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Expert Review

Gene Therapy of the Other Genome: The Challenges of Treating Mitochondrial DNA Defects

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Abstract. Human mitochondrial DNA is a 16.5 kb circular DNA molecule located inside the mitochondrial matrix. Although accounting for only about 1% of total cellular DNA, defects in mitochondrial DNA have been found to have major effects on human health. A single mtDNA mutation may cause a bewildering variety of clinical symptoms mainly involving the neuromuscular system at any age of onset. Despite significant advances in the understanding of mitochondrial DNA defects at a molecular level, the clinical diagnosis of mtDNA diseases remains a significant challenge and effective therapies for such diseases are as yet unavailable. In contrast to gene therapy for chromosomal DNA defects, mitochondrial gene therapy is a field that is still in its infancy and attempts towards gene therapy of the mitochondrial genome are rare. In this review we outline what we believe are the unique challenges associated with the correction of mtDNA mutations and summarize current approaches to gene therapy for the "other genome".

KEY WORDS: delocalized cations; DQAsomes; gene therapy; liposomes; mitochondria; mitochondrial DNA delivery; mitochondrial targeting; nonviral vectors.

INTRODUCTION

The mitochondrion is a fascinating mammalian cell organelle that plays a critical role in the life cycle of the cell. It is composed of hundreds of different proteins that are encoded on two separate genomes. The majority of mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol, directed to the mitochondria by specific targeting sequences and then inserted into their intended compartment in the mitochondrion by a complex import machinery that is largely conserved across species (1,2). Additionally, just a few but nonetheless important proteins are encoded on mitochondrial DNA (mtDNA) and expressed exclusively in the mitochondrial matrix. Long recognized as the site of ATP production in the animal cell these organelles are also key players in the apoptosis pathway for programmed cell death in addition to being involved in thermogenesis and calcium homeostasis. The more recent discovery of mitochondrial DNA associated diseases and the proposed role of mtDNA mutations in aging have led to renewed interest in these organelles and the genome they carry. The use of mitochondrial DNA as a marker for matrilineal lineage based studies of the global dynamics of human population has also raised awareness of mitochondrial DNA outside the field of biology. It is therefore not surprising that the mitochondrion in health and more so in dysfunction has been the focus of much research. From a clinical stand point the treatment of mitochondrial dysfunction poses a daunting challenge. Mitochondrial dysfunction may be due to mutations/deletions in either the nuclear genes encoding mitochondrial proteins or due to mutations/deletions in mtDNA. The treatment of mitochondrial dysfunction due to nuclear gene defects is approachable by conventional paradigms applicable to most nuclear gene defects but further discussion of such approaches is beyond the scope of this review. This review is limited to a discussion of the specific challenges associated with gene therapy for diseases caused solely by defects in the mitochondrial genome.

THE MITOCHONDRIAL GENOME AND THE COMPLEX GENETICS OF MITOCHONDRIAL DNA MUTATIONS

The mitochondrial genome is a circular molecule with a size and copy number dependent on species and tissue type (3,4). Human mitochondrial DNA is a circular molecule, 16,569 bp in size and devoid of introns, that codes for 13 polypeptides, 22 tRNAs and 2 rRNAs. The 13 polypeptides are all subunits of the oxidative phosphorylation (OXPHOS) enzyme complexes. Seven polypeptides are subunits of complex I, one is part of complex III, three are subunits of complex IV and two are part of ATP synthase (5). Unlike nuclear genes that are diploid in all somatic cells (with the exception of genes on the x and y chromosomes of males), mitochondrial genes are polyploid There are 2–10 copies of mitochondrial DNA per mitochondrion with often several hundred mitochondria per cell (3,4). The high incidence of

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mutations in mtDNA is believed to be largely due to the sensitivity of mtDNA to oxidative damage by reactive oxygen species (ROS) formed in the matrix as a byproduct of oxidative phosphorylation. A poorly developed and inaccurate DNA repair machinery has often been cited as the reason for the subsequent accumulation of mtDNA mutations (5). However, it has been shown recently that in addition to recombination (6,7), mammalian mitochondria also seem to possess other types of DNA repair activity similar to nuclear DNA repair systems (8). Mutations in mitochondrial genes on one or more of these multiple copies of mitochondrial DNA lead to the coexistence of mutated copies and wild type copies in a condition known as heteroplasmy (9). When the percentage of mutant mitochondrial DNA reaches a certain level the cell shifts from a normal phenotype to the diseased phenotype. This phenomenon is called the threshold effect (10,11). It is not hard to imagine the complexity in the genetics of diseases caused by such a heteroplasmic distribution of mutated genes within an individual mitochondrion as well the mitochondrial population of a cell. Therefore, to say that mitochondrial genetics is nonmendelian is in our opinion a gross understatement. In addition to heteroplasmy and the threshold effect, maternal inheritance and stochastic segregation further differentiate mitochondrial genetics from nuclear genetics and add to the complexity of mtDNA diseases (12,13).

THE COMPLEXITY OF MITOCHONDRIAL DNA DISEASES

The existence of mitochondrial DNA diseases was revealed for the first time in 1988 with the publication of two papers, one in Science and the other in Nature. The first described an association between a mitochondrial missense mutation and maternally transmitted Leber's hereditary optic neuropathy (14). The second showed the presence of mitochondrial DNA deletions in patients with spontaneous mitochondrial encephalomyopathies (15). Since 1988, the number of diseases found to be associated with defects of the mitochondrial genome has grown significantly (12,16,17). Metabolically active tissues poorly tolerate a decrease in cellular respiration and currently there are several tissue-specific syndromes associated with mtDNA defects. These tissue systems include in decreasing order of vulnerability; the brain, skeletal muscle, heart, kidney and liver (18). Therefore, "typical" groups of mtDNA diseases are neuromuscular diseases and neurodegenerative diseases and prominent clinical signs often involve the visual system. Ptosis, restriction of eye movement, optic atrophy, pigmentary retinopathy, sudden or subacute visual loss, and hemianopia are particularly noteworthy (18). Unfortunately such symptoms are also seen in the case of diseases that are not linked with mtDNA and hence clinical profiles remain difficult to identify (13,19). There is still insufficient data on the correlation of particular mtDNA mutations and the set of symptoms that are clinically recorded. In other words the presence of a particular mutation in two different patients does not necessarily result in both the patients displaying the same symptoms. The percentage of mutated DNA as well as the presence or absence of additional mutations and the particular subtype of mitochondrial DNA all influence the manifestation of clinical symptoms. Thus in the same way that mitochondrial genetics is far removed from established paradigms so too is the clinical diagnosis of mitochondrial DNA diseases. The situation is further compounded by the lack of detailed clinical epidemiology and a suitable classification system for such diseases (13). This has far reaching consequences not only for patient care but also for the allocation of research funding needed to combat these diseases (13).

THE COMPLEXITY OF STRATEGIES APPLICABLE TO THE TREATMENT OF MITOCHONDRIAL DNA DEFECTS

Any mutation in mtDNA ultimately affects the normal functioning of the respiratory chain and leads to defects in the final common pathway of oxidative metabolism. Therefore, the correction of these disorders by the administration of alternative metabolic carriers of energy does not appear feasible (20). A gene therapeutic approach would seem to be the most appropriate alternative. Given the complex role that mitochondrial DNA mutations play in the manifestation of mitochondrial disease, the approaches to treat these conditions are quite diverse. In addition to introducing wild type genes to compensate for the mutations, strategies can be designed to repair mutations in the existing mtDNA or prevent the mutated mtDNA copies from replicating. For a permanent cure of mtDNA diseases, the balance between mutated and healthy mtDNA needs to be shifted below the threshold level required for the phenotypic expression of the disease with the ultimate goal to completely eradicate the mutated portion of the mitochondrial genome. In our opinion, this might only be achievable by the combination of two approaches, i.e., by combining an "antigenomic" strategy (21–24) aimed at the selective inhibition of mutated mtDNA with a "genomic" approach aimed at the replenishment of mitochondria with healthy mtDNA. Depending on the proportion between mutated and healthy mtDNA circles, an antigenomic approach alone might be sufficient to give healthy mtDNA the necessary replicative advantage leading to the complete eradication of mutated DNA. All currently available strategies as summarized in Table I can be separated based on the intended subcellular disposition of the therapeutic nucleic acid.

Strategies Based on the Delivery of DNA into the Nucleus

Due to the widely advanced development of nucleus targeting viral and non viral vectors (25–28), it is not surprising that many efforts to correct mitochondrial DNA defects have relied on the delivery of the therapeutic DNA to the nucleus. An obvious strategy to correct mitochondrial DNA mutations would be to deliver a construct of the wild type mitochondrial gene fused to a sequence encoding a mitochondrial targeting sequence into the nucleus, followed by nuclear cytosolic expression and subsequent import of the gene product into the mitochondria. This "indirect" approach to mitochondrial gene therapy is known as allotopic expression (29,30). The feasibility of allotopically expressing mtDNA encoded proteins was demonstrated for the first time in 1988 using an yeast model (31). More recently though in 2002 the feasibility of applying such an approach to mammalian cells was reported (32). A T > G transversion

Table I. Summary of Current Approaches Directed at the Manipulation of the Mitochondrial Genome

Therapeutic Molecule	Subcellular Destination	Delivery System Used	Intended Expression Product	Long-term Goal	Expected Impact on Heteroplasmy	Comments
Gene construct	Nucleus	Viral transfection vector	Mitochondrial protein (allotopic expression)	Gene replacement	No	Proof of principle demonstrated in isolated organelles, in cell culture and *in vivo* (31–39). Applicable only to proteins that can be successfully imported from cytosol. Achieving stable long-term expression and appropriate mitochondrial regulation of expression might pose a challenge
Gene construct	Nucleus	Viral transfection vector	Mitochondrially targeted nuclease	Degradation of mutated mtDNA	Yes	Proof of principle established in cell culture (45,46). Applicable only to limited mutations. If found necessary, the achievement of stable long-term expression will be challenging
Gene construct	Nucleus	Viral transfection vector	Mitochondrially targeted repair enzyme	Repair of mutated mtDNA	Yes	Proof of principle demonstrated in cell culture (47–51). The achievement of stable long term expression and suitable control of gene expression will be challenging
Gene construct	Nucleus	Viral transfection vector	Mitochondrially targeted tRNA (allotopic expression)	Gene replacement	No	Proof of principle established in cell culture. Only applicable to correction of tRNA mutations (40–44). The need to cotransfect yeast import factors is an added complexity that may further complicate strategies needed to achieve stable expression and regulation
Gene construct	Mitochondrion	DQAsomes	Mitochondrial protein	Gene replacement	Yes	Proof of principle demonstrated in isolated organelles and in cell culture (74,88–92). DNA functionality yet to be demonstrated. Further development and optimization depends on the availability of suitable reporter gene systems to show functionality
Oligonucleotide	Mitochondrion	PEI, liposomes, DQAsomes	N/A	Gene silencing	Yes	Proof of principle demonstrated in isolated organelles and in cell culture (63–66, 68–71, 74). Functionality yet to be demonstrated
Peptide nucleic acid	Mitochondrion	Conjugated TPP	N/A	Inhibition of replication of mutated mtDNA	Yes	Proof of principle demonstrated in an organelle free system (67)
PNA-alkylating agent conjugates	Mitochondrion	None	N/A	Inhibition of replication of mutated mtDNA	Yes	Proof of principle demonstrated in an organelle free system (72). As yet inactive in intact mitochondria

at nt 8993 in mtDNA that encodes ATPase 6 is known to cause impaired mitochondrial ATP synthesis in two related mitochondrial disorders: neuropathy, ataxia and retinitis pigmentosa and maternally inherited Leigh syndrome. Manfredi *et al*. designed a construct encoding an amino-terminal mitochondrial targeting signal appended to a wild type ATPase 6 gene recoded to be compatible with the universal genetic code. The construct was then transfected into human cells, where the allotopically expressed wild-type ATPase 6 protein was imported into and processed within mitochondria followed by incorporation into complex V. Allotopic expression of stably transfected constructs in cytoplasmic hybrids (cybrids) homoplasmic with respect to the 8993T->G mutation resulted in significantly improved recovery of these cybrids after growth in selective medium as well as a significant increase in ATP synthesis (32). The ATPase 6 gene has also been incorporated into chromosome 1 in the nucleus of Chinese hamster ovary cells suggesting the feasibility of long-term expression of such transferred genes (33). The allotopic approach has also been applied to the rescue of cybrids with the mtDNA point mutation (G11778A in the gene encoding the ND4 subunit of Complex I) that is responsible for Lebers hereditary optic neuropathy (34).

In addition to the need to recode the wild type mitochondrial gene sequence for efficient translation in the cytosol, it is debated that the allotopic approach might be limited to only those proteins that are amphiphilic enough to permit uptake into the matrix via the protein import machinery. This problem may be overcome by using an approach that cannot strictly be classified as allotopic. This revolutionary approach involves substituting proteins with those from other species and was made feasible after the discovery that the internal rotenone-insensitive NADH-quinone oxidoreductase (Ndi1) of *Saccharomyces cerevisiae*, a single polypeptide enzyme, is able to carry out the electron transport function of the mitochondrial proton-translocating NADH-quinone oxidoreductase (complex I), which consists of at least 43 different subunits. This yeast gene can be stably transfected into human cells and results in the functional expression of a protein that is able to restore normal NADH dehydrogenase function in human cells that lack the essential mitochondrial DNA (mtDNA)-encoded subunit ND4 (35–37). Further, these researchers have been able to show the applicability of this technique in an *in vivo* model (38). More recently this technique was reported to be feasible for the putative restoration of complex III function too (39). The cyanide-insensitive alternative oxidase from Ciona intestinalis, was successfully expressed in cultured human cells where it was shown to be localized to the mitochondria and take part in electron transport.

The approach to allotopic therapy differs slightly based on whether the therapeutic gene codes for RNA or protein. While mammalian mitochondria are capable of protein import there is no natural mechanism for import of tRNA or rRNA from the cytosol. However, during the end of the 1990s, a natural tRNA import pathway was identified in yeast and was subsequently adapted to drive the import of tRNAs from the cytosol into the mitochondria of mammalian cells (40–42). Cytoplasmic tRNAs modified with the correct amino acylation identity can be targeted to the mitochondrial matrix, where they participate in mitochondrial translation. Human mitochondria, which do not normally import tRNAs, are able to internalize yeast tRNA derivatives *in vitro* in the presence of an essential yeast import factor (40) and synthetic tRNA molecules engineered with the appropriate yeast aminoacylation identity (43). In the most recent development nuclear DNA-encoded tRNAs targeted into mitochondria were shown to rescue a mitochondrial DNA mutation associated with the MERRF syndrome in cultured human cells (44). There still does not exist a strategy for importing rRNA into mammalian mitochondria but this may be of little therapeutic significance given that most of the known mtDNA mutations affect tRNAs. Further it might be interesting to note that successful allotopic expression of all 13 mtDNA-encoded polypeptides will render mtDNA-encoded RNA redundant.

Gene therapeutic strategies for mitochondrial disease have not been limited to the delivery of therapeutic mitochondrial genes but have also involved the delivery of constructs designed to express enzymes capable of acting on mitochondrial DNA. For example, restriction endonucleases specific for sites found only in the mutant forms of mtDNA have been proposed as a way of selectively degrading mutant mtDNA (45). In patients with neuropathy, ataxia and retinitis pigmentosa (NARP), the T8399G mutation creates a unique *PstI* restriction site that is not present in wild-type human mitochondrial DNA. A DNA construct engineered with a COX subunit VII mitochondrial leader sequence upstream of the *PstI* gene and expressed in cultured human cells resulted in the degradation of mtDNA harboring *PstI* sites. The targeted enzyme was able to achieve a selective accumulation of wild type mtDNA when expressed in a heteroplasmic rodent cell line containing one mtDNA haplotype with two sites for *PstI* and another haplotype having none (45). Similarly, Leigh's disease is caused by the mt T8993G mutation that results in a unique restriction site for the enzyme *SmaI*. When transiently expressed as a fusion with a mitochondrial targeting sequence in cybrids carrying the mutant mtDNA, mitochondria targeted by the *SmaI* enzyme showed specific elimination of the mutant mtDNA (46). A repopulation by the wild-type mtDNA was observed which led to the restoration of both the normal intracellular ATP level and normal mitochondrial membrane potential (46). While such strategies are limited to mutations that generate unique restriction sites they might potentially be employed for the effective treatment of at least some mitochondrial disorders.

Another fascinating strategy for preventing the accumulation of mtDNA mutations would be to increase the DNA repair capacity in mitochondria, i.e., to support mitochondria in maintaining the heteroplasmic balance in favor of healthy mtDNA. 8-oxoguanine DNA glycosylase/apurinic lyase (OGG1) is known to be involved in repair of oxidative damage to mtDNA (47). A tetracycline-regulated vector containing the gene for OGG1 downstream of a manganese-superoxide dismutase mitochondrial localization sequence was transfected into HeLa cells and resulted in an eightfold increase in the amount of OGG1 protein in mitochondria. Functional studies showed that cells containing recombinant OGG1 were more proficient at repairing oxidative damage in their mtDNA, and this increased repair led to increased cellular survival following oxidative stress (48). In a separate

study human OGG1 was also shown to have a protective effect against oxidative DNA damage in oligodendrocytes (49). Mitochondrial overexpression of the enzyme O(6)-Methylguanine-DNA methyltransferase (MGMT) that prevents certain types of alkylation damage was shown to increase cell survival after treatment with DNA alkylating agents (50,51). Such DNA repair strategies could potentially be combined with allotopic expression systems to maintain the heteroplasmic balance in favor of wild type mtDNA. Considering the possible role of mtDNA mutations in the aging process (52) one might also be tempted to speculate on the far reaching consequences of such a "genome-maintaining strategy".

Strategies Based on the Delivery of Nucleic Acids into Mitochondria

Assuming the allotopic expression of all 13 polypeptides is eventually made possible, or that suitable replacement proteins are discovered in other species, there remains the need to have the exogenous therapeutic genes placed under the control of a proper regulatory mechanism required for normal functioning. A hypothesis termed colocation for redox regulation (CORR) is one of several theories that seek to provide a reason for the evolutionary preservation of organelle genomes (53). CORR proposes that the colocation of mtDNA and its products is essential for the rapid control of gene expression by the redox state in the mitochondrial matrix. Redox control of synthesis de novo is suggested as the common property of mtDNA encoded proteins thus leading to the possibility that it is biologically essential that these proteins be synthesized at the site of their action; i.e., in the mitochondrial matrix (53). Under the presumptions of CORR, it is imaginable that once a mitochondrial gene is taken out of its natural environment in the mitochondrial matrix and transferred to the nucleus, the redox state that is responsible for its regulation in the matrix is no longer able to exert its effect. Considering that in addition to long-term expression a gene therapeutic strategy needs to preserve the appropriate regulation of the transferred genes to effect a permanent cure, if CORR is true, allotopic expression may not be feasible as a permanent cure for mtDNA defects. Gene therapy designed to introduce a therapeutic mitochondrial gene into its natural location in the matrix, might turn out to be the only approach to achieve a permanent cure of mtDNA diseases. This strategy has been termed "direct" mitochondrial gene therapy (54,55). It might also be possible to introduce entire copies of wild-type mtDNA (isolated from an individual's healthy tissues), into mitochondria of (the same patient's) affected tissues. The procedure can figuratively be compared with replacing a mutated chromosome from the nucleus with the wild-type counterpart. Given the structural complexity and size of the nuclear genome, such a "therapeutic chromosome exchange" is beyond reach of current biomedical means. In contrast, however, to introduce a 16.5 kb DNA circle free of any histone-like proteins into cells and into mitochondria is in our opinion an objective realistic enough to be worth pursuing. Additionally, the smaller size and more primitive organization of the mitochondrial genome and associated expression machinery might make it easier to achieve reliable long-term expression from therapeutic plasmid like constructs. However for the present the major hurdle remains the delivery of DNA into mitochondria. What follows is a brief overview of current strategies to achieve the delivery of exogenous DNA into mammalian mitochondria.

Several physical methods of introducing DNA into mitochondria have been described. Electroporation is routinely employed to introduce DNA up to several kilo base pairs in size into isolated yeast mitochondria and has been used to transform mammalian mitochondria as well (56,57). Biolistic bombardment has achieved success in isolated yeast mitochondria, living yeast cells and most recently in algae (58–60). In mammalian systems, physical methods like electroporation and biolistic bombardment are as yet restricted to the use of isolated mitochondria thereby making their use in clinical treatment dependent on some sort of intracellular mitochondrial delivery strategy. Such an approach may not seem far fetched in the light of a report by Clark and Shay that mammalian cells endocytose isolated mitochondria (61). A more recent and highly interesting observation has been that mammalian cells seem capable of direct mitochondrial transfer (62).

The demonstration that the conjugation of a mitochondrial leader sequence (MLS) peptide could direct the import of 17 and 322 bp DNA sequences into the matrix of isolated mitochondria provided a completely new way to introduce exogenous DNA into isolated, energized, mammalian mitochondria (63,64). This method, which essentially involves hitchhiking the mitochondrial import pathway was used in the first demonstrations of oligonucleotide delivery to mitochondria of living cells (65,66). First, Geromel *et al.* covalently conjugated the MLS peptide of the matrix protein ornithine transcarbamylase (OTC) to a 22-mer fluorescein labeled oligonucleotide (66). Cationic liposomes prepared from trimethyl aminoethane carbamoyl cholesterol iodide (TMAEC-Chol) were used as a carrier to deliver the peptide-DNA conjugate into cultured skin fibroblasts where upon the conjugates displayed a sub cellular staining pattern that suggested association with mitochondria. Next, in a more comprehensive study, Flierl *et al.* not only demonstrated association with mitochondria but also the localization of cell-internalized oligonucleotides inside the mitochondrial matrix (65). The ability to deliver such short oligonucleotides has opened the door to the design of oligonucleotide based antisense approaches for mitochondrial gene therapy. Antisense therapy could also be achieved by the use of DNA analogs like peptide nucleic acids (PNAs) (21). For the delivery of the PNAs into mitochondria of living cells the PNAs were conjugated with triphenylphosphonium (TPP) cations (67). Although the PNA-TPP conjugates appeared to be imported into the mitochondrial matrix in living cells, alterations in the level of heteroplasmy could not be demonstrated when using cells heteroplasmic for the A8344G MERRF (Myoclonus epilepsy and ragged red fibre syndrome) mutation and the corresponding TPP-PNA-MERRF (67). Chimeric RNA/DNA oligonucleotides (ONs) and short single-stranded (SS) ONs used to direct site-specific single-nucleotide changes in chromosomal DNA of eukaryotic cells are also being explored for use in correcting point mutations of mtDNA (68–71). Alternatively nucleic acid analogs like PNAs could be used for selectively directing DNA alkylating reagents to the site of mutated mtDNA circles (72). However, this approach failed to

work with isolated mitochondria as well as mitochondria in cells. As a possible reason for this failure, the limited accessibility of mtDNA to some alkylating reagents has been discussed (72). Nevertheless, taken together, the aforementioned results strongly support the applicability of short nucleic acid analogs in the gene therapeutic treatment of the subset of mitochondrial disorders that are caused by point mutations in mtDNA.

One of the biggest challenges in mitochondrial gene therapy is the mitochondria-specific delivery of large DNA constructs capable of the expression of a therapeutic gene within mammalian mitochondria. While plant mitochondria are known to have DNA import capability (73), until recently there was still doubt as to the feasibility of achieving the import of plasmid sized DNA into mammalian mitochondria. However, two recent papers have described promising results in the quest to deliver large gene constructs into mammalian mitochondria. First it was shown that the combination of a mitochondria-specific delivery system and a conjugated MLS sequence localized to a single 100-bp site on a 5.4 kb plasmid could direct the specific association of the plasmid with mitochondria in live mammalian cells (74). This is to our knowledge the first demonstration of its kind. A more recent paper described the existence of a natural competence in mammalian mitochondria. Isolated mammalian mitochondria were shown to be capable of importing naked linear DNA which then resulted in both template driven DNA synthesis and RNA transcription of the construct (75). This study was performed only with isolated mammalian mitochondria but if the proposed mitochondrial competence can be utilized in live cells this will provide a valuable alternative to the use of MLS sequences to drive the mitochondrial import of therapeutic DNA constructs.

In what would appear to be a slightly more ambitious approach to the correction of mtDNA defects, healthy mitochondria isolated from the patient's own cells could be used to repopulate cells in diseased tissue. This direct mitochondrial transfer can be achieved by physical means such as direct microinjection (76) or by so called biological methods like ovum transplantation and cytoplast fusion and would result in the complete replacement of mutated mtDNA (77,78). This technique is being increasingly used to generate cellular models of mtDNA deficiencies and for the study of mitochondrial function. An even more novel method based on the use of *Escherichia coli* bacteria has recently been proposed for the transfer of DNA into isolated mammalian mitochondria (79). Using a suitable *E. coli* strain transformed with a plasmid construct carrying an origin of DNA transfer sequence, the authors transferred the plasmid DNA into mitochondria by conjugation between *E. coli* and mitochondria (79). The mitochondria had been engineered to contain T7 RNA polymerase and robust levels of T7 transcription were detectable from the transferred T7 plasmid (79).

THE NEED FOR MITOCHONDRIAL GENE DELIVERY SYSTEMS

The largest DNA molecule introduced by electroporation into isolated mitochondria has been in the region of 7.2 to 16.5 kbp (56,80). The largest DNA molecule introduced into isolated mitochondria using a conjugated leader peptide sequence has been 322 base pairs in size (63). Short oligonucleotides (18–22 bp) coupled to mitochondrial leader sequences have been introduced into mitochondria of living mammalian cells (65,66). Most recently the feasibility of achieving the leader peptide mediated mitochondrial import of plasmid DNA in living mammalian cells has been demonstrated (74). With the exception of our DQAsome mediated delivery studies other successful delivery of DNA to mitochondria has been achieved with the use of TMAEC-Chol cationic liposomes (66) and cationic polyethylenimine (65); i.e., systems not designed in any specific way to target mitochondria. In the case of using TMAEC-Chol cationic liposomes for the oligonucleotide-MLS peptide delivery, the fluorescence of labeled oligonucleotide-MLS conjugates became associated with mitochondria (66), but oligonucleotide internalization into the mitochondrial matrix was not established (65). In our studies we observed that the same conjugate used by Geromel *et al.* showed no significant mitochondrial localization when delivered intracellularly by Lipofectin (74). Our data correspond with those of Flierl *et al.* who reported that attempts to utilize cationic liposomes for the delivery of MLS-oligonucleotide complexes into mitochondria of living myoblasts failed with three different commercially available cationic liposome formulations (65). It was found instead that using branched chain polyethylenimine (PEI) was more successful (65). Interestingly, it was found that only mitochondria in close proximity to the fluorescently labeled MLSoligonucleotide aggregates rapidly developed high levels of fluorescence (65). Such observations might be interpreted to suggest that the successful targeting of the nucleic acids to mitochondria had more to do with the targeting effect conferred by the MLS rather than any specific targeting on the part of the delivery system (liposome or polycation). Even if the presence of MLS peptides at oligonucleotides actively contributes to a "targeting effect" and subsequently mediates the transport of the complex across the mitochondrial membranes, the question arises whether conjugated MLS peptide alone would be sufficient to "drag" the larger plasmid DNA molecule to mitochondria. We therefore believe that a mitochondria-specific delivery system is essential for efficient delivery of plasmid DNA to mitochondria.

Strategies for nuclear gene therapy benefit from the availability of a wide variety of gene delivery systems that are broadly classified into viral or nonviral transfection systems. Viral systems are still the most efficient gene delivery vectors for nuclear gene therapy and are currently in clinical trials. There does not however seem to be much interest in the application of viral systems to mitochondrial gene therapy. The viral life cycle is well known to be closely associated with mitochondrial function (81) and it is also known that certain viral particles display a marked mitochondrial localization (82,83). We believe that it is not unreasonable to speculate on the eventual availability of a viral gene delivery vector for transfection of mammalian mitochondria. There has been a claim of the development of such a technique but published experimental data to support this claim are yet unavailable (84). Nevertheless, the successful development of such a method to transform mammalian mitochondria will represent a significant step towards effective treatments for mitochondrial DNA disorders.

In contrast to nuclear therapy, non viral gene delivery systems have had a head start in mitochondrial gene therapy (54,85,86). Much of our research has been focused on the development of novel mitochondria-specific non viral gene delivery vectors. We have so far designed two nonviral mitochondria specific delivery systems and are actively developing others (54,87). The first and best characterized system is based on the use of vesicles prepared from mitochondriotropic quinolinium compounds. These vesicles called DQAsomes (pronounced dequa somes) have been developed for the exclusive purpose of transporting DNA specifically to the immediate vicinity of the mitochondria of a live cell (54,88,89). Our strategy involves using a mitochondria specific delivery system to deliver a MLS peptide-DNA conjugate across the plasma membrane, through the cytosol and to the immediate site of the mitochondria of a living cell. The mitochondrial leader sequence peptide conjugated to the DNA will then permit uptake of the delivered DNA into the mitochondria via the mitochondrial protein import machinery. In a series of publications we have shown that DQAsomes fulfill all prerequisites for a mitochondriotropic DNA delivery system. They bind and protect DNA from nuclease digestion, are potentially endosomolytic and release their DNA cargo at the site of mitochondria inside living mammalian cells (90,91). In our most recent study we have shown that the use of DQAsomes to deliver a fluorescent oligonucleotide conjugated to a MLS peptide results in an association of the fluorescence with mitochondria of the cell in a manner that strongly suggests successful internalization of the oligonucleotide into the matrix (74). DQAsomes were also shown to be more efficient at delivering DNA to mitochondria than Lipofectin (a representative nonviral vector designed for gene delivery to the nucleus)(74). Most importantly, we have shown that DQAsomes can mediate the mitochondrial association of plasmid DNA conjugated to an MLS sequence (74).

Given the relative ease with which liposome formulations can be modified with various targeting ligands, mitochondriotropic moieties synthesized with suitable linker motifs could well be used to produce mitochondriotropic liposomes. As a first step in this direction we have described liposomes prepared with a novel lipid consisting of a mitochondriotropic triphenyl phosphonium cation conjugated to a stearyl anchor (87). These liposomes have been shown to be mitochondria specific in their subcellular distribution and preliminary data show that these liposomes can be formulated to bind DNA and hence putatively serve as mitochondria-specific DNA delivery systems (Boddapati, Weissig, to be published elsewhere). Using similar approaches we postulate that almost any nanoparticulate carrier can be suitably modified to produce a mitochondriotropic delivery system. Figure 1 is a schematic representation of approaches to

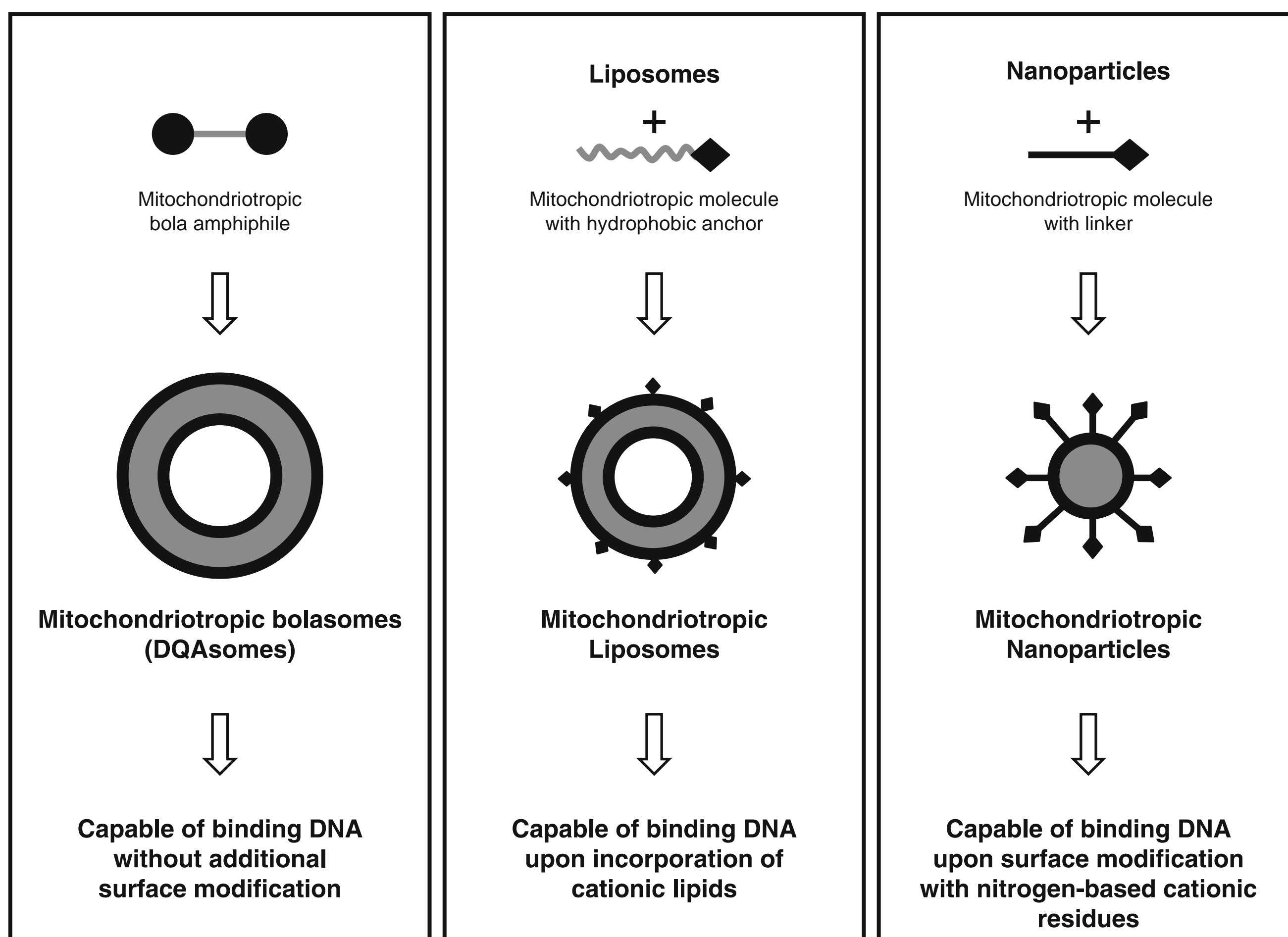

Fig. 1. Schematic approaches for the development of mitochondria-specific nonviral DNA delivery systems.

designing mitochondriotropic carrier systems with currently available technology. However since we have so far only been able to use fluorescently labeled DNA that is unable to translate a detectable product, further development or optimization of mitochondriotropic delivery systems would be futile without the facility to demonstrate and measure expression of the delivered DNA.

THE NEED FOR MITOCHONDRIAL REPORTER GENES AND PHYSIOLOGICAL MODELS OF MITOCHONDRIAL DISEASE

Evaluating the effectiveness of mitochondria-specific systems in delivering DNA into mitochondria depends largely on physical tracking of DNA. Direct labeling of DNA with contrast agents for visualization either by fluorescence or electron microscopy is an indirect way of showing mitochondrial targeting of the gene (65,66,92). In addition to using commercially available fluorescence labeling kits, we have employed a novel protocol for selectively staining free pDNA in cultured cells (92). These techniques serve to demonstrate the physical location of the DNA but do not prove that the DNA has reached its site in a transcriptionally active state. Additionally while protein expression from even a single copy of successfully delivered construct is detectable, physical labeling techniques are much less sensitive in demonstrating the location of delivered DNA. While any new nonviral transfection system (i.e., cationic lipids, polymers and others) aimed at the nuclear-cytosolic expression of proteins can be systematically tested and subsequently improved by utilizing anyone of many commercially available reporter gene systems (reviewed in 93) such a methodical approach cannot currently be applied to the development of mitochondrial gene delivery systems.

Recently the green fluorescent protein (GFP) gene recoded for expression in yeast mitochondria has been shown to result in functional expression of the protein when inserted into a construct derived from the yeast mitochondrial genome (94). The success of this strategy with yeast strongly suggests that it is not unreasonable to expect that a GFP gene recoded for exclusive expression in mammalian mitochondria and inserted into an equivalent mammalian expression construct will result in functional expression if successfully delivered into mammalian mitochondria. Such a construct should not be expressed by the nuclear-cytosolic transcription and translation system and any detected expression would be indicative of mitochondrial expression. Further data to support the choice of GFP as a reporter gene have been recently described by Lightowlers and coworkers (75). In addition to reporting the existence of a natural competence of mitochondria they report template driven DNA synthesis as well as transcriptional activity from a minimal construct designed for expression in the rat mitochondria. This linear construct comprised a rat non coding region analogous to the D-loop region and a mitochondrial recoded GFP under the control of the rat mitochondrial heavy strand promoter as well as a luciferase gene under the control of the rat mitochondrial light strand promoter. In their experiments they have found that for such a construct in a cell free system the heavy strand promoter displays more robust activity and that the GFP gene probably produces a more stable transcript. While it was already known that the only requirements for mitochondrial transcription are the presence of a D-loop and the supercoiled form of the DNA (95) there remains the need to produce constructs that can be propagated by current bacterial cloning techniques to produce a readily available construct similar to those currently available for nuclear expression. A series of papers by Charles Coutelle's laboratory described a feasible approach for the design of such a construct based on the use of a *Cre recombinase* excisable bacterial vector backbone (96–98), however, so far no mitochondrial reporter system has become commercially available.

An alternative, albeit more complex approach to testing mitochondria specific transfection systems would be the use of knockout models of mitochondrial genes derived from Rho0 cells (99). Rho0 cells are cells devoid of mitochondrial DNA and mammalian Rho0 cell lines were first described in the late eighties (100). Jazayeri *et al.* describe the stable expression of a dominant negative form of mitochondria-specific DNA polymerase-gamma (POLGdn) to reversibly deplete mitochondrial DNA (mtDNA) from human HEK293 cells (99). Long term (20 days) expression of POLGdn completely eliminated mtDNA from the cells, resulting in Rho0 cells which when fused with human platelets, yielded clonal cybrid cell lines that were populated exclusively with donor-derived mtDNA. In addition to the authors proposal to use such cell lines to study mtDNA function regulation and mutation it is possible that such techniques can be used to generate cellular models for evaluating mitochondria-specific DNA delivery systems. Murine models of mitochondrial mutations have also been described (101,102), further improving the feasibility of rescue of phenotype models to evaluate mitochondrial DNA delivery systems. Nevertheless, looking back over the extensive development of nonviral

Table II. Summary of Attempts to Develop Mitochondrial Reporter Constructs

Reporter Gene	Expression System	Cloning System	Construct	Comments
Ornithine Transcarbamylase	Mouse Mitochondria	*Escherichia coli*	Minicircle constructs ranging from 3 to 8 kb	Expression of synthetic human OTC gene demonstrated in *E. coli* (96–98). No results so far with mammalian expression
Green fluorescent protein	Yeast mitochondria	Yeast	Plasmid construct of 5 kb	Protein expression demonstrated in yeast (94)
Green fluorescent protein and luciferase	Rat mitochondria	*E. coli*	Linear construct of 1 kb	Transcription and template driven DNA synthesis demonstrated in isolated mammalian mitochondria (75)

nuclear-targeted transfection systems during the last 15 years, it appears to us that the availability of a reliable reporter gene (that can be used with any cell line and the expression of which can easily be detected via colorimetric or other methods) is an absolute necessity for starting, widening and intensifying efforts towards making mitochondrial gene therapy as feasible as nuclear-cytosolic gene therapy already is. Table II is meant to serve as a summary of studies that might eventually be the basis of the development of a mitochondrial reporter construct.

PERSPECTIVES

Mitochondrial gene therapy is a complex field that is still in its infancy and efforts undertaken during the last decade to correct defects in the mitochondrial genome have faced more complex hurdles and have consequently proven to be more challenging than strategies directed at the nuclear genome. In this review we have tried to describe the unique problems that need to be solved to make mitochondrial gene therapy clinically feasible. In light of the many different approaches towards the treatment of mtDNA diseases and the eventual availability of the requisite research tools and research funding we optimistically believe in the feasibility of gene therapy of the mitochondrial genome.

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