained in the mtDNA are not hard and fast. For example, the mtDNA of the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* contain the ATP9 gene, which encodes subunit *c* of ATP synthase, whereas subunit *c* is nucleus encoded in mammals. Even more striking has been the finding that some organisms among the algae, ciliates, apicomplexans, and flowering plants lack mtDNA-encoded COX II, COX III, and/or ATP6, all highly hydrophobic proteins. Because these three subunits are necessary for the functioning of COX and ATP synthase, respectively, it is almost certain that these genes have been transferred to the nuclear DNA in these organisms. In fact, the nucleus-encoded genes specifying COX II and COX III (Perez-Martinez *et al.*, 2000, 2001; Watanabe and Ohama, 2001), and, as reported herein and elsewhere (Funes *et al.*, 2002), ATPase 6, have been identified in algal species, including *C. reinhardtii*.

It is noteworthy that the mitochondrial genetic code of *C. reinhardtii* is identical to the universal nuclear code (Boer and Gray, 1988). Presumably, the presence of a nuclear DNA-compatible genetic code allowed for a more facile transfer of mtDNA-encoded genes to the nucleus, but this does not mean that all of these genes can be transferred. As discussed in some detail by Gonzalez-Halphen (Perez-Martinez *et al.*, 2000, 2001; Funes *et al.*, 2002), COX II, COX III, and ATPase 6 all reside at the lower end of the scale of "meso-hydrophobicity" of mtDNA-encoded polypeptides, whereas COX I and cytochrome *b*, which have never been found to be nucleus-encoded in any examined organism, reside at the high end. Thus, the relatively lower degree of hydrophobicity of ATPase 6, coupled with a compatible genetic code, fortuitously allowed for the transfer, and ultimate retargeting back to mitochondria, of *CrATP6*.

Although mammals and algae are separated by more than a billion years of evolution, we were able to demonstrate that the nucleus-encoded *C. reinhardtii* ATPase 6 polypeptide can function in human mitochondria. In particular, the algal *CrATP6* gene, when expressed in human cybrids harboring a homoplasmic pathogenic mutation in the analogous mtDNA-encoded *MTATP6* gene, enabled these cells to be viable under growth conditions (i.e., galactose plus oligomycin) that killed the untransfected mutant cells, and this rescue of viability was almost certainly due to an increase in the ability of the cybrids to produce enough ATP to be maintained in tissue culture. We note, however, that the

complementation of function by CrATP6F was only moderate, because the transfected cells still grew at a rate almost 10-fold lower than that of wild-type cybrids. Much of this reduced rate was likely to have been due to the presence within the homoplasmic mutant cybrid mitochondria of complex V holoproteins containing the "endogenous" mutated human ATPase 6 subunit. Given the competition between the human and *C. reinhardtii* ATPase 6 subunits for assembly into complex V holoproteins, we deem it rather remarkable that the algal ATPase 6 could replace its human homolog as part of a chimeric F_0F_1 -ATP synthase, even if only partially. On the other hand, the recessive nature of the NARP/MILS T8993G mutation implies that even a partial rescue of function could be of clinical utility.

We recently showed that allotopic expression of the human *MTATP6* gene could rescue deficiencies in cell growth and in ATP synthesis in cybrids harboring homoplasmic levels of the 8993G mutation (Manfredi *et al.*, 2002). The work reported herein extends the potential application of successful allotopic expression of "cognate" gene products (e.g., an allotopically expressed recoded human mitochondrial gene targeted to human mitochondria) to the expression of homologous gene products across species boundaries, similar to the rescue of deficiencies in human (Bai *et al.*, 2001) and hamster (Seo *et al.*, 1998) rotenone-sensitive complex I by the expression of the nucleus-encoded rotenone-insensitive NADH quinone oxidoreductase gene (*NDI1*) from *S. cerevisiae*. The ability to perform this type of "xenotopic" or "trans-kingdom" allotopic expression may be particularly useful in efforts to import highly hydrophobic proteins into human mitochondria (and, equally important, into mitochondria from model organisms, such as mice). For example, the MTS of CrATP6 might be able to direct the importation into mitochondria of a (normally mtDNA-encoded) human cytochrome *b* subunit engineered specifically for allotopic expression in cells from patients harboring mutations in this polypeptide (Rana *et al.*, 2000). Numerous obstacles need to be overcome if allotopic expression is to be adapted as an approach to gene therapy for mitochondrial diseases (Funes *et al.*, 2002; Manfredi *et al.*, 2002), but the results reported herein extend the range of possibilities for those efforts to succeed.

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