Limitations of Allotopic Expression of Mitochondrial Genes in Mammalian Cells

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ABSTRACT

The possibility of expressing mitochondrial DNA-coded genes in the nuclear-cytoplasmic compartment provides an attractive system for genetic treatment of mitochondrial disorders associated with mitochondrial DNA mutations. In theory, by recoding mitochondrial genes to adapt them to the universal genetic code and by adding a DNA sequence coding for a mitochondrial-targeting sequence, one could achieve correct localization of the gene product. Such transfer has occurred in nature, and certain species of algae and plants express a number of polypeptides that are commonly coded by mtDNA in the nuclear-cytoplasmic compartment. In the present study, allotopic expression of three different mtDNA-coded polypeptides (ATPase8, apocytochrome *b*, and ND4) into COS-7 and HeLa cells was analyzed. Among these, only ATPase8 was correctly expressed and localized to mitochondria. The full-length, as well as truncated forms, of apocytochrome *b* and ND4 decorated the periphery of mitochondria, but also aggregated in fiber-like structures containing tubulin and in some cases also vimentin. The addition of a hydrophilic tail (EGFP) to the C terminus of these polypeptides did not change their localization. Overexpression of molecular chaperones also did not have a significant effect in preventing aggregations. Allotopic expression of apocytochrome *b* and ND4 induced a loss of mitochondrial membrane potential in transfected cells, which can lead to cell death. Our observations suggest that only a subset of mitochondrial genes can be replaced allotopically. Analyses of the hydrophobic patterns of different polypeptides suggest that hydrophobicity of the N-terminal segment is the main determinant for the importability of peptides into mammalian mitochondria.

SINCE the primordial endosymbiont invaded an evolving eukaryotic cell more than a billion years ago, the mitochondrial genome has been shedding its genes, some of which were relocated to the nuclear genome. During evolution, some of these fragments mutated codons and acquired mitochondrial targeting sequences so that they could actually be correctly translated in cytoplasmic ribosomes and targeted to mitochondria. In most animal species, genes controlling mitochondrial DNA (mtDNA) maintenance and expression are all encoded by the nuclear genome. Also encoded by nuclear DNA are a large number of subunits of the oxidative phosphorylation (OXPHOS) system, which modulate the function of the catalytic subunits encoded by the mtDNA. The nuclear genome of a modern *Homo sapiens* has >600 recognizable mitochondrial DNA-derived fragments that have migrated from the mitochondria, mostly as DNA fragments in recent evolution (Woischnik and Moraes 2002). It could be inferred that the transfer of genes from the mitochondria to the nucleus is a constant process during evolution. However, by the time the metazoan mitochondrial genome was stabilized ~800 million years ago, the gene content of the organellar genome remained relatively unchanged (Saccone *et al.* 2002). The very compact size of modern animal mtDNA suggests that it was advantageous for the mitochondria to transfer its genes to the nuclear genome, retaining only genes coding for polypeptides that were extremely hydrophobic and that would have difficulties being expressed in the cytosol and imported into the mitochondria. However, in certain species, such as the alga *Chlamydomonas reinharditi* and some legumes, some of these genes coding for hydrophobic peptides migrated to the nucleus, undergoing some subtle mutations to reduce their hydrophobicity so that their products could be correctly targeted to mitochondria (Perez-Martinez *et al.* 2000; Daley *et al.* 2002).

With the discovery of mtDNA mutations associated with human diseases, the replacement of defective genes became an important goal for mitochondrial geneticists. Unfortunately, it is still not possible to introduce foreign genes into the mitochondria of animal cells, even though a successful procedure has been developed for *Saccharomyces cerevisiae* (Bonnefoy and Fox 2002). To circumvent this problem, the possibility of allotopic expression of mitochondrial genes in the nucleus became an attractive idea.

Allotopic expression of mitochondrial genes has achieved some success in *S. cerevisiae* (Farrell *et al.* 1988), but some limitations were observed (Farrell *et al.* 1988; Law *et al.* 1990; Claros *et al.* 1995). More

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recently, successful allotopic expression of the mitochondrial ATP6 (Manfredi *et al.* 2002) and ND4 (Guy *et al.* 2002) in human cells was reported. In the present study we explored the generality of such an approach.

MATERIALS AND METHODS

Plasmid constructions: The full-length apocytochrome *b* gene was reengineered by amplifying nine independent fragments by proofreading PCR (using Vent polymerase, New England Biolabs, Beverly, MA). All the oligonucleotide primers were designed to alter AUA codons to AUG and UGA to UGG (a total of 29 codons had to be modified; Figure 1A). Adjacent amplicons had 16–18 overlapping nucleotides, so that they could be combined in pairs and reamplified using external primers. A sequence coding for a hemagglutinin (HA) tag was included 3′ of the stop codon. The final assembled construct was cloned in a *Hin*dIII site downstream of a cytochrome *c* oxidase subunit VIII mitochondrial targeting sequence ($C8^{TS}$) in a pcDNA3 plasmid (De Giorgi *et al.* 1996). The final construct was sequenced for accuracy. A clone with all the necessary modifications but with a PCR-introduced mutation was identified. Site-directed mutagenesis (Quickchange; Stratagene, La Jolla, CA) was used to correct this nucleotide position. Truncated forms of apocytochrome *b* were produced by cloning intermediate assembled amplicons downstream of $C8^{TS}$ as described above. ATPase 8 was produced similarly using four overlapping amplicons to adapt the genetic code to the universal format and to introduce a *Hin*dIII site 5′ of the initial AUG (a total of 7 codons had to be modified in the A8 gene; Figure 1B). Removal of a stop codon by Quickchange and fusion to pEGFP-N1 (CLONTECH, Palo Alto, CA) produced the cytochrome *b* (cyt *b*) fused to green fluorescent protein (GFP).

ND4 constructs were produced by oligonucleotide tiling by Integrated DNA Technologies (Coralville, IA). Appropriate restriction endonuclease sites were introduced in the synthetic gene for cloning and deletions. Codon usage was adapted to human ribosome preference tables as well as modified at 36 positions due to mitochondrial *vs.* universal code differences. The mitochondrial targeting sequence of the nicotinamide nucleotide transhydrogenase (NNT^{TS}) was included in the synthetic gene. A *Hin*dIII site was added downstream of the targeting sequence for replacement with the $C8^{TS}$. NNT^{TS}ND4 and $C8^{TS}$ND4 were cloned in the multiple cloning site/*Eco*RI of both pcDNA3.1 and pEGFP-N2 (CLONTECH). Truncated forms of ND4 were produced by digestion with *Age*I and subsequent religation, resulting in an NNT^{TS}ND4 lacking 184 amino acids. Digestion with *Nhe*I and subsequent religation produced an NNT^{TS}ND4 lacking 284 amino acids. *Age*I and *Nhe*I deletions were performed in pcDNA3 constructs and, in the case of *Age*I, also transferred to a pEGFP-N1 construct.

The P1 targeting sequence was amplified from a $P1^{TS}$A6 construct (a gift of Giovanni Manfredi, Cornell University) with polymerase Platinum Pfx (Invitrogen Life Technologies, Carlsbad, CA). The amplicon was flanked by two *Hin*dIII sites that we used to ligate it to other vectors. The $P1^{TS}$ was added to the red fluorescent protein (RFP) expressing vector PdsRed-N1 at its *Hin*dIII. The $P1^{TS}$ was added to the ND4 construct in a similar manner. $P1^{TS}$ was also added to the *Hin*dIII site downstream of a $C8^{TS}$ in a $C8^{TS}$ND4 construct to generate a $C8P1^{TS}$ND4.

As controls for the *in vivo* studies, we used a human lysyl-$tRNA^{Lysine}$ synthetase fused to the $C8^{TS}$, enhanced green fluorescent protein (EGFP) fused to $C8^{TS}$ and NNT^{TS} and RFP fused to $P1^{TS}$. A construct containing the subunit β of F_1 ATPase was used as a control in the *in vitro* import assays. The sequence of all constructs was confirmed by DNA sequencing.

***In vitro* mitochondrial import:** *In vitro* mitochondrial import assays were performed by radiolabeling *in vitro* translated polypeptides with ^{35}S-*trans* label (ICN Biomedicals, Irvine, CA) using a TNT reticulocyte or wheat germ transcription-translation kit (Promega, Madison, WI). The labeled product was incubated with freshly isolated rat liver as described (Cavadini *et al.* 2002). The final reaction was analyzed by PAGE and fluorography (Cavadini *et al.* 2002). HSP70 and GroEl/ES were purchased from Sigma-Aldrich (St. Louis).

Transient cell transfection and protein visualization: COS-7 cells were transfected with FuGENE 6 transfection reagent as recommended by the manufacturer (Roche Biochemicals, Indianapolis). Coverslips were placed on the bottom of six-well dishes and cells seeded at ~40–60% confluence. Six microliters of FuGENE 6 and 1–2 μg of the different plasmids were used. Between 24 and 48 hr later cells were fixed with 2% paraformaldehyde and processed for indirect immunofluorescence as described below.

For colocalization studies, MitoTracker (CMX-Ros; Molecular Probes, Eugene, OR) was applied to the cells (250 nm) 30 min before fixation. Mitotracker was removed and cells were washed with PBS before fixation. Cells were fixed on the coverslips with 2% paraformaldehyde in PBS, permeabilized with methanol, and incubated overnight with primary antibody anti-HA high-affinity rat monoclonal antibody (clone3F10) from Roche Biochemicals. The antibody at a concentration of 100 ng/μl was diluted 1:200 with 5% BSA solution in PBS. The antibody was placed on top of the coverslips and incubated overnight at 4°. The secondary antibody was labeled goat anti-rat IgG Alexa Fluor 488 (Molecular Probes; A-11006). This antibody was resuspended in 5% BSA PBS 1:200 dilution. The secondary antibody was placed on top of the coverslips for 2 hr after washing them with PBS for 10 min. Finally, coverslips were washed twice for 10 min with PBS and mounted using Molecular Probes prolong antifade kit (P7481). Fluorescence was inspected with a Carl-Zeiss confocal microscope.

Additional antibodies were monoclonal anti-β-tubulin Cy3 conjugate clone TUB2.1 (Sigma C4585), monoclonal antivimentin Cy3 conjugate clone V9 (Sigma C9080), and anti-HSP70 SPA-810 (Stressgen, Victoria, British Columbia, Canada).

Stable transfections: A full-length apocytochrome *b* gene containing the $C8^{TS}$ and no HA tag was cloned in a pIRES puromycin vector (BD Biosciences CLONTECH). Osteosarcoma cells containing a mitochondrial genome with a 4-bp deletion in the N terminus of the apocytochrome *b* gene (Rana *et al.* 2000) were transfected with lipofectamine (Life Technologies). After 48 hr, cells were selected for puromycin resistance. Clones isolated by the plastic ring method were expanded and analyzed for their oxygen consumption and complex II + III activity as described (Barrientos *et al.* 1998).

RESULTS

Construction of reengineered mitochondrial genes for allotopic expression: The concept of allotopic expression of mitochondrial genes has important implications for the development of therapeutic approaches to patients with mitochondrial DNA mutations. A major roadblock to this approach is the highly hydrophobic nature of mitochondrial-encoded polypeptides. Figure 1 illustrates the Kyte and Doolittle hydrophobicity profile of selected mitochondrial proteins when compared with other artificially targeted polypeptides used in this

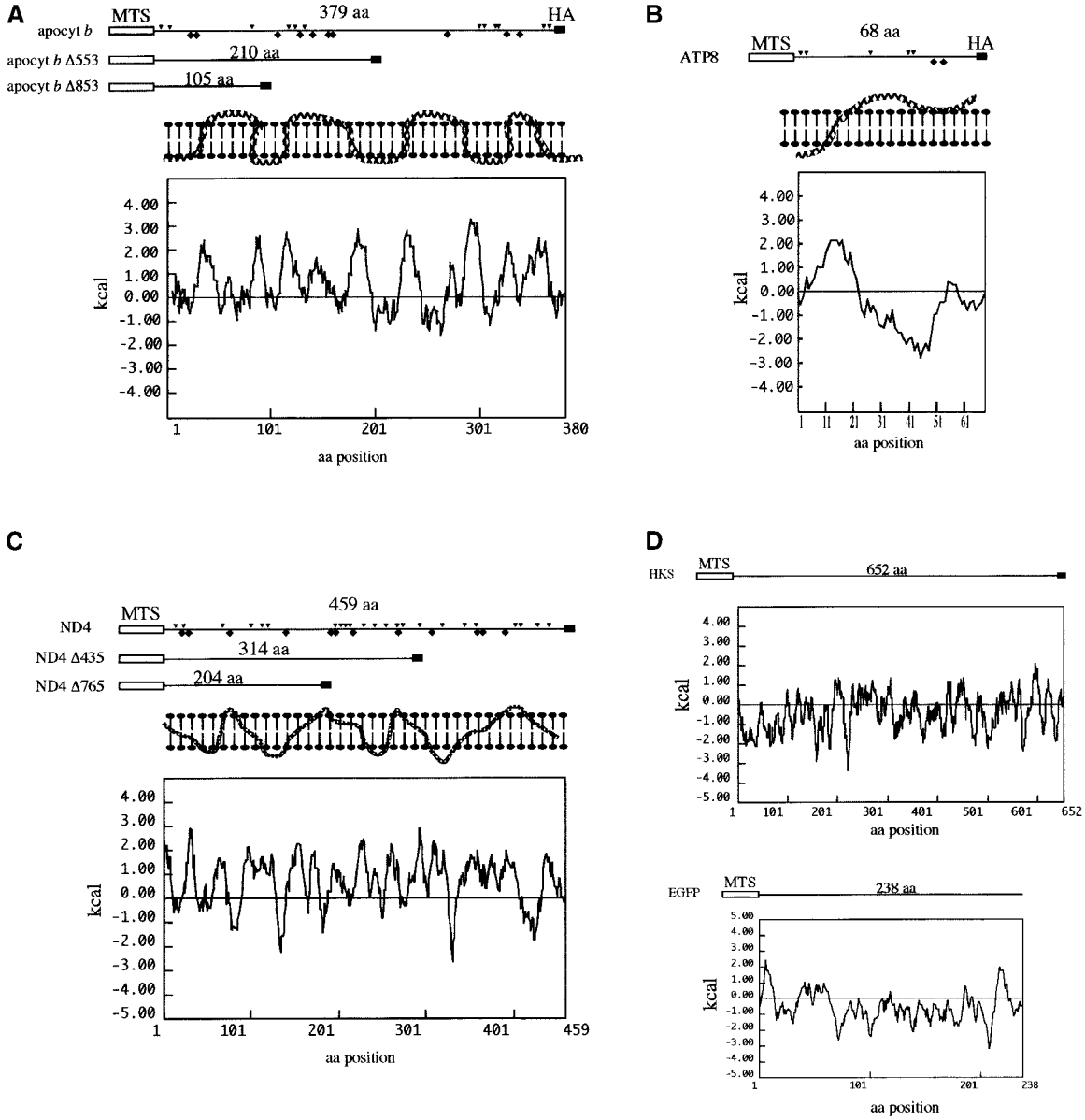

FIGURE 1.—Hydrophobicity profiles of polypeptides used in allotopic expression studies. The Kyte and Doolittle plots illustrate the highly hydrophobic character of the mitochondrial-coded apocytochrome *b* (A), ND4 (C), and to a lesser extent ATPase 8 (B). For comparison purposes the profiles of the two controls used in the *in vivo* import experiments, mitochondrial-targeted Lysyl-tRNA^{Lys} aminoacyl synthetase (HKS) and EGFP, are also depicted (D). A high positive score indicates a high hydrophobicity index. The codons modified to conform to the universal genetic code are shown in the linear representation of the full-length gene constructs. ▼, the approximate position of ATA codons that were altered to ATG. ◆, the approximate position of TGA codons that were altered to TGG.

study as controls [mitochondrial-targeted lysyl-tRNA^{Lys} synthetase (HKS) and mitochondrial-targeted EGFP]. To test the limits of the nuclear/cytoplasmic allotopic expression approach we selected three mitochondrial genes, namely ATPase 8 (a subunit of complex V), cytochrome *b* (a subunit of complex III), and ND4 (a subunit of complex I).

To be translated correctly by cytoplasmic ribosomes, mitochondrial genes must have their ATA codons mutated to ATG (ATA codes for isoleucine in cytosol and methionine in mitochondria). In addition, TGA codons must be mutated to TGG (TGA is a stop codon in the cytosol and codes for tryptophane in mitochondria). These alterations were performed by overlapping PCR in the case of ATP8 (7 alterations) and cyt *b* (29 alterations) and by producing a synthetic gene in the case of ND4 (36 alterations; see MATERIALS AND METHODS for details). Truncations were produced by digestion of the final gene product (in the case of ND4) or by cloning intermediates in the gene construction process (in the case of cyt *b*). Constructs either were fused to the EGFP coding sequence or had an HA tag for immunocy-

tochemical detection. Both fusions were at the C terminus of the recoded gene. Five different mitochondrial targeting sequences were used: (1) a targeting sequencing of the subunit VIII of cytochrome *c* oxidase ($C8^{TS}$); (2) a double-targeting sequence containing C8 and the subunit IX of ATPase of *Neurospora crassa* in tandem ($C8S9^{TS}$); (3) a targeting sequence from the multiple membrane-spanning domains NNT^{TS}; (4) a targeting sequence from the P1 isoform of the ATPase synthase subunit c ($P1^{TS}$); and a double-targeting sequence containing C8 and P1 ($C8P1^{TS}$).

The rationale behind the targeting sequences selection was the following. $C8^{TS}$ has been used extensively to import foreign polypeptides into mitochondria (De Giorgi *et al.* 1996). Reports using the yeast system indicated that tandem targeting sequences improve the mitochondrial import process. Because the $S9^{TS}$ is longer than $C8^{TS}$ and also has been extensively used for import of foreign polypeptides into mitochondria, we fused $S9^{TS}$ to $C8^{TS}$. NNT^{TS} is able to import NNT to the inner mitochondrial membrane. NNT contains 10 membrane-spanning domains (Holmberg *et al.* 1994), and its targeting sequence should be adapted for the import of large and hydrophobic passengers. $P1^{TS}$ has been used recently to import recoded ATPase6 (Manfredi *et al.* 2002) and ND4 (Guy *et al.* 2002) into mitochondria. Therefore, it was important to test this targeting sequence and compare the results with our additional constructs. A $C8P1^{TS}$ targeting sequence could potentially improve import as explained for the $C8S9^{TS}$ targeting sequence. Figure 2 illustrates the constructs used in this study.

***In vitro* import of reengineered mitochondrial polypeptides:** We tested whether reengineered ATP8 and cyt *b* (full-length, cyt *b*Δ553, and cyt *b*Δ853) could be imported into mitochondria *in vitro*. Constructs were incubated with a rabbit reticulocyte transcription/translation system in the presence of [^{35}S]methionine. As shown in Figure 3A, ATP8 was efficiently synthesized and imported into isolated rat liver mitochondria. Treatment with proteinase K did not affect the mature portion of the polypeptide localized in the mitochondrial matrix. The removal of the targeting sequence by mitochondrial peptidases was also observed. On the other hand, apocytochrome *b* and its truncated forms were not efficiently translated in the *in vitro* system. These translation products could be observed only when membranes were added to the transcription/translation reaction, indicating that their high hydrophobicity prevented translation at appreciable levels (Figure 3B). Similar results were observed for the truncated forms of apocytochrome *b* (not shown).

Unfortunately, the use of microsomes precludes import assays, as the polypeptide remains embedded in

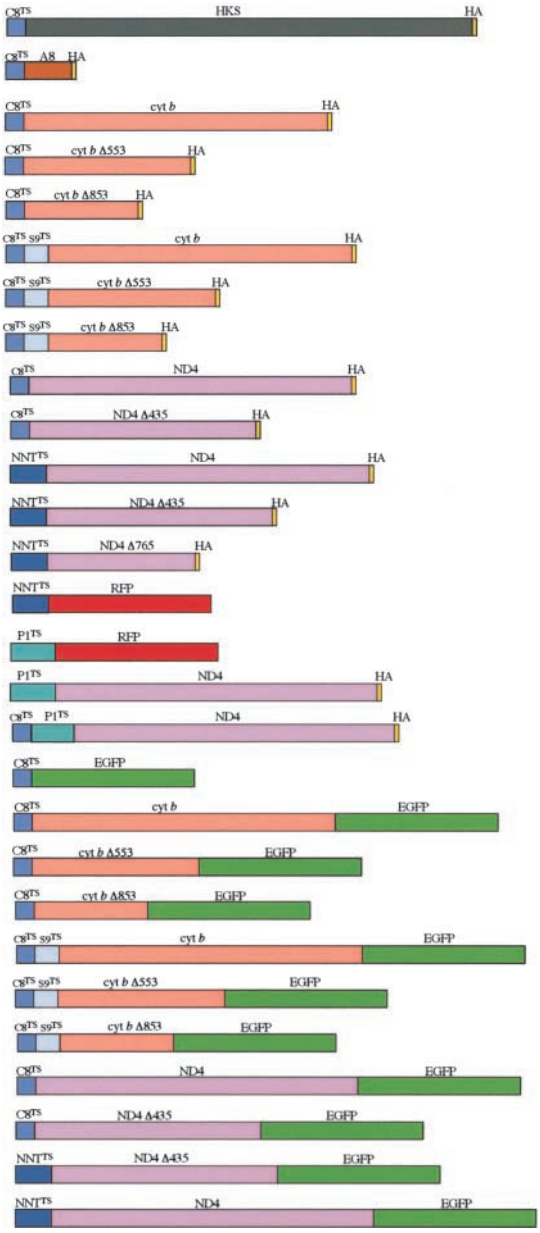

Figure 2.—Mitochondrial-targeted reengineered constructs used in this study. For allotopic expression of mitochondrial genes in the nuclear/cytoplasmic compartment, we recoded incompatible codons and appended a mitochondrial targeting sequence to the N terminus of the different polypeptides. Three different mitochondrial genes were reengineered: ATP synthase subunit VIII (A8), apocytochrome *b* (cyt *b*), and NADH dehydrogenase subunit IV (ND4). Truncated forms of apocytochrome *b* (Δ553 and Δ853) and ND4 (Δ435 and Δ765) were also constructed. Five different mitochondrial targeting sequences were used: human cytochrome oxidase subunit VIII targeting sequence ($C8^{TS}$), a tandem presequence containing $C8^{TS}$ followed by the *N. crassa* ATP synthase subunit IX targeting sequence ($C8S9^{TS}$), the human NNT^{TS}, the P1 isoform of the subunit c of ATP synthase ($P1^{TS}$), and a tandem presequence containing $C8^{TS}$ followed by $P1^{TS}$ ($C8P1^{TS}$). Constructs had an HA tag, an EGFP, or an RFP tail for detection. Control constructs are also depicted. HKS is a human lysyl-$tRNA^{Lys}$ synthetase.

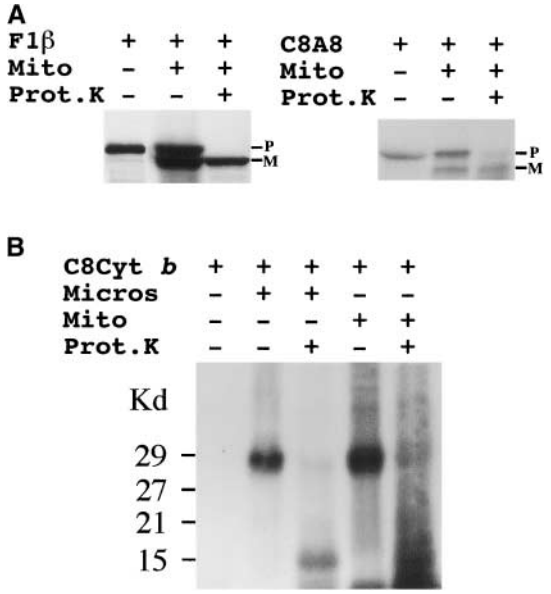

FIGURE 3.—ATPase8 can be efficiently imported into mitochondria *in vitro*. [^{35}S]methionine-labeled A8 produced by *in vitro* transcription/translation was incubated with rat liver mitochondria and assayed for import in the absence and presence of proteinase K and PAGE (A, right). The nuclear-coded subunit β of F_1 ATPase was used as a positive control (A, left). Due to its high hydrophobicity, apocytochrome *b* was not produced at a detectable level, unless microsome membranes were added to the transcription/translation mix (B). The apparent molecular weight of apocytochrome *b* in PAGE is abnormal due to its hydrophobicity, migrating faster than the predicted molecular weight. When microsomes were replaced by freshly prepared rat liver mitochondria, apocytochrome *b* was stabilized, but was still susceptible to proteinase K, indicating random association with mitochondrial membranes (B, last lane on the right).

the lipid bilayer. We attempted to circumvent the need for microsomes by attempting a coupled transcription-translation-import system using freshly purified mitochondria instead of microsomes during the translation reaction. Although the presence of mitochondrial membranes stabilized apocytochrome *b*, it did not protect the polypeptide from proteinase K digestion, suggesting that apocytochrome *b* was randomly embedded in the mitochondrial membranes (Figure 3B). The alternative use of wheat germ instead of a rabbit reticulocyte system, as well as T7 or SP6 promoters, did not improve the yield in the absence of membranes (not shown). We also attempted to stabilize the apocytochrome *b* by adding excess molecular chaperones to the *in vitro* protein synthesis reaction. The addition of HSP70 and GroEL/ES proteins to the translation mix had a very small effect on the yield of apocytochrome *b* in the absence of membranes (not shown). In conclusion, the high hydrophobicity of apocytochrome *b* or its truncated forms precluded *in vitro* import assays and suggested that the efficiency of *in vivo* translation/import of the allotopically expressed gene would be low.

***In vivo* import of reengineered ATP8:** We analyzed the ability of the reengineered gene products to localize to mitochondria *in vivo*. COS-7 and HeLa cells were transfected with the different constructs and analyzed either live (in the case of EGFP fusion proteins) or after fixation by immunodetection of the HA tag. The localization pattern of the different peptides in COS cells was essentially identical to the pattern in HeLa cells.

Immunostaining for HA showed that ATP8 was properly located in the mitochondria, colocalizing with the mitochondrial potentiometric dye, Mitotracker CMX-ROS (Figure 4). The localization of ATP8 having the $C8^{TS}$ was similar to nonhydrophobic mitochondrial-targeted constructs (*e.g.*, mitochondrial-targeted HKS-HA), albeit the number of transfected cells with detectable staining was greatly reduced with the ATP8 construct (~70% or less). The intensity of the staining was also reduced by ~60% (Figure 4). The reduced intensity likely is responsible for the reduced number of cells staining for HA.

Allotopic expression of reengineered apocytochrome *b* and ND4 did not lead to mitochondrial import *in vivo* but led to cellular toxicity: In contrast to ATP8, the different forms of apocytochrome *b* and ND4 showed a different localization pattern. Highly expressing cells showed a fiber-like structure and a loss of mitochondrial membrane potential, suggesting that the expression of these polypeptides was toxic to the cells (Figure 4). In cells expressing low levels of apocytochrome *b*, full length and truncated, we observed a punctate pattern that seemed to be in contact (but not colocalizing) with the Mitotracker staining (see white arrowheads in Figure 4). The staining for ND4 constructs showed a diffused, fiber-like staining that in some cases appeared to also localize to discrete structures that could be mitochondrial surfaces. ND4 and truncated forms did not colocalize with Mitotracker and appear to cause loss of mitochondrial membrane potential in regions positive for HA (Figure 4). Cells staining strongly positive for HA after transfection with the apocytochrome *b* and ND4 constructs showed a fiber-like appearance (Figures 4 and 5).

To better characterize the unexpected localization of the allotopic-expressed apocytochrome *b* forms in fiber-like structures, we double stained transfected cells with HA and either vimentin or tubulin. We observed a consistent colocalization pattern with tubulin, suggesting that most of the expressed protein aggregates with tubulin and possibly other cytoskeleton components. Vimentin is also a major component of the apocytochrome *b* fibers, but in contrast to tubulin, it was observed mostly in the heavy bundles of cells expressing high levels of apocytochrome *b* or its fragments (Figure 5).

A hydrophilic EGFP tail at the C terminus does not improve the importability of apocytochrome *b* and ND4 forms but decorates import pores: EGFP constructs

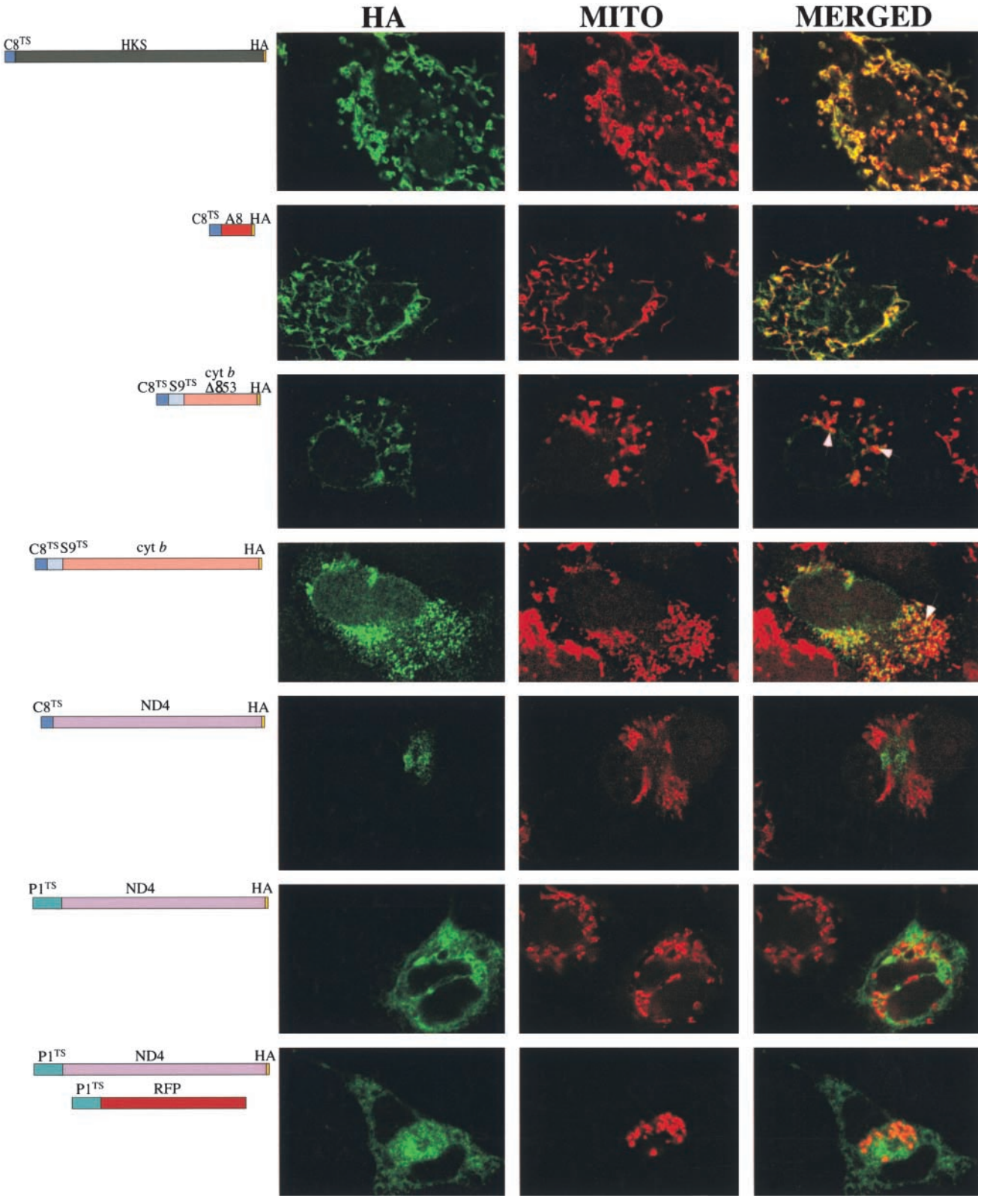

FIGURE 4.—ATPase 8 can be efficiently imported into mitochondria *in vivo*, whereas apocytochrome *b* and ND4 cannot. COS-7 cells were transfected with expression vectors containing the indicated constructs. Forty-eight hours after transfection cells were incubated for 30 min with Mitotracker CMX-Ros (Molecular Probes) as described in MATERIALS AND METHODS. Cells were fixed and stained with antibodies against HA. In the last three panels at the bottom, Mitotracker was not used, and the red staining was derived from the cotransfection of a $P1^{TS}$-RFP. Arrowheads show HA staining at the outer surface of mitochondria.

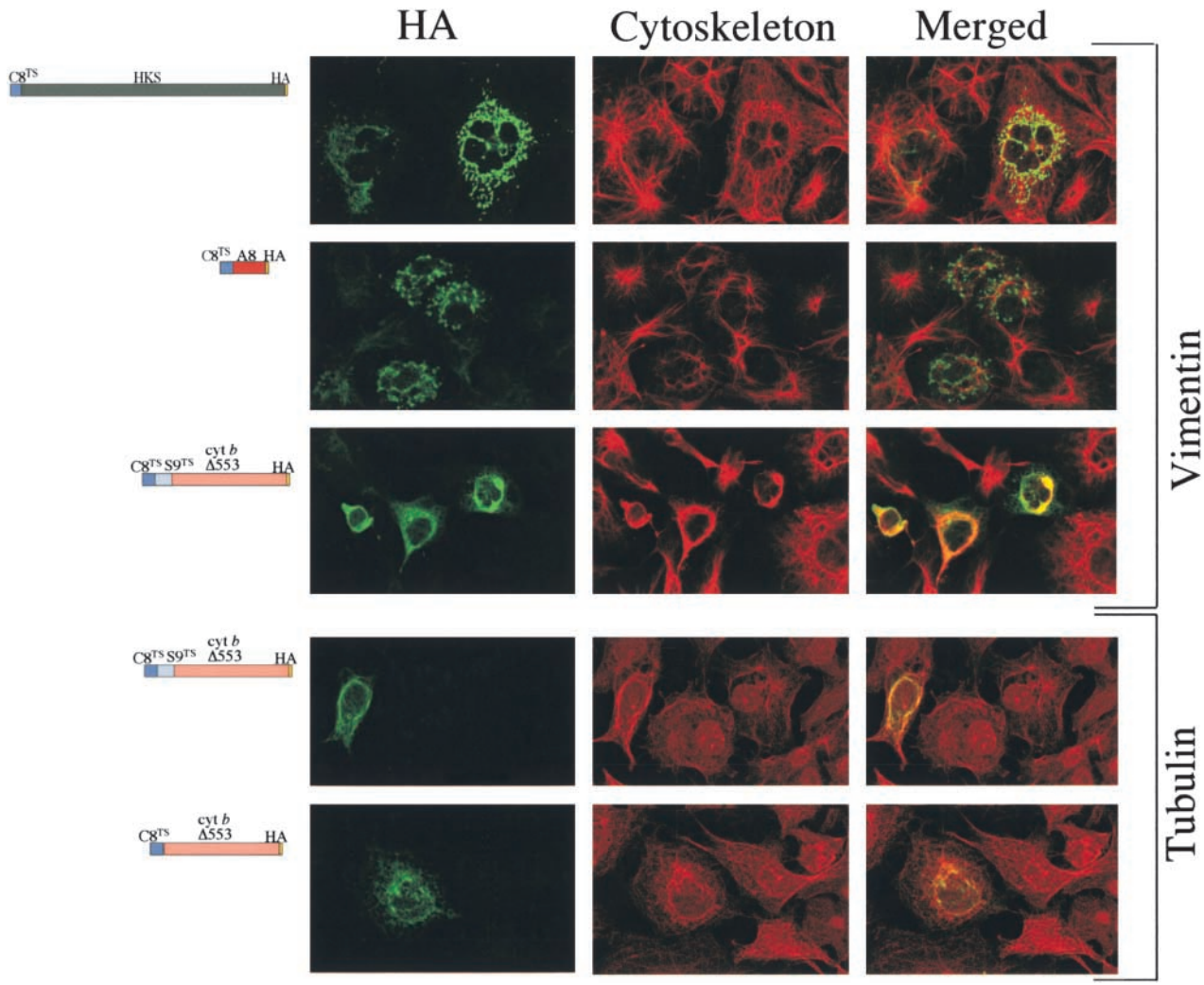

FIGURE 5.—Apocytochrome *b* and ND4 aggregate and associate with the cytoskeleton in the cytosol. COS-7 cells were transfected with expression vectors containing the indicated constructs. Forty-eight hours after transfection cells were stained for HA and vimentin or tubulin. In both apocytochrome *b* and ND4 (not shown) allotopic expression, a fiber-like structure was decorated by the anti-HA antibody. These fibers contained both tubulin and vimentin.

were produced and analyzed to assess the effect of a hydrophilic tail (238 amino acids) in the mitochondrial import process. The different apocytochrome *b* forms as well as different ND4 peptides fused to EGFP did not localize to internal mitochondrial compartments (Figure 6), even though the structure of the "fibers" was different from that of the heavier bundles observed with the HA constructs. These observations suggest that a hydrophilic tail is not sufficient to promote efficient mitochondrial import and may alter the characteristic association with cytoskeleton components.

Careful examination under the confocal microscope showed that the apocytochrome *b*-EGFP fusion protein localized to the surface of the mitochondria (Figure 7A). These observations confirmed the observations made with HA staining, indicating that although the targeting sequences are doing their job, the precursor protein is not import competent, probably because it aggregates before or during translocation through the TOM and TIM import complexes (Figure 7B). These live images also illustrate that the mitochondrial import machinery engaged in apocytochrome *b* import is not equally distributed around the mitochondrial surface, but rather clustered in specific patches or regions. It is unclear at this point if this localization reflects the location of all TOM/TIM complexes or a subset of TOM/TIM complexes that are in contact with ribosomes (KELLEMS *et al.* 1974, 1975) and consequently available to cotranslational import (FUJIKI and VERNER 1993), arguably the only mechanism by which hydrophobic proteins could be imported.

The effect of different targeting sequences: Although the $C8^{TS}$ has been shown to support import of different polypeptide "passengers," it was possible that it was not "strong" enough for the import of highly hydrophobic polypeptides. We followed an approach used to improve

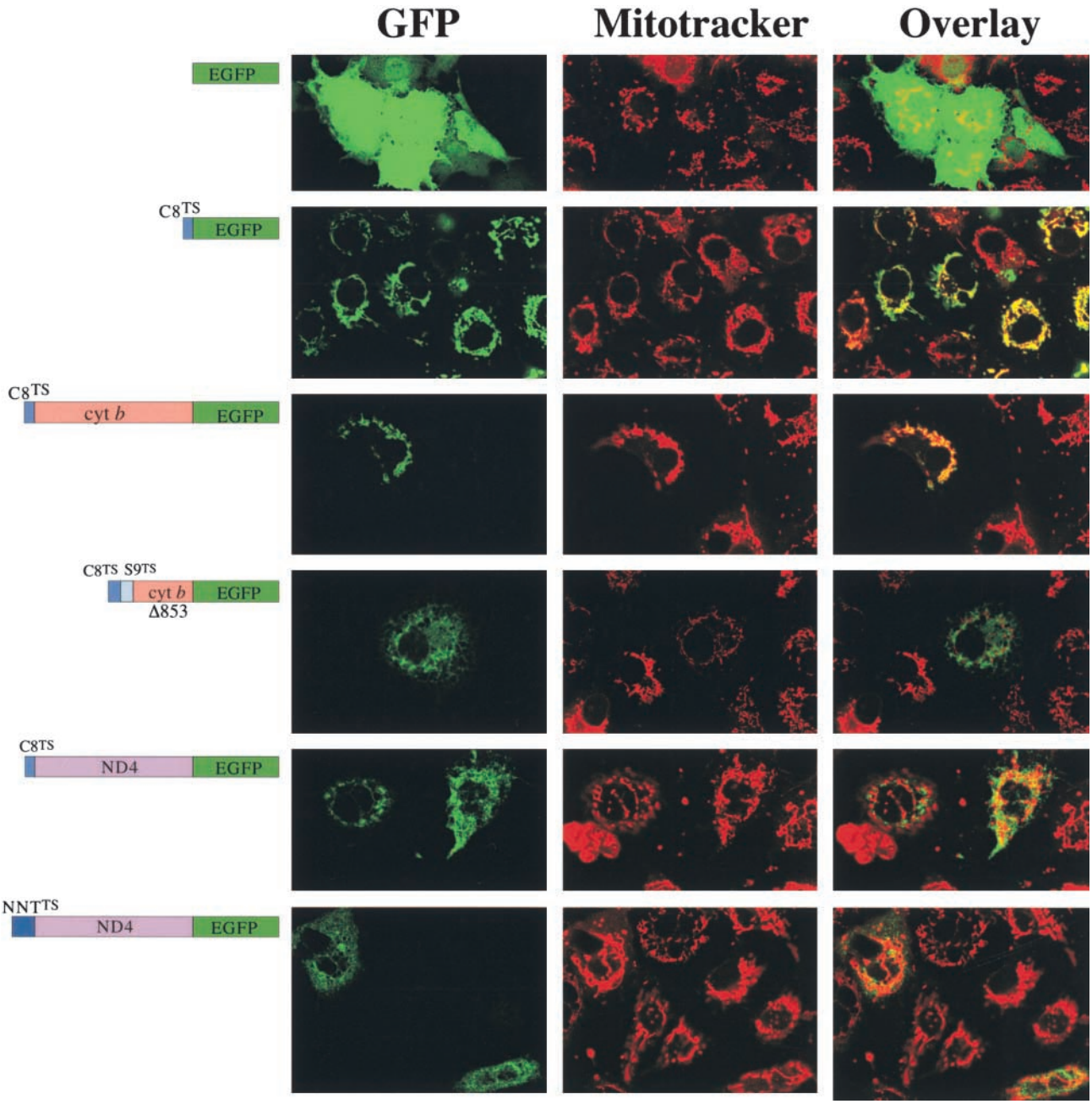

FIGURE 6.—A hydrophilic tail cannot compensate for the import defect of apocytochrome *b* and ND4. COS-7 cells were transfected with expression vectors containing the indicated constructs. Forty-eight hours after transfection cells were incubated with Mitotracker and visualized live. The presence of a hydrophilic tail, in the form of EGFP, did not improve mitochondrial targeting for the ND4 and apocytochrome *b* constructs.

allotopic expression of mitochondrial genes in yeast (GALANIS *et al.* 1991) and included an additional targeting sequence in front of the existing $C8^{TS}$. The final constructs had a tandem $C8S9^{TS}$ that mildly improved the targeting of apocytochrome *b* to the exterior of mitochondria (Figure 4). We next attempted to improve the efficiency of the import process by using the targeting sequence of a large hydrophobic mitochondrial protein that is naturally expressed from the nuclear-cytoplasmic compartment. The NNT has 10 membrane-spanning domains and a well-defined 43-amino-acid targeting sequencing. Once again, the use of NNT^{TS} with apocytochrome *b* or ND4 constructs was not sufficient to promote an efficient mitochondrial import. The NNT^{TS}, however, was able to import a less hydrophobic passenger, such as the RFP (not shown). Finally, we used $P1^{TS}$ that has been used for both ATP6 and ND4 import (GUY *et al.* 2002; MANFREDI *et al.* 2002). Although $P1^{TS}$ was very efficient in importing RFP, it was unable to promote import of ND4 (Figure 4). A tandem $C8P1^{TS}$

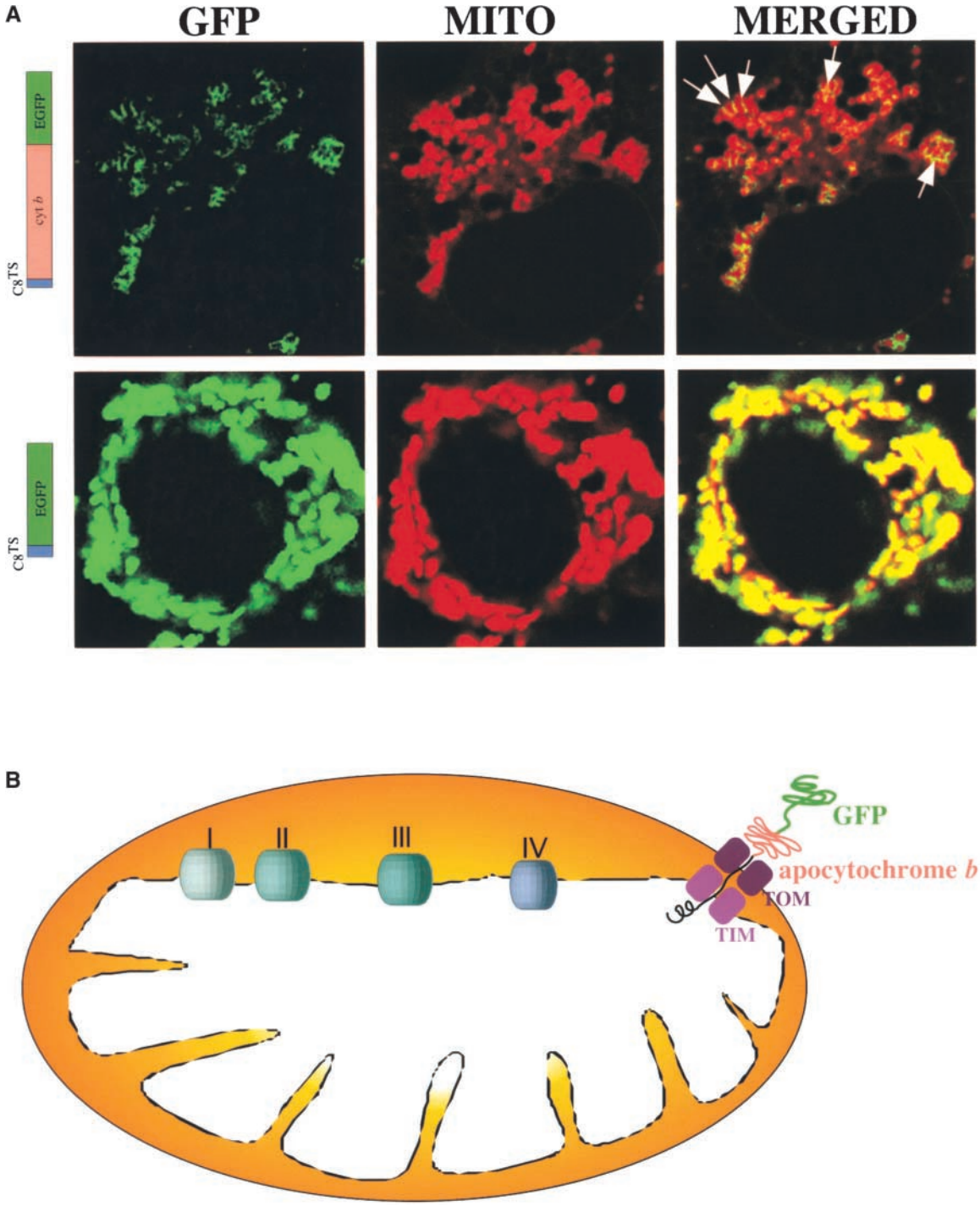

FIGURE 7.—Apocytochrome *b*-EGFP fusion protein localizes to the external mitochondrial surface. A careful examination of cells transfected as in Figure 6 showed that the allotopically expressed apocytochrome *b* is targeted to mitochondria, but cannot be internalized (A). This staining pattern is consistent with a block in the translocation of the polypeptide through the mitochondrial import pores (B).

was equally unable to promote ND4 import into mitochondria (Figure 4).

The transient expression of all tested constructs, including the import-competent ATP8, was significantly lower than that of constructs expressing less hydrophobic polypeptides. To get a more precise estimate, we cotransfected 1 μg of each construct containing the same targeting sequence ($P1^{TS}$) and two different "passengers," RFP and ND4-HA. Figure 8 shows that ND4-HA was detected in only ~1 cell for every 100 cells that were RFP positive. The figure also illustrates how examination at low magnification of thick optical slices could suggest colocalization of RFP and HA (Figure 8A). However, examination of the same cell from Fig-

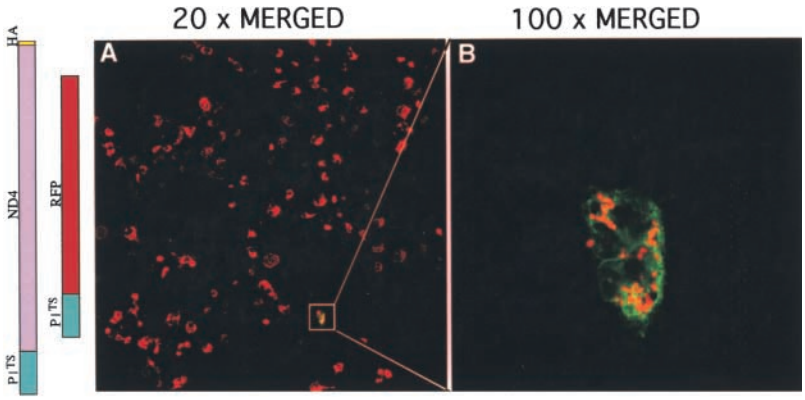

FIGURE 8.—Expression levels of allotopically expressed ND4. COS cells were cotransfected with 1 μg each of $P1^{TS}$ND4 and $P1^{TS}$RFP. After 48 hr the cells were fixed and examined for expression and subcellular localization of the products of two constructs. A shows that $P1^{TS}$RFP is expressed at markedly higher levels than $P1^{TS}$ND4. B shows that these two gene products do not colocalize.

ure 8A with a 0.5-μm optical slice under a ×100 oil objective clearly shows that HA and RFP do not colocalize (Figure 8B).

The effect of overexpressing molecular chaperones on allotopic expression of mitochondrial genes: Preimport aggregation of highly hydrophobic polypeptides would not be surprising, considering that they are probably inserted in the mitochondrial inner membrane cotranslationally when expressed from the mitochondrial genome. To maintain such polypeptides in an import-competent structure in the cytosol, we attempted to overexpress a constitutively active form of the heat shock factor 1 (HSF1). HSF1 is a transcription factor that activates the expression of several heat shock proteins (WAGSTAFF *et al.* 1998), many of which function as molecular chaperones for polypeptide precursors destined to mitochondria (*e.g.*, cytosolic HSP70). Coexpression of a constitutively active form of HSF (WAGSTAFF *et al.* 1998) resulted in a dramatic increase in HSP70 expression (Figure 9). Although there was a slight improvement in mitochondrial localization in double-transfected cells (*i.e.*, HSF and apocytochrome *b*), mutated HSF expression was unable to preclude the formation of apocytochrome *b* fibers in highly expressing cells or to direct the polypeptides to the inside of mitochondria. The main determinant for mitochondrial "capping" or fiber formation seems to be the levels of expression (Figure 9).

Allotopic expression of apocytochrome *b* does not restore function in an apocytochrome *b* null mutant cell line: Despite our immunocytochemical observations, it is possible that the full-length apocytochrome *b* construct can be correctly expressed and imported at low levels, which could be relevant in restoring function. We took advantage of a cell line containing homoplasmic levels of a apocytochrome *b* 4-bp deletion (RANA *et al.* 2000) to test this possibility. Osteosarcoma cybrids harboring the mutated mitochondrial apocytochrome *b* gene were transfected with a full-length apocytochrome *b* allotopic construct, cloned in bicistronic pIRES-puromycin vector. Because the promoterless puromycin resistance gene is located downstream of the gene of interest, this vector assures that the vast majority of clones resistant for the selection marker would express the gene of interest. We analyzed 14 puromycin-resistant clones for respiration. None of them showed significant oxygen consumption activity, which was similar to mock-transfected controls (not shown).

DISCUSSION

Allotopic expression of mitochondrial genes: Although mtDNA of animal cells usually codes for the same 13 polypeptides, some species are able to express some of these from the nucleus. Two genes constitute exceptions that have never been found to be expressed from the nucleus, cytochrome oxidase subunit I and apocytochrome *b*.

In the late 1980s, the group of Phillip Nagley in Australia described allotopic expression of two *S. cerevisiae* genes, ATPase 8 and ATPase 9, using the nuclear-encoded *N. crassa* ATPase 9 targeting sequence (GEARING and NAGLEY 1986; FARRELL *et al.* 1988; LAW *et al.* 1988, 1990; GALANIS *et al.* 1990, 1991; LAW and NAGLEY 1990). Only allotopically expressed ATPase8 could restore respiration in a mutant strain. The *S. cerevisiae* ATPase 9 could be imported only when it had additional amino acids from the *N. crassa* ATPase 9, following the targeting sequence (LAW *et al.* 1988). In the mid-1990s, Claros and colleagues analyzed the ability of *S. cerevisiae* mitochondria to import cytochrome *b* and its truncated forms by fusing them to a mitochondrial maturase that could function as a selection marker (CLAROS *et al.* 1995). Their conclusions from that study were that the full-length apocytochrome *b* could not be imported, even though smaller fragments of up to four transmembrane domains could. However, even two transmembrane domains could not be imported efficiently. They attributed the difficulties of apocytochrome *b* to being imported to high mesohydrophobicity, which basically describes how dense the hydrophobic residues are for a certain peptide segment (CORRAL-DEBRINSKI *et al.* 1999).

Our studies showed that even two transmembrane domains could not be imported efficiently in mammalian cells. Our system differs from the ones used by

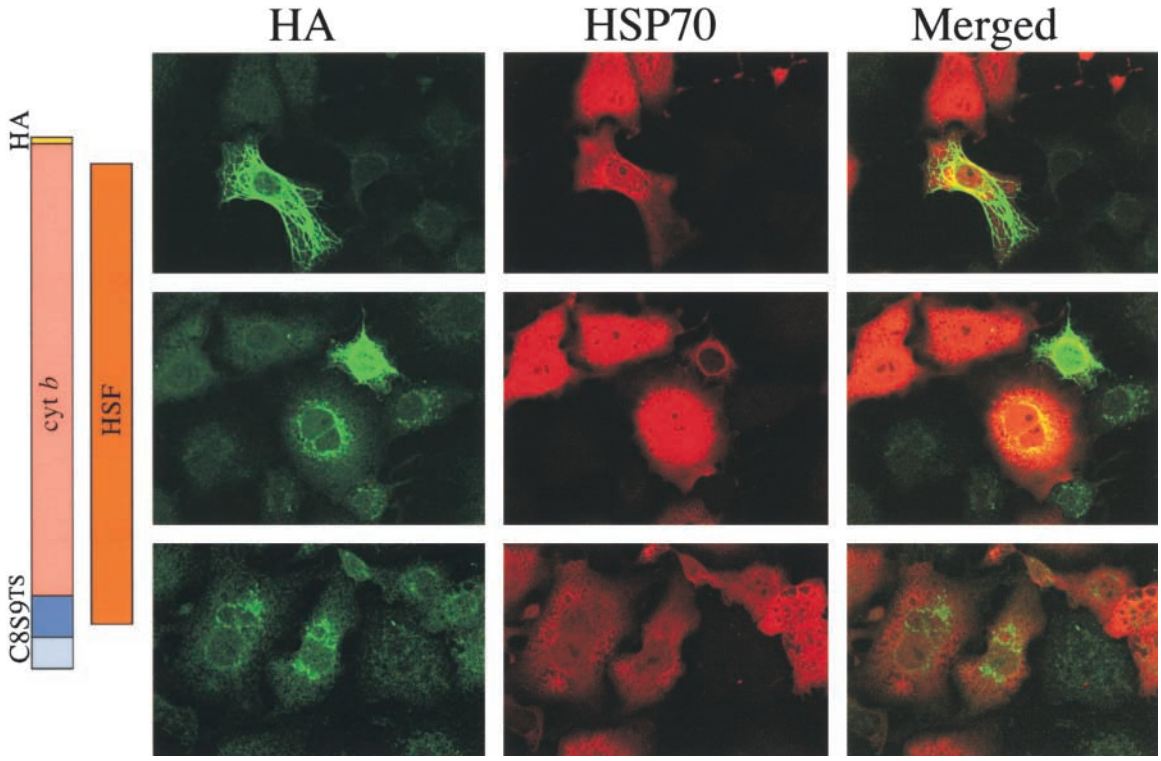

Figure 9.—Overexpression of molecular chaperones does not promote efficient apocytochrome *b* and ND4 import into mitochondria. Aggregation of preimport mitochondrial precursors is inhibited by cytosolic molecular chaperones. We coexpressed allotopic apocytochrome *b* constructs and a constitutively active form of HSF, a strong inducer of chaperone expression. Increased chaperone expression (measured as HSP70) did not have a major effect on mitochondrial targeting of apocytochrome *b* constructs.

Claros and colleagues in that in the latter a maturase tail was fused in frame and the selection system could be much more sensitive than that of our direct visualization approach. It is possible that the small hydrophobic moiety was rapidly degraded, but the maturase moiety was still able to be imported and to function as a selection marker. Although we cannot rule out the sensitivity issue as the main reason for the differences, a hydrophilic EGFP tail did not improve mitochondrial import in our system, even though it reduced aggregation with the cytoskeleton. However, in general, our conclusions were similar to theirs in that we believe that the hydrophobic segment remains stuck on mitochondrial membranes, possibly at the import pores (Corral-Debrinski *et al.* 1999).

We were successful in expressing and targeting ATP8 allotopically. The gene product seems to be correctly processed and imported into the mitochondria. However, as mentioned above, we were not able to do the same with different segments of apocytochrome *b* or ND4. These genes may not be able to remain import competent due to their high mesohydrophobicity and may need to be inserted into a membrane immediately, probably cotranslationally (Lithgow 2000).

Limitations of allotopic expression as a therapeutic approach: Several lines of evidence suggest that the number of mitochondrial genes limited by the allotopic expression approach is high. ATP8, which was correctly imported into mitochondria, had levels of expression that were drastically reduced when compared with less-hydrophobic controls. An additional problem of allotopic expression, even when feasible, is the competition with endogenous mutant proteins. This aspect will not be important when the mutation affects the ability of the affected protein to interact with the complexes. However, these are likely to be the minority, and competition for assembly will be an issue to be dealt with. Previous reports suggest that because a small number of corrected complexes would be necessary to improve OXPHOS, this may not be a major problem (Manfredi *et al.* 2002).

Manfredi and colleagues showed that allotopic expression of ATPase6 was able to improve ATP synthesis in human cells with an ATP6 pathogenic mutation (T8993G NARP mutation). The $C8^{TS}$ was able to direct ATPase6 to mitochondria and it was correctly processed by mitochondrial peptidases (Manfredi *et al.* 2002). We have transfected cells with the $P1^{TS}A6$ construct (a kind gift of Giovanni Manfredi, Cornell University) and found that the gene product colocalized with Mitotracker (not shown). Owen *et al.* 2000 showed that A6 and ND6 fused to GFP were not efficiently expressed from the nucleus and were not able to localize to mitochondria when a $C8^{TS}$ or a $S9^{TS}$ was used. The same group

later reported the allotopic expression of ND4, but their results were dramatically different from ours (Guy *et al.* 2002). ND4 expressed from adeno-associated virus (AAV) vectors showed essentially 100% transfection efficiency and the expression levels were reported as strong and mitochondrial localized. We do not have an explanation for these differences at this point. Their system differs from ours in two aspects: (1) the expression vector was the AAV, and although AAV may be able to deliver the gene to more cells, the localization of the gene product should not be influenced by the vector and (2) they expressed their construct in osteosarcoma cells. In our hands, 143B-derived osteosarcoma cells have a very low efficiency in expressing transfected plasmids. For this reason we used COS-7 and HeLa cells. Interestingly, their results with an ND4-GFP fusion were similar to ours, where the GFP showed a punctate signal but did not colocalize well with Mitotracker.

In addition to the importability and competition issue, the import of highly hydrophobic proteins is also limited by the significantly lower level of expression when compared with less hydrophobic polypeptides. Moreover, high levels of expression, when achieved, lead to a toxic effect at the mitochondrial and cellular levels. The combination of these three factors plus the fact that most pathogenic mitochondrial mutations affecting single genes are missense mutations, which are still expressed from the mitochondrial ribosomes, poses a strong barrier to allotopic expression. However, improvement in mitochondrial function in cells stably expressing reengineered ATP6 (Manfredi *et al.* 2002) and ND4 (Guy *et al.* 2002) has been reported.

The role of N terminus hydrophobicity in mitochondrial import: What limits the passenger protein for correct targeting? As mentioned above, Claros *et al.* (1995) have introduced the concept of mesohydrophobicity as an important factor for mitochondrial import competency. According to Claros and colleagues, mesohydrophobicity, which describes the average hydrophobicity in a window of 60–80 amino acids, together with a calculation of the most hydrophobic 17-amino-acid segment (H17), could predict importability of hydrophobic peptides (Claros *et al.* 1995; Corral-Debrinski *et al.* 1999). Using their algorithm (Claros 1995), we analyzed this correlation to predict the mitochondrial importability of the tested polypeptides in mammalian cells (Figure 10A). We found that C8^{TS}cyt*b*, C8^{TS}ND4, and C8^{TS}ATP6 fell above the limit for importability in yeast, whereas C8^{TS}A8 fell within importable limits (Figure 10A). This prediction agrees with our results in that we were not able to detect fully imported polypeptides after transient transfections with C8^{TS}cyt*b* and C8^{TS}ND4. However, C8^{TS}ATPase6 was shown to be imported in mammalian cells, albeit at low efficiency (Manfredi *et al.* 2002). The *C. reinhardtii* ATPase6 also has a mesohydrophobicity that falls in the top right corner of the graph of Figure 10A, and it is a nuclear-encoded mitochondrial protein. Interestingly, the *C. reinhardtii* A6 has also been found to improve ATPase function in human cells with the mtDNA NARP mutation (Ojaimi *et al.* 2002). Therefore, the mesohydrophobicity analyses may not be 100% accurate for mammalian import and may be species specific. The mesohydrophobicity × H17 graph also predicts that the smallest forms of truncated apocytochrome *b* and ND4 should be imported, which they were not (Figure 10A).

Studies with legume species have been very informative in this area. Legumes have two cytochrome oxidase subunit II genes, one encoded by the mitochondrial DNA and one by nuclear DNA. This phenomenon is more likely to occur in these species where the genetic code of mitochondria is not different from the nuclear. In the mitochondria of legumes, there is a significant decrease in hydrophobicity in the nuclear-encoded protein compared with the organelle-encoded protein. By a series of elegant *in vitro* import experiments, Daley *et al.* (2002) showed clearly that a reduction in local hydrophobicity, particularly in the first transmembrane domain, was necessary for import to take place. In addition, they also noted that a legume COXII-specific targeting sequence could not be replaced by other targeting sequences, suggesting that an optimized balance between local hydrophobicity and the targeting sequence was necessary for efficient mitochondrial import (Daley *et al.* 2002). We correlated the results from our studies with the mesohydrophobicity of the first 100-amino-acid segment of different proteins. The results of these analyses fit well with the available information on mammalian allotopic expression of mitochondrial genes. By setting an arbitrary threshold, C8^{TS}A6 could be importable, as was shown by Manfredi *et al.* (2002). It also fit with our data on the different forms of apocytochrome *b* and ND4 (Figure 10). The only discrepant finding remaining, when this model is applied, relates to the report of Guy *et al.* (2002), suggesting that ND4 could be efficiently imported. As mentioned above, we do not have an explanation for this difference.

Can allotopic expression be improved? We reasoned that increased expression of molecular chaperones could improve importability of highly hydrophobic peptides. Chaperones, such as the cytosolic HSP70, are required to maintain mitochondrial precursors in a loosely folded, nonaggregated conformation. This function is necessary for translocation of mitochondrial precursors through the mitochondrial import apparatus (Lithgow 2000). In an attempt to upregulate molecular chaperones, we expressed a constitutively active mutant form of HSF1. Deletion of the second leucine zipper from HSF1 allows the protein to trimerize and exposes its transcriptional activation domain, even in the absence of a stressful stimulus (Wagstaff *et al.* 1998). Hence, this form of HSF1 is constitutively active and is able to activate several HSP promoters. However, expression of the constitutively active HSF1 provided only a modest

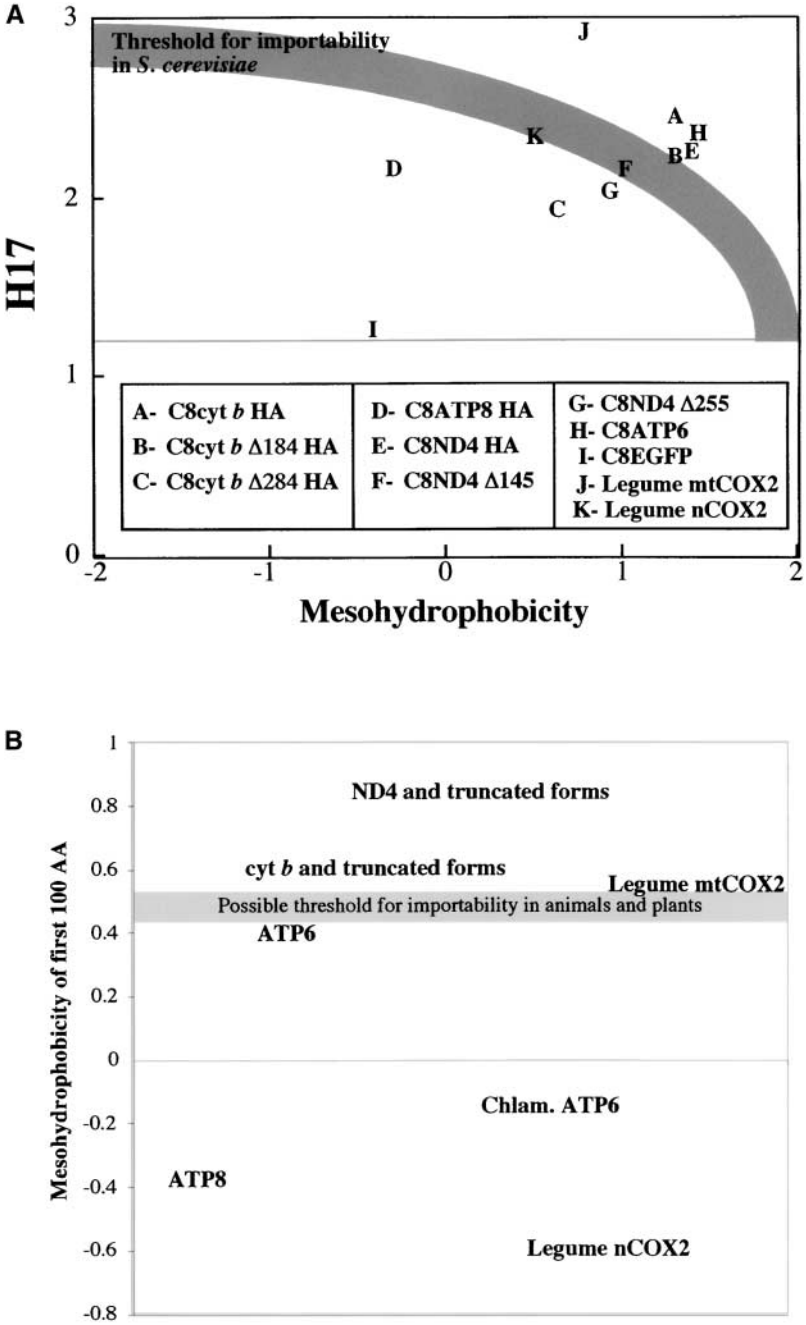

FIGURE 10.—Likelihood of mitochondrial import based on a mesohydrophobicity index. (A) A plot developed by Claros and colleagues (CLAROS 1995) and used to estimate mitochondrial importability in yeast. By this approach, some of the apocytochrome *b* and ND4 truncated products should be importable. However, a legume nuclear-coded COXII falls in the threshold for importability, and ATP6 would not be importable. (B) The mesohydrophobicity of the first 100 amino acids is a better predictor for importability in mammalian cells, as was suggested for legumes (DALEY *et al.* 2002). Mesohydrophobicity, which is the average regional hydrophobicity over an extended region of the sequence [in this case, 100 amino acids (aa)], was calculated using MitoProtII (Version 1.0, CLAROS 1995). The graph in A was also produced with MitoProtII. H17 is calculated as the maximum local hydrophobicity of a segment with a scanning window length of 17 amino acids that possess the highest mean hydrophobicity per residue.

improvement in the mitochondrial colocalization of reengineered apocytochrome *b*, and most (if not all) of it remained outside the organelle.

Corral-Debrinski and colleagues have recently shown that overexpression of the gene PSE1 can help import cytochrome *b* segments fused to a maturase. Pse1p is a member of the karyopherin family, which is composed of nuclear/cytoplasmic proteins involved in mRNA export from the nucleus. These researchers proposed that the effect of Pse1p could be to improve, either directly or indirectly, the coupling between mRNA-specific localization/translation and mitochondrial import processes—in other words, to enhance the translation of hydrophobic peptides in ribosomes in contact with mitochondria (cotranslational import; FUJIKI and VERNER 1993; CORRAL-DEBRINSKI *et al.* 1999). It is interesting to note that our EGFP fusion proteins decorated patches of mitochondrial membranes that may constitute sites of attachment to ribosomes, which are known to occur in yeast mitochondria (KELLEMS *et al.* 1974, 1975). It is unclear how improved delivery of mRNAs coding for hydrophobic proteins to mitochondrial-associated ribosomes would improve import, even though it could be solely a numerical improvement (CORRAL-DEBRINSKI *et al.* 1999).

C. reinhardtii expresses several genes in the nuclear/

cytoplasmic compartment that are commonly expressed in the mitochondrial genome of other species, including COXII, COXIII, and ATPase6 (Perez-Martinez *et al.* 2000, 2001). In addition to changes in codon and acquisition of a targeting sequence and reduction of their hydrophobicity (Perez-Martinez *et al.* 2000), Darwinian selection found an additional way around import barriers, such as splitting COXII into two fragments (Perez-Martinez *et al.* 2001).

In conclusion, our results indicate that although the allotopic expression approach can work for a few selected genes, for most mitochondrial genes it remains an extremely challenging task that faces a significant number of hurdles before it can be applied in genetic therapy. Nevertheless, research in optimization approaches may be able to overcome these problems in the future. The search for amino acid changes that reduce hydrophobicity of the N terminus without affecting biologic activity is likely to improve the feasibility of the allotopic approach for mitochondrial genes.

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