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Forum Review

Approaches and Limitations to Gene Therapy for Mitochondrial Diseases

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ABSTRACT

Mitochondrial dysfunction may be caused by mutations in either the nuclear and/or the mitochondrial genome. Since 1988, mitochondrial DNA mutations have been linked to retinopathies, myopathies, neurodegenerative diseases, and possibly normal aging. Adequate drug therapies for these disorders have yet to be discovered. Therefore, gene therapy must be considered as a possible alternative. In this review, we will discuss the possibilities and the problems associated with gene therapy for mitochondrial disorders. Antioxid. Redox Signal. 3, 451–460.

INTRODUCTION

MITOCHONDRIA are small organelles located in the cytoplasm of eukaryotic cells. They are the sole site of oxidative phosphorylation for the generation of ATP, the main energy source of the cell. Any given somatic cell may contain 10–2,000 mitochondria, each carrying two to 10 copies of the mitochondrial genome, depending upon the energy requirements of the respective organ (48, 49). For example, greater numbers of mitochondria are seen in metabolically active organs such as the brain, skeletal muscle, and the liver. Mutations in mitochondrial DNA (mtDNA) often occur by point mutation and deletion (Table 1). Typically, there is a mixed population of mutant and wild-type forms, a condition known as heteroplasmy (1). If wild-type to mutant levels of mitochondria fall, ATP levels may become compromised to a level below a threshold for normal cellular functions. When that happens, clinical manifestations may become evident.

Defects in oxidative phosphorylation may be caused by mutations in the nuclear DNA (nDNA) and/or the mtDNA (Table 1 and Fig. 1). The ~16.5-kb genome of human mtDNA contributes to transcripts encoding 13 polypeptides of the oxidative phosphorylation pathway. Likewise the mitochondrial genome contributes all the tRNAs, as well as two rRNAs, used for translation of the polypeptide transcripts. All of the other polypeptides involved in the production of ATP (~100 in total) are derived from the nDNA.

Metabolic therapies for treating patients with defects in oxidative phosphorylation have not been encouraging. Objective improvements have been reported on clinical examination, yet giving alternate metabolic fuels cannot bypass the final common pathway of oxidative phosphorylation, where the defect is present. Given the limited options for therapy for these defects, gene therapy appears to be a logical alternative. In this review, we will discuss advances and problems associated with the development of gene therapies for defects in oxidative metabolism.

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TABLE 1. CHARACTERISTICS OF NUCLEAR AND MITOCHONDRIAL MUTATIONS

	Nuclear	*Mitochondrial*
Mode of inheritance	Mendelian	Maternal
Protein characteristics	Variable	Highly hydrophobic
Threshold effect	None	Present
Common mutation types	Point mutations (missense, nonsense), single base deletions (frame shift)	Large deletions, insertions, point mutations, tRNA mutations
Age-related	Rare	Common

GENE THERAPY FOR DEFECTS IN MITOCHONDRIAL GENES ENCODED BY nDNA

We have recently seen advances in gene therapy for a considerable number of nuclear gene defects. Viral vectors used in this effort have included adeno-associated virus (AAV), adenovirus (Ad), several retroviruses, and others. Currently available therapeutic vectors of these types can be used for many of the nuclear defects included in oxidative metabolism. In these instances, a leader sequence is already included in the gene, and import of the protein occurs naturally as it would in cells without a defect.

Several groups have pursued gene therapy strategies to target diseases associated with nDNA mutations causing mitochondriopathies. Correction of ornithine-δ-aminotransferase deficiency has been corrected in a Chinese hamster ovary cell line using retroviral vectors (23). Recombinant Ad has also been used in correcting secondary metabolic defects in ornithine transcarbamylase (OTC) deficiency in spf^{ash} mice, a model for the human X-linked inherited urea cycle disorder (56). Intravenous injection of recombinant Ad expressing mouse OTC dramatically increased both mitochondrial and liver OTC concentration and activity. However, a phase I human study with an Ad vector resulted in severe inflammatory toxicity, coagulopathy, adult respiratory distress syndrome, and the death of one of the volunteer subjects (reported at NIH Recombinant DNA Advisory Committee).

Our group has recently created one such vector using AAV, which has the capacity to infect both proliferating and nonproliferating cells without triggering an acute inflammatory response (32) (Fig. 2). We designed recombinant AAV (rAAV) vectors to express pyruvate dehydrogenase E1α (PDH). PDH is a pivotal enzyme subunit in metabolism, used in the decarboxylation of pyruvate to acetyl-CoA and carbon dioxide. In our studies, we have observed *in vivo* gene expression of PDH fused to green fluorescent protein (GFP), in cells of the CNS, specifically rat spinal cord neurons with expression patterns similar to native PDH. Several groups have previously shown strong expression using rAAV in the basal ganglia, cerebral cortex, cerebellum, and several other key

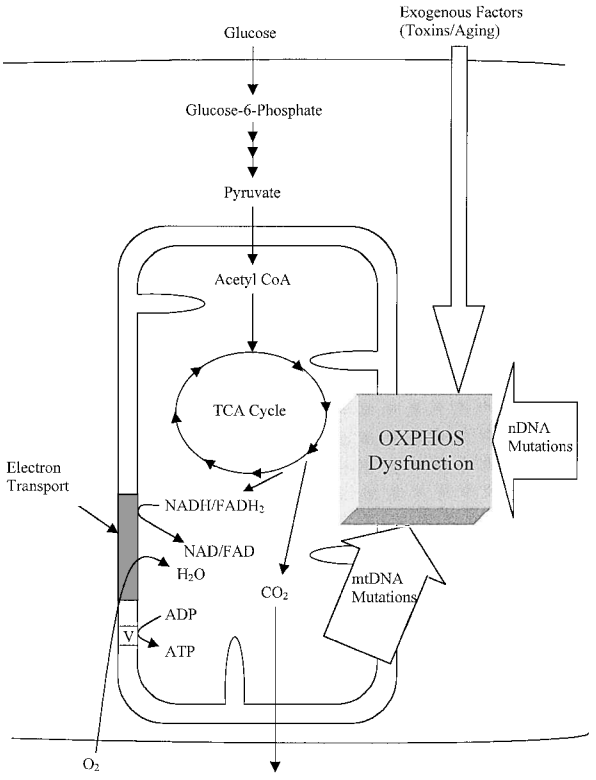

FIG. 1. Basic oxidative phosphorylation and events causing dysfunction. OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid.

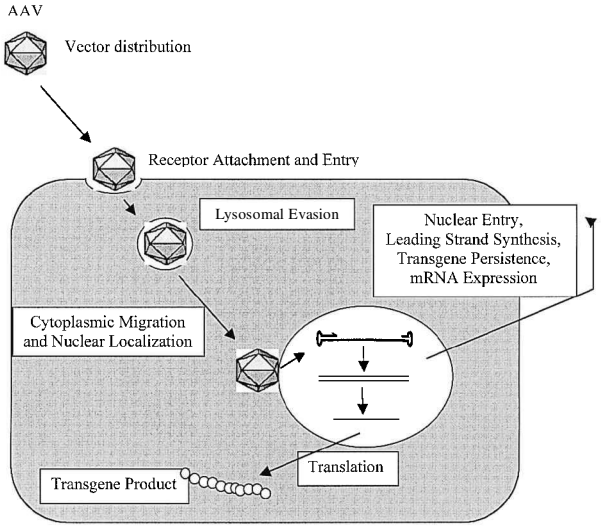

FIG. 2. Simplified mechanisms of AAV gene therapy.

areas of the brain by direct injection (27, 28, 55). Similar experiments using rAAV have shown high transduction efficiencies in muscle (21), liver (42), and the eye (11, 13), all essential target organs for treatment of mitochondriopathies. Earlier data have shown increased PDH complex (PDC) activity in PDH E1α patients by transfection of a normal PDH E1α gene (4), suggesting that a rAAV-based method to deliver the gene could be successful. Seo et al. have used a similar AAV strategy to complement defects in complex I defects in human cells by transduction with the NADH-quinone oxidoreductase (NDI1) gene of *S. cerevisiae*, achieving nearly 100% transduction efficiencies in their cell lines (40). In addition to AAV's ability to transduce various tissue types efficiently, expression has been shown to be long-term, life-long in mice, and has not been implicated as the causative agent for any pathology.

Collectively, these studies have progressed to a point of a promising therapy for some of the nuclear-derived mutations of mitochondriopathies. Unfortunately there remain several hurdles to accomplish gene therapies for mutations in mtDNA safely in humans.

GENE THERAPY STRATEGIES FOR mtDNA MUTATIONS ENCODING PROTEINS

The first strategy that met with some success in yeast was reported by Nagley et al. (30). They complemented an ATPase defect in subunit 8 by nuclear expression of the gene fused to another leader sequence of another normally mitochondrially translocated protein. This strategy is called allotypic expression (Table 2). For this idea to become a reality, mitochondrial proteins must be expressed and targeted to

TABLE 2. MODALITIES OF CORRECTION FOR MITOCHONDRIAL MUTATIONS

	Advantages	*Disadvantages*
Allotypic expression	Cellular processed protein (use of same nuclear-encoded protein machinery for translation and import); use of current gene therapies for continual expression	Hydrophobicity of proteins to be imported; inability to treat tRNA mutations; gene therapy problems (administration, toxicity, control of expression)
Inteins	Possibility to overcome import protein hydrophobicity; use of cotranslational import?	Same as above
PNAs	Highly specific binding to DNA and RNA, cause selective advantage to normal mtDNA and/or transcripts	Binding to nDNA; cell type- and concentration-dependent; administration *in vivo*
Peptide-DNA conjugates	Possibility to introduce entirely new mitochondrial genomes	Cleavage of conjugated peptide may pose problems; administration *in vivo*
Cytoplasts	Derived from patient, therefore no immune complex problems; *ex vivo* growth of normal mitochondria limitless?	Readministration to host; fusion of cytoplasts to all cell types? Only heteroplasmy percentage can change
DQAsomes	Ability to transfect mitochondria directly	Administration *in vivo*; still in its infancy

the correct mitochondrial compartment for complementation of the given defect. The genetic code differs between the nucleus and the mitochondria in humans. For example, UGA encodes a stop in the human nuclear code, as opposed to a tryptophan in mitochondria (2). Likewise, AUA changes from Ile to Met, and AGA and AGG from Arg to stops. Advances in PCR technologies and DNA sequencing can overcome this hurdle, but import of the proteins appears to be a larger problem.

One theory that has been proposed to explain the evolutionary advantage for maintaining the 13 genes within the mitochondrial genome is based on the extreme hydrophobicity of these proteins that could prevent efficient import (7). Others have suggested that these proteins may be toxic to the cell in the cytoplasm (19), although this second idea has little evidence supporting it. On the other hand, it is clear from studies of allotypic expression of mitochondrial proteins that they are very hydrophobic and may be very resistant to the unfolding process that will be necessary for translocation. A measure of hydrophobicity of the proteins encoded by the mitochondria in yeast has shown that those still expressed by mtDNA are among the most hydrophobic (7).

Our findings have unfortunately suggested that the hydrophobicity of the proteins may be a limiting factor for successful gene therapy. We have tried to replicate the studies of Nagley in allotypic expression of both of the mitochondrial genes ATPase subunit 6 and NADH dehydrogenase subunit 6 in human embryonic kidney cells, 293s. We have found that even short segments of either of these genes are not translocation-competent. We used as few as the first 88 N-terminal amino acids fused to either the leader of COX8, a 25-amino acid leader, or ATP9, a 66-amino acid leader sequence. Neither of these sequences appeared to be translocated into the mitochondria. Also, these proteins had been fused in-frame with GFP, another hydrophilic protein, increasing the overall hydrophilicity of these chimeras. Duplication of the leader sequence, ATP9, was suggested to help import; it also failed. It seems that even these short segments are too hydrophobic to be translocated into mitochondria. As stated previously, GFP fused to these proteins we would expect to assist in import and not decrease. We have no evidence of GFP inhibiting import because fusion of the full-length PDH gene to GFP was successful. This is not to say that import of these peptides is not possible.

To combat this problem, it had been suggested to increase the overall hydrophilicity of the protein. One interesting idea has been the use of inteins (Table 2 and Fig. 3). Inteins, which were discovered in 1990, are self-splicing "introns of proteins" (35). Protein splicing involves the posttranslational excision of an internal splicing region (the intein) from a precursor protein with subsequent ligation of the remaining external protein fragment (the extein). Six inteins are known to exist in eukaryotes, including two in yeast (34). Exteins have also been discovered that are encoded from two different mRNAs, yet ligate to a single protein (54). These have been called split inteins. The only requirement of splicing is that the amino acid residue that is immediately C-terminal to the intein must be a cysteine, a serine, or a threonine. Inteins can be used to increase the overall hydrophilicity of the protein. The amount of "cargo" the inteins carry does not limit maturation of the protein (35).

Using this approach, the major requirement would be to make the catalytic (self-splicing) activity of the intein conditional, in order to

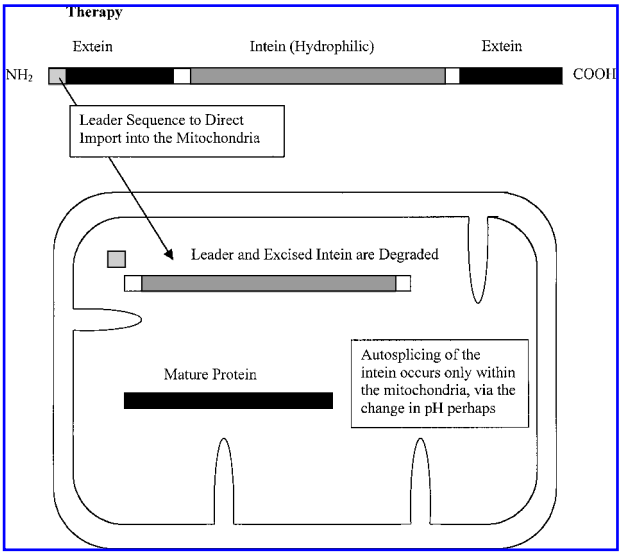

FIG. 3. Proposed mechanism of intein import for mitochondrial gene therapy.

control where in the cell the precursor protein folds into a catalytically active, splicing form. Several groups have reported pH-sensitive inteins, some of which may be optimal at the intramitochondrial pH (53).

Inteins can be used to decrease the speed of translation, and this could improve the import of these hydrophobic proteins if insertion is cotranslational. However, experimental evidence has not been always consistent with this idea. Williams et al. have shown that an intein fused to a mitochondrial targeting sequence is spliced prior to being imported into the mitochondria (52). Although the intein was found to be spliced in the cytoplasm, small amounts were found to be imported into the mitochondria. This lends support to the possibility of cotranslational import, or cytosolic chaperones preventing the folding of the intein until translocation is complete. In this experiment, translation of this intein was not modified to slow translation, which can be accomplished by increasing the amounts of rare tRNAs used in translation or by increasing the amount of superfluous sequence of the intein. This seems feasible for a C-terminal portion of a split intein, although one would expect that the N-terminus would still have difficulty importing because the leader sequence would be the only sequence before translation of the allotypic protein. Nevertheless, the data suggest that import into the mitochondria may be flexible.

Classical import of mitochondrial proteins, in yeast studies, has been long known to need a basic, amphipathic helix as a leader sequence to direct nuclear encoded mitochondrial proteins to the translocase in the outer mitochondrial membrane (TOM) (25). Several experiments have proven that the leader sequence is efficient in importing various reporter genes, including GFP and the enzyme dihydrofolate reductase (18, 36). However, nuclear encoded mitochondrial proteins tend to be more hydrophobic than polypeptides that reside in the cytoplasm. These proteins could not be expected to stay soluble. Further requirements must be made for successful import. Cytosolic chaperones, such as Hsp70 and mitochondrial stimulating factor (MSF), are available to prolong import competence (29). MSF can readily bind to several mitochondrial precursor proteins and can prevent and even reverse their aggregation to transfer them to the TOM complex (14). The TOM complex, located in the outer membrane, is one of three translocases described in mitochondrial membranes (31). TOM cooperates with two distinct complexes in the inner membrane, the translocase of the inner membrane (TIM) 23 complex and the TIM 22 complex. Precursors with an N-terminal presequence are imported via the TIM 23 complex, whereas mitochondrial carrier proteins require the TIM 22 complex. Members of the mitochondrial carrier family include at least 34 members in yeast, such as ADP/ATP carrier (33). The carrier proteins are synthesized in the cytosol without a cleavable presequence, but do carry internal targeting signals within the "mature" part of the polypeptide. The specific route by which the substrate reaches the inner membrane is still uncertain. It has been suggested that translocation across the outer membrane and insertion into the inner membrane occur at contact sites, preventing misfolding and aggregation of unshielded hydrophobic precursors. The internal insertion signals resemble positively charged presequences of matrix-targeted precursors, suggesting an element of conservation of the import signals (3). Members of the TIM complexes are conserved throughout eukaryotes, including humans (47). Further understanding of the TIM complexes will eventually provide insight into import and sorting of the more hydrophobic proteins encoded by nDNA. This information may give us the necessary tools to achieve allotypic expression. Mitochondrial proteins expressed from the nucleus may then be modified to include internal import signals for correct translocation of the precursor polypeptides while retaining enzyme activity.

IMPORT OF NUCLEIC ACIDS INTO MITOCHONDRIA

The underlying challenge of targeting and uptake across both mitochondrial membranes is no different when trying to introduce nucleic acids (RNA or DNA) into the mitochondrial matrix. Whereas the outside membrane is permeable to molecules with molecular masses of

up to 5 kDa, the inner membrane is far more selective. However, import of nucleic acids to mitochondria *in vivo* may be necessary for therapy of most all mutations in mtDNA. Diseases associated with mutations in tRNAs would otherwise need allotypic expression of all of the mitochondrial proteins encoded in the mtDNA. Conversely, successful import of even very small antisense DNA or RNA oligonucleotides against mutant mtDNA sequences [such as those associated with myoclonic epilepsy and ragged red fibers (MERRF) (41) and mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) (12)] has the potential to inhibit replication of these mtDNA molecules. Replication of mutated genomes would then decrease, allowing wild-type, unaffected genomes to have a selective advantage in repopulating the mitochondria.

Peptide nucleic acids (PNAs) have recently been tested as a potential antisense agent to inhibit the turnover of mutant mtDNA (46) (Table 2). DNA synthesis from a runoff replication template containing the MERRF mutation was blocked by PNA binding, whereas the wild-type template, differing by a base pair, produced full-length replication products. PNAs are novel, synthetic DNA-like molecules in which the chains of pyrimidine and purine bases are linked by an aminoethyl backbone (8). PNAs bind to DNA and RNA very specifically and are resistant to nuclease and proteases (9, 10). Chinnery et al. have reported successful import of PNAs into isolated mitochondria and human myoblasts upon conjugation of the biotinylated PNA molecule to the signal sequence from cytochrome *c* oxidase (COX) subunit VIII (5). Yet, not unlike other protocols, problems have persisted. PNA uptake is cell type-specific as well as concentration-dependent. To use this strategy *in vivo* could therefore be quite challenging.

Nevertheless, the *ex vivo* generation of cytoplasts for reinjection into patients may be useful (20) (Table 2). In this case, heteroplasmic cells can be taken from patients and proliferated selectively inhibiting turnover of mutant mtDNA. Readministration of cytoplasts has not yet been accomplished, but because cytoplasts are derived from the patient to be reintroduced, there will not be an immunological response. Cytoplasts can also be generated by the treatment of cells with cytochalasin (16), but the combination of PNAs may facilitate the process. PNAs can generate antisense activity against nDNA because a fraction of PNAs localize to the nucleus (5), even when conjugated to the COX VIII presequence, but this is manageable when used *in vitro*.

RNA molecules may also be imported into the mitochondria. It has been reported that a 5S ribosomal RNA may be imported, but its function is unknown (26). Another is the nuclear-encoded RNA component of the mitochondrial RNA processing enzyme (RNase MRP), which may be involved in maturing RNA primers for mtDNA replication (24). The MRP RNA contains a putative targeting region, which when deleted abolishes import. However, the mitochondrial location of this RNA is still disputed and no further reports have become available. Mammalian mitochondria do not import nucleic acids for intramitochondrial translation because all the tRNAs are encoded in the mtDNA. In other species that do import tRNAs, the number of imported species varies from one in yeast to the totality in many protozoan species (15, 45). In *S. cerevisiae*, there is a highly specific process, because only one of two cytosolic $tRNA^{lys}$ isoacceptors is targeted to the mitochondria. It has been shown that import of the tRNA requires the presence of the precursor of mitochondrial lysyl-tRNA synthetase (MSK), as well as the maintenance of the inner membrane potential (44). Additionally, mutants defective in the protein translocation pathway do not import the $tRNA^{lys}$ (43). These data strongly suggest that MSK is likely to act as a carrier for mitochodrial translocation of the tRNA. Kolesnikova *et al.* have recently begun to exploit this import pathway as a possible therapy for tRNA import into human cells (22). This group has suppressed mutations in mtDNA with use of the imported yeast tRNA. In their experiments, the aminoacylation identity of the anticodon was changed and the imported lysine isoacceptor was then able to be charged with tRNAs other than $tRNA^{lys}$. It appears that although aminoacylation is a prerequisite of import, the identity of the tRNA is of less importance. The tRNA import selectivity observed was also

found to be equally similar in human cells and mitochondria. Further manipulation of this system could be useful in replacing mutant tRNAs or suppressing nonsense mutations.

Evidence suggests that the amount of nucleic acid introduced into mitochondria is not limited to the short length of tRNAs. The rate-limiting step for functional translocation is linked to the diameter of the molecule imported (39). Seibel et al. have shown mitochondrial uptake of 17-bp and 322-bp DNA molecules by chemical cross-linking the OTC signal peptide to the DNA. Although this strategy again holds promise for antisense, it has been stressed that a DNA fragment of 300 bp in length is sufficient to allow construction of a transcribable mitochondrial tRNA gene, composed of coding sequence flanked by the nucleotides responsible for RNA processing and a mitochondrial promoter. Further studies by Seibel *et al*. are promising, having stated completion of an artificial mitochondrial plasmid containing the complete information for replication, accomplished using mitochondrial extract *in vitro*, and transcription (38). Attempts to achieve replication or transcription of this plasmid have failed so far, but this may be tied to the conjugated amino acid sequence of the mature OTC protein necessary for cleavage of the presequence. A cross-linker that is unstable in the mitochondrial matrix could potentially alleviate this problem, but introduction of this product *in vivo* could be troublesome.

Certain properties are necessary in generating a mitochondria-specific vector. First, the vector needs to be endocytosed into several different cell types. Second, transport of the vector must be directed to the mitochondria. And third, the vector must be translocated into the mitochondria. Although PNAs and peptide-DNA conjugates both have the second and third abilities listed, neither appears to have the ability for endocytosis in various cell types. Although cationic lipids have also been shown to vary greatly in their ability to transfect cells, a new type of cationic lipid has been produced to selectively release DNA at mitochondria-like membranes. Termed DQAsomes, these cationic lipids are made of dequalinium, which has the intrinsic property to accumulate in mitochondria in response to the electrochemical gradient, and may serve to deliver DNA to mitochondria in living cells (50, 51) (Table 2). Studies suggest that after endocytotic uptake DQAsome/DNA complexes will release DNA to a lesser extent, if at all, upon contact with anionic lipids in the cytosol. However, cardiolipin-rich mitochondrial membranes can displace the DNA from its carrier. Although studies using DQAsomes are in their infancy, if proven efficient and not toxic, their use as a mitochondrial transfection vector may have potential.

REGULATION OF GENE EXPRESSION

Regulation of nuclear-expressed mitochondrial genes, probably the last worry in developing a gene therapy for mitochondriopathies, may not be a problem after all. As dynamic as the two genomes of the mitochondria and nucleus are to work in a concerted effort to produce the majority of the cell's energy supply, they may internally regulate themselves depending on expression of the transgene. Transfection studies of PDH, using the cytomegalovirus or Rous sarcoma virus promoters, failed to complement enzyme activity completely (4). This is believed to be caused by the strength of the promoter, because transfection with the stronger cytomegalovirus, chicken β-actin hybrid promoter, achieved 100% complementation (37). Similarly, if mutant proteins are part of a complex, such as PDH E1α, the wild-type transgene must compete with the endogenous mutant form (so long as the ability remains intact) (6). Overexpression of the gene PDH, even stimulating transcription of other genes in the PDC, will be regulated by the cell's ability to phosphorylate and dephosphorylate the complex to activate/inactivate the complex. Overexpression seems to be impossible for PDH.

Studies suggest that oxidative phosphorylation genes are regulated by energy demand of the cell (17). Experiments done in cells from patients with mitochondrial myopathies had increased levels of mtDNA transcripts and increased nuclear oxidative phosphorylation gene transcripts. Similar changes were also observed in ADP/ATP nucleotide transporter knockout mice (D.C. Wallace, personal communication). In fact, *in vitro* expression of ri-

bozymes to PDH E1α have not been able to effectively decrease mRNA transcript levels of PDH (personal observations). Collectively, these observations suggest that tissues naturally attempt to compensate for oxidative phosphorylation defects associated with either mtDNA or nDNA mutations. Similarly, the expectation would be that expression of a transgene (if accomplished) would be regulated systemically to generate ATP levels adequate for cell survival.

CONCLUSION

Gene therapy is still relatively new to clinical medicine. The unexpected problems in clinical trials and the inadequacy of animal models, combined with the problems discussed here, make it unlikely that clinical trials for mutations in mtDNA will soon be forthcoming. Unless educated risks are taken, however, it is unlikely that progress toward a definitive therapy will be made. Allotypic expression of proteins that are normally mitochondrially encoded holds great promise, because other species have evolved to use the nuclear encoded expression of some of the mammalian mtDNA proteins.

ACKNOWLEDGMENTS

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ABBREVIATIONS

AAV, adeno-associated virus; Ad, adenovirus; COX, cytochrome *c* oxidase; GFP, green fluorescent protein; MERRF, myoclonic epilepsy and ragged red fibers; MSF, mitochondrial import stimulating factor; MSK, mitochondrial lysyl-tRNA synthetase; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; OTC, ornithine transcarbamylase; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase (E1α); PNA, peptide nucleic acid; rAAV, recombinant adeno-associated virus; RNase MRP, mitochondrial RNA processing enzyme; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

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