Genetic Correction of Mitochondrial Diseases

Using the Natural Migration of Mitochondrial Genes to the Nucleus in Chlorophyte Algae as a Model System

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ABSTRACT: Mitochondrial diseases display great diversity in clinical symptoms and biochemical characteristics. Although mtDNA mutations have been identified in many patients, there are currently no effective treatments. A number of human diseases result from mutations in mtDNA-encoded proteins, a group of proteins that are hydrophobic and have multiple membrane-spanning regions. One method that has great potential for overcoming the pathogenic consequences of these mutations is to place a wild-type copy of the affected gene in the nucleus, and target the expressed protein to the mitochondrion to function in place of the defective protein. Several respiratory chain subunit genes, which are typically mtDNA encoded, are nucleus encoded in the chlorophyte algae *Chlamydomonas reinhardtii* and *Polytomella* sp. Analysis of these genes has revealed adaptations that facilitated their expression from the nucleus. The nucleus-encoded proteins exhibited diminished physical constraints for import as compared to their mtDNA-encoded homologues. The hydrophobicity of the nucleus-encoded proteins is diminished in those regions that are not involved in subunit–subunit interactions or that contain amino acids critical for enzymatic reactions of the proteins. In addition, these proteins have unusually large mitochondrial targeting sequences. Information derived from these studies should be applicable toward the development of genetic therapies for human diseases resulting from mutations in mtDNA-encoded polypeptides.

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INTRODUCTION

Since pathogenic mutations in the human mitochondrial genome (mtDNA) were first described,[1,2] more than 100 point mutations and numerous mtDNA rearrangements associated with mitochondrial diseases have been reported.[3] Despite the molecular genetic characterization of these mutations, no effective therapies exist for mitochondrial diseases. Allotopic expression is one potential approach to gene therapy for human diseases resulting from mutations in mtDNA-encoded protein genes. This entails the functional expression of a mitochondrial gene that has been relocated in the nucleus.[4,5] This strategy would allow the expression of a mitochondrial protein from the nucleus of cells harboring an mtDNA-encoded mutant form of the protein. The allotopic expression of a mitochondrial gene was pioneered with the *atp8* gene of yeast.[4] Some eukaryotes contain nucleus-encoded genes that are normally found in the mtDNA in the vast majority of organisms. The characteristics of these relocated genes are discussed here. These characteristics suggest genetic modifications that may facilitate allotopic expression of a mitochondrial gene.

MITOCHONDRIAL GENOMES AND MITOCHONDRIAL GENE MIGRATION

The endosymbiotic event that gave rise to mitochondria was followed by a massive migration of genes to the nucleus,[6] a process that continues to this day, as exemplified by organisms that contain the same protein encoded in both the mitochondrial and the nuclear genomes, such as, subunit ATP9 of *Neurospora crassa,*[7] and the COX II subunit of legumes.[8] Most nucleus-encoded proteins destined for the mitochondrial matrix or inner membrane, the innermost mitochondrial compartments, require a mitochondrial targeting sequence (MTS). This is generally a small, N-terminal cleavable presequence of 20 to 40 residues, capable of forming an amphiphilic alpha-helix that is recognized by the mitochondrial import apparatus. After import, the MTS is usually removed by a mitochondrial processing peptidase.

The migration of mitochondrial genes to the nucleus gave rise to the present highly reduced mtDNAs that encode a limited set of protein and RNA components.[6] Mitochondria that possess a complete set of oxidative phosphorylation (OX-PHOS) complexes—I, II, III, IV, and ATP synthase—usually contain the genes *nad1, nad2, nad3, nad4, nad4L, nad5, nad6, cox1, cox2, cox3, cob, atp6*, and *atp8* that encode highly hydrophobic membrane proteins with two to seventeen transmembrane stretches.[6] In the mtDNA of green algae *Chlamydomonas reinhardtii*, *Chlamydomonas eugametos*, and *Polytomella parva,* members of the "Reinhardtii" clade,[9] there is a conspicuous absence of the *nad3*, *nad4L, cox2*, *cox3*, *atp6*, and *atp8* genes encoding essential membrane proteins that participate in OX-PHOS.[10–12]

Several hypotheses have been proposed to explain why mitochondrial genomes preserve a limited set of genes encoding OX-PHOS components (reviewed in Ref. 13). These include (1) the presence of some organellar proteins in the cytoplasm may have

detrimental effects on the cell; (2) highly hydrophobic cytoplasm-synthesized organellar proteins could be misrouted to other cell compartments, such as the endoplasmic reticulum; (3) the retention of genes in the mitochondrial genome may allow them to be rapidly regulated by the organelle redox state; and (4) highly hydrophobic proteins may not be readily imported into mitochondria, and must be synthesized *in situ* to be properly inserted into the inner mitochondrial membrane. Additionally, in some organisms the evolution of a mitochondrial genetic code different from the nuclear code may inhibit the functional expression of mitochondrial genes relocated in the nucleus. Also, mitochondrial genes in some organisms have acquired complex processing requirements, such as mRNA editing, that would prevent their expression from the nucleus. As outlined below, we favor the hypothesis that hydrophobicity[14] is the ultimate limiting step for the functional relocation to the nucleus of mitochondrial genes encoding polytopic membrane proteins.

MIGRATION OF MITOCHONDRIAL GENES TO THE NUCLEUS IN *CHLOROPHYTE ALGAE*

In members of the genera *Chlamydomonas* and *Polytomella*, some mitochondrial genes that are retained in the mtDNA of the vast majority of eukaryotes have migrated to the nucleus. Among these are *cox2*, encoding subunit II of cytochrome *c* oxidase (COX II);[15] *cox3*, encoding subunit III of cytochrome *c* oxidase (COX III);[16] *atp6* encoding subunit ATP6 of F_1F_0-ATP synthase;[17] and *nad4L*, encoding subunit NAD4L of the NADH-ubiquinone oxidoreductase [GenBank accession number AY216718]. These four subunits are essential components of their corresponding enzyme complexes.

Mitochondrial genes adapted for expression in the nucleus exhibit several distinct features that facilitate nuclear expression.[18] A number of modifications that occur in the chlorophyte algal genes *cox2a*, *cox2b, cox3*, *atp6,* and *nad4L* contribute to their ability to be expressed from the nucleus and are described below:

(1) *Acquisition of promoters.* Relocated genes must acquire promoters and other regulatory elements to be expressed in the nucleus. The chlorophyte algal promoters that regulate the expression of the relocated mitochondrial genes have yet to be characterized.

(2) *Acquisition of polyadenylation signals.* The polyadenylation signal of *C. reinhardtii* nuclear genes is present in the relocalized mitochondrial genes.

(3) *Acquisition of introns.* Several introns with orthodox splicing sites are present in some of the relocalized chlorophyte mitochondrial genes. The *cox2a* and *cox3* genes of both algae, and *cox2b* and *atp6* of *C. reinhardtii* contain introns, while *nad4L* of *C. reinhardtii* and *cox2b* of *Polytomella* sp. lack introns.

(4) *Change in codon usage.* The chlorophyte algae of the "Reinhardtii" clade exhibit a biased codon usage in their nuclear genes. The relocated mitochondrial genes of *C. reinhardtii* and *Polytomella* sp. exhibit codon usage patterns similar to other nuclear genes and that are distinct from mtDNA-encoded genes.

(5) *Acquisition of a region encoding a MTS. C. reinhardtii* MTSs vary in size. COX IIA, COX III, ATP6, and NAD4L exhibit unusually large MTSs of 143, 119, 107, and 133 residues, respectively. Shorter MTSs of 30 to 70 residues are associ-

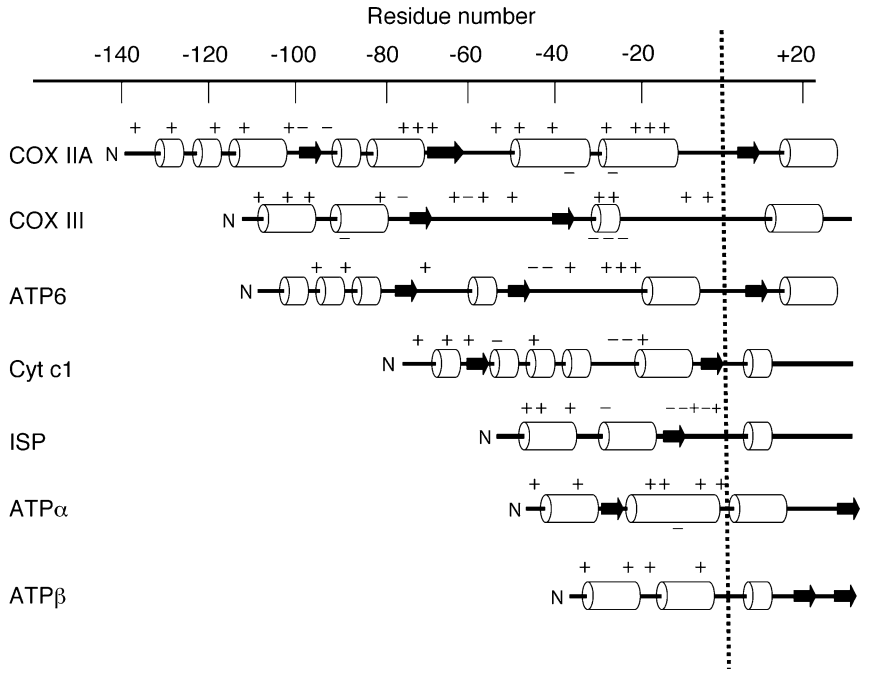

FIGURE 1. Secondary structure analysis of different *C. reinhardtii* mitochondrial targeting sequences. Predicted alpha helices are depicted as *cylinders*, beta sheets as *bold arrows*, and random coils as *straight lines*. The *vertical dashed line* separates the MTSs from the mature portions of the proteins. Residues are indicated as *negative numbers* for MTSs and as *positive numbers* for the N-terminal regions of the mature proteins. The *C. reinhardtii* nucleus-encoded mitochondrial proteins analyzed were COX IIA of cytochrome *c* oxidase [GenBank AF305080]; COX III of cytochrome *c* oxidase [AF233515]; ATP6 of the F_0 sector of F_1F_0-ATP synthase [AF411119]; cytochrome c_1 (Cyt c_1)[AF245393]; the Rieske-type iron-sulfur protein (ISP) [X91795]; ATPalpha, the alpha subunit of F_1F_0-ATP synthase [X94149]; and ATPbeta, the beta subunit of F_1F_0-ATP synthase [X61624].

ated with proteins targeted to the mitochondrial matrix. As with other MTSs, it is predicted that these long MTSs form amphiphilic α-helices (FIG. 1) that are important for binding with the mitochondrial outer membrane receptors (reviewed in Ref. 19). Long presequences may improve the efficiency of import of nucleus-encoded, highly hydrophobic proteins into mitochondria.[20–22]

(6) *Diminished hydrophobicity.* The membrane domains of nucleus-encoded mitochondrial proteins are less hydrophobic and are spaced further apart than their mtDNA-encoded counterparts. Both mesohydrophobicity (*meso*H), a measure of the distance between hydrophobic domains, and the maximum hydrophobicity of the putative transmembrane segments (<H>) are predictors of the likelihood that a protein could be imported into the mitochondrion.[21,23,24] The nucleus-encoded COX IIA, COX IIB, COX III, ATP6, and NAD4L of chlamydomonad algae exhibit reduced *meso*H and <H> compared to their mtDNA-encoded counterparts, which

presumably facilitates their import into mitochondria.[24] In particular, hydropathy analysis of the algal COX III and ATP6 sequences showed that the decrease of mean hydrophobicity occurs mostly in those transmembrane regions of the protein that are not involved in subunit–subunit interactions or in the function of the subunit.[16,17] A similar phenomenon is observed for the relocated mitochondrial *sdh3* gene of angiosperms.[25]

In leguminous species that express both mitochondrial and nuclear *cox2* genes, the nucleus-encoded COX II proteins have a lower hydrophobicity than the mtDNA-encoded COX II. A chimeric protein, consisting of mitochondrial COX II with the MTS of the nuclear COX II subunit, was not imported into mitochondria.[26] However, this protein could be imported after the introduction of two amino acid substitutions in the first transmembrane alpha-helix, which introduced fewer hydrophobic residues in the nucleus-encoded COX II.[26] Therefore, structural changes that diminish hydrophobicity can allow import into mitochondria of a nucleus-encoded protein that is normally mtDNA encoded. These results support the hypothesis[14,16,21] that hydrophobicity limits the functional relocalization of mitochondrial genes to the nucleus.

(7) *Fragmentation of genes*. The splitting of mitochondrial genes may be another mechanism that can facilitate their migration to the nucleus. In both *Polytomella* sp. and *C. reinhardtii*, the *cox2* gene encoding COX II of cytochrome *c* oxi-

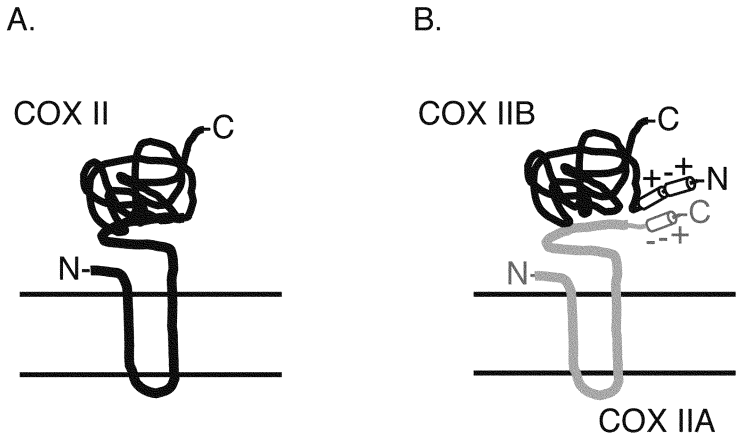

FIGURE 2. Cytochrome *c* oxidase subunit II from *Chlamydomonas* and *Polytomella* is a heterodimer. Most COX II subunits are single polypeptides encoded by single mitochondrial genes (**A**). In Chlamydomonad algae (**B**), COX IIA (*gray*) is encoded by the nuclear gene *cox2a* and corresponds to the N-terminal, hydrophobic half of a canonical COX II protein. COX IIB (*black*) is encoded by the nuclear *cox2b* gene and corresponds to the soluble C-terminal half of a conventional COX II protein.[15] The chlorophyte COX IIA and COX IIB subunits contain unique C- and N-terminal extensions, respectively, not present in orthodox COX II subunits, that may interact through their highly charged α-helices (represented as *cylinders*), to assemble and stabilize the two COX II proteins in the mature cytochrome *c* oxidase complex. N represents the N-terminus of the protein; C represents the C-terminus.

dase has not only relocated to the nucleus but has been split into two separate nuclear genes, *cox2a* and *cox2b.*[15] *cox2a* encodes COX IIA, corresponding to the N-terminal half of a typical single-polypeptide COX II, including the two transmembrane regions. *cox2b* encodes COX IIB, equivalent to the C-terminal domain of an orthodox COX II, which is located in the intermembrane space. COX IIA and COX IIB assemble noncovalently to give a heterodimeric COX II in the mature cytochrome *c* oxidase complex (FIG. 2). *cox2a* and *cox2b* genes are also present in the chlorophyte alga *Scenedesmus obliquus*, but in this alga *cox2b* has migrated to the nucleus, while *cox2a* was retained in the mtDNA. This indicates that the division of *cox2* into two genes occurred in the mtDNA of an ancestral chlorophyte.[27] The fragmentation of a highly hydrophobic protein containing two or more putative transmembrane stretches into simpler protein modules may facilitate their import into mitochondria.[15,21] Proper assembly of the protein modules may be mediated by interactions between charged N- and C-terminal extensions (FIG. 2).

(8) In conclusion, structural modifications have occurred during the relocation of mitochondrial genes to the nucleus. The diminished mesohydrophobicity seems to be of particular importance for hydrophobic mitochondrial OX-PHOS proteins whose genes are localized in the nucleus.

ALLOTOPIC EXPRESSION OF MITOCHONDRIAL GENES AND ITS APPLICATIONS TO HUMAN MITOCHONDRIAL GENE THERAPY

Human mitochondrial and *C. reinhardtii* ATP6 subunits have been allotopically expressed in human cells with mutations in the mtDNA-encoded *atp6* gene.[28,29] Remarkably, expression of *C. reinhardtii* ATP6 improved ATP synthesis in human cells, despite the evolutionary distance between green algae and vertebrates. A similar approach was used in cells harboring a mutation in the *nad4* gene using a nucleus-encoded human NAD4 subunit of complex I.[30] However, these investigations revealed that the allotopic expression of highly hydrophobic human mitochondrial OX-PHOS subunits is inefficient and still must be optimized. Some features of the *cox2*, *cox3*, *atp6,* and *nad4L* genes that are naturally nucleus-localized in some organisms could be used to enhance the efficiency of allotopic expression of mitochondrial genes in humans with mitochondrial diseases.[31,32] The hydrophobicity of the human proteins could be diminished in the same regions where hydrophobicity has been reduced by evolution in chlamydomonad algae. Site-directed mutagenesis that diminishes hydrophobicity of some residues in certain transmembrane stretches, and the addition of appropriate MTSs, might prove sufficient to improve the efficiency of allotopic expression. For hydrophobic proteins with multiple membrane helices, nuclear expression of mitochondrial genes could be accomplished as two or more nuclear genes with each gene encoding a subset of the membrane-spanning domains of the protein. N-Terminal and C-terminal extensions could be added to facilitate functional assembly of split proteins in the mitochondrial inner membrane. Alternatively, insertion of inteins, self-splicing intervening protein sequences, in the allotopically expressed proteins may facilitate their association in the mitochondrial inner membrane.[33]

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