Defects of mitochondrial (mt) DNA cause a diverse group of incurable, progressive diseases that often lead to severe disability and premature death. Most patients with pathogenic mtDNA defects have a mixture of mutant and wild-type mtDNA (heteroplasmy), and the clinical defect is only expressed when the percentage of mutant mtDNA exceeds a critical threshold. Since mtDNA is continually replicating and being turned over, we have proposed an approach to the treatment of these disorders that utilizes sequence-specific antigenomic peptide nucleic acids (PNAs) to hybridize and specifically inhibit the replication of mutant mtDNA under physiological conditions. By allowing the selective propagation of wild-type molecules, it may be possible to correct the cellular biochemical defect and to prevent the progression of disease. This paper summarizes the experimental progress in this area, including the cellular uptake of PNA molecules and their import into mitochondria both in vitro and in cell culture by the addition of a nuclear-encoded mitochondrial targeting sequence. The possibilities of extending this strategy to the treatment of mtDNA deletion disorders are discussed.

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phenotype (Lightowlers et al., 1997). Although this threshold level is dependent upon both the nature of the mutation and the tissue affected, the vast majority of pathological mtDNA mutations investigated are highly recessive and biochemical dysfunction is only apparent with low levels (<10%) of wild-type mtDNA (Boulet et al., 1992; Chomyn et al., 1992; Attardi et al., 1995).

Despite an increased awareness of their clinical importance and considerable progress towards understanding the relationship between pathogenic defect and clinical phenotype, effective biochemical and pharmacological treatments are not available (Taylor et al., 1997a).

**Approaches to treatment**

Notwithstanding problems unfamiliar to conventional somatic gene therapy approaches, a number of experimental strategies are currently being pursued towards achieving a gene therapy for mitochondrial diseases (Chrzanowska-Lightowlers et al., 1995; Taylor et al., 1997a). These include introducing modified genes into mitochondria (Seibel et al., 1995; Collombet et al., 1997) and novel approaches that aim to correct the genetic defect in situ (Clark et al., 1997; Shoubridge et al., 1997). These authors have shown that by inducing focal damage to mature muscle that contains high levels of a pathological tRNA mutation, the satellite cell population that proliferates in response to the insult can contain remarkably low levels of the mutant mtDNA. As such, subsequent analysis of the regenerated mature muscle reveals much lower levels of mutation, consistent with an increase in the number of muscle fibres that have normal cytochrome c oxidase (COX) activity (an enzyme of the mitochondrial respiratory chain that is in part encoded by the mitochondrial genome). A treatment that stimulates the proliferation of satellite cells to regenerate and to form fully functional muscle fibres is potentially of therapeutic importance.

Another possible approach has become evident following recent cell culture studies in which the mitochondrial inhibitor oligomycin (in conjunction with galactose) has been used to select for wild-type cells over those containing the T8993G ATPase subunit 6 mutation. Using cybrid lines containing heteroplasmic levels of this mutation, Manfredi et al. (1999) have demonstrated that these conditions are able to induce a significant shift in the level of mtDNA heteroplasmy in favour of the wild-type genome, suggesting a basis for pharmacological intervention in ATPase gene disorders.

A strategy that we are currently pursuing makes fundamental use of the heteroplasmic nature of the majority of pathogenic mtDNA mutations, and the fact that the mitochondrial genome is continuously replicating and thus being turned over (Gross et al., 1969). Because mutant mtDNA is functionally recessive, the specific inhibition of mutant mtDNA replication by suitable antigenomic agents could theoretically correct the imbalance of the ratio of mutant to wild-type mtDNA that causes the clinical disease. We postulate that if the selective binding of an antigenomic agent was able to sterically inhibit the mitochondrial DNA polymerase $\gamma$-initiated replication of the mutant genome, this might allow the selective propagation of wild-type genomes to the extent that the relative amount of wild-type mtDNA would be sufficient to restore normal biochemical function to the cell and to arrest the progression of disease (Taylor et al., 1997a). This approach depends on the designing of suitable antigenomic molecules, as an opportunity already exists for the binding of sequence-specific agents to mutant mtDNA. Capitalizing on the extensive displacement of the two separate strands of mtDNA that occurs during the replication of the mitochondrial genome (Clayton, 1991), it has been possible
In-vitro manipulation of mtDNA heteroplasmy to design an antigenomic molecule that binds selectively to a mutant mtDNA strand.

**Antigenomic PNA therapy for mtDNA disorders**

We designed peptide nucleic acids (PNAs) complementary to human mtDNA sequences, and assessed their ability to inhibit mtDNA replication in an in-vitro assay. PNAs are polyamide–nucleic acid derivatives in which the deoxyribose phosphate backbone of DNA is replaced by an uncharged N-(2-aminoethyl)-glycine polymer (Nielsen et al., 1991). PNAs bind complementary DNA and RNA strands with higher affinities than an equivalent oligodeoxynucleotide (Egholm et al., 1993). Mutation of a single base within a PNA:DNA duplex can disrupt the thermal stability of the complex by up to 20°C (Ørum et al., 1993). This characteristic is particularly important as it confers upon PNA the ability, at a given temperature, to selectively discriminate between two nucleic acid templates that differ by a single base substitution. Coupled with enhanced stability in serum and cell extracts (Demidov et al., 1994), these properties make PNAs therapeutically attractive for antisense and antigene strategies to modulate gene expression (Hanvey et al., 1992; Knudsen and Nielsen, 1996; Good and Nielsen, 1998).

A PNA was designed to be complementary to a human mtDNA template containing the A8344G tRNA<sup>r</sup> point mutation associated with myoclonus, epilepsy and ragged-red fibre (MERRF) disease, with the base substitution in the middle of the sequence. This PNA is 100% complementary to the mutant sequence, and so would hybridize at physiological temperature. In theory, the mismatched base present in the wild-type mtDNA sequence ought to disrupt the stability of the PNA:DNA binding to such an extent that at physiological temperature it would not hybridize. In an in-vitro mtDNA replication run-off assay, this PNA was shown under physiological conditions to specifically inhibit the replication of the mutant (G8344) but not wild-type (A8344) template (Taylor et al., 1997b). Moreover, the inhibitory effects of this PNA were maintained in the presence of saturating concentrations of E.coli single-stranded binding protein (SSB), a protein analogous to the mitochondrial SSB that binds single-stranded DNA during replication.

**Cellular uptake and targeting of PNA to mitochondria**

Although these in-vitro data clearly indicate the potential of this approach for providing antigenomic gene therapy for mtDNA point mutation disorders, a fundamental prerequisite is good cellular uptake of these molecules and accurate targeting and delivery of PNA into the mitochondrial matrix. Previous reports had suggested that unmodified antisense PNAs were poorly imported across cell membranes, on account of their low phospholipid permeability (Hanvey et al., 1992; Wittung et al., 1995), or were otherwise sequestered away from the nucleus in cytoplasmic endosomes (Bonham et al., 1995). Using fluorescence confocal microscopy to visualize a biotinylated PNA, we have investigated the ability of several cell lines to import PNA into the cytoplasm. These molecules were rapidly taken up by a number of cell lines in a time- and temperature-dependent manner during which the PNA was localized and concentrated in the nucleus (Taylor et al., 1997b; Chinnery et al., 1999). In order to target the PNA to the correct intracellular location, we have utilized the protein import machinery to deliver PNA into mitochondria. Previous studies have shown that the addition of a nuclear-encoded N-terminal mitochondrial leader sequence to a nucleic acid will promote targeting to mitochondria isolated from yeast (Vestweber and Schatz, 1989) or rat liver.
Figure 1. Deleted mitochondrial genomes contain just one of two similar sequences found in the intact genome. The ‘common deletion’ (ΔmtDNA4977) is flanked by a perfect 13 bp direct repeat present in the normal mitochondrial genome (Schon et al., 1989), one copy of which is lost with the missing segment and one copy of which remains in the deleted circle.

Figure 2. Once the deletion has been defined, an antigenomic peptide nucleic acid (PNA) can be constructed that binds co-operatively across one copy of the repeat sequence only when deletion has taken place.

(Seibel et al., 1995). By synthesizing a PNA–peptide conjugate that contains the N-terminal leader sequence of cytochrome c oxidase subunit VIII, we have been able to demonstrate targeting of antigenomic PNA molecules to the matrix compartment within isolated rat liver mitochondria in vitro and cultured human myoblasts in vivo (Chinnery et al., 1999).

Treatment of mtDNA deletion disorders
Although much attention has focused on the growing number of pathological mtDNA tRNA abnormalities associated with disease (Schon et al., 1997), it is large-scale rearrangements that represent the major subset of pathogenic mtDNA mutations, accounting for some 60% of reported mtDNA gene defects (Chinnery and Turnbull, 1997). Gross deletions of mtDNA have been observed at high levels in progressive external ophthalmoplegia (PEO), Kearns–Sayre syndrome (KSS) and Pearson marrow/pancreas syndrome (Holt et al., 1988; Moraes et al., 1989; Rötig et al., 1991). In addition, somatic mtDNA deletions have been shown to accumulate with normal
In-vitro manipulation of mtDNA heteroplasmy

human ageing, particularly in post-mitotic tissues such as brain and skeletal muscle (Cortopassi and Arnheim, 1990; Corral-Debrinski et al., 1992). The level of mutant mtDNA has been shown to clonally expand within individual cells to a level past the threshold required to cause a focal biochemical abnormality (Brierley et al., 1998).

Mitochondrial DNA deletions often occur at the site of perfect repeat sequences of 4–13 nucleotides in length; such repeats that are remarkably common in the mitochondrial genome. One of these repeats is always present in the deleted molecule (Schon et al., 1989; Mita et al., 1990) and, as such, the repeat sequence and either the 3' or 5' flanking sequence will be common to both the wild-type and the deleted molecule (Figure 1). This makes the deleted genome refractory to the antigenomic approach for treatment in which a potential therapeutic molecule is designed to selectively hybridize to the aberrant molecule at physiological temperatures and salt conditions. Although there is no unique sequence in deleted mtDNA that can be targeted to give selective hybridization, we believe that it is possible to exploit the close proximity of the 3' and 5' flanking regions in the deleted genome as a template for selective hybridization (Figure 2). To achieve this goal, it must be established that the 5' and 3' complementary regions of an antigenomic PNA molecule can act co-operatively to increase the binding affinity of such a molecule to a deleted mtDNA template, as compared to either the 3' or 5' regions alone. This would involve bridging the repeat sequence, and experiments are currently being attempted in our laboratory to determine binding co-operativity using both thermal melt spectrophotometry and surface plasmon resonance technology (Jensen et al., 1997).

Conclusions

The unrelenting growth in the field of diagnostic mitochondrial medicine over the past 10 years impels us to consider possible options for treatment. The absence of specific biochemical and pharmacological therapies reinforces the need to develop realistic gene therapies. In the case of mitochondrial disease, it is the important balance between the level of wild-type versus mutant mitochondrial genomes that determines clinical phenotype. As such, a subtle manipulation of mtDNA heteroplasmy has the potential to redress the imbalance and so to reverse the biochemical and clinical abnormality. In view of the in vitro and cell uptake data available, the sequence-specific inhibition of mutant mDNA replication by antigenomic PNAs promises to achieve important goals to this end. We look forward to the next 10 years.

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References


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