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Treatment of Mitochondrial Disease

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Defects of the mitochondrial genome are widely recognized as important causes of disease in man. Patients may present at any age with clinical symptoms that vary from acute episodes of lactic acidosis in infancy to severe neurodegenerative illness in adulthood. While modern molecular genetic techniques have facilitated major advances in the diagnosis and characterization of specific molecular defects, treatment for the majority of patients remains supportive in the absence of definitive biochemical therapies. As a consequence, the possibilities for mitochondrial DNA gene therapy must be considered. In this review, we will evaluate the current biochemical strategies available to clinicians for the management of patients with mitochondrial disease and examine the possible approaches to the gene therapy of mitochondrial DNA defects.

KEY WORDS: Mitochondrial DNA; mutations; gene therapy; DNA replication; peptide nucleic acids; selective hybridization.

INTRODUCTION

Mitochondrial DNA and Mitochondrial Function

Crucial to understanding the difficulties and possibilities of treating patients with mitochondrial respiratory chain disease is a knowledge of key factors in the pathogenesis of these disorders. The major function of mitochondria is to generate energy for cellular processes in the form of ATP by oxidative phosphorylation. This, the final metabolic pathway in oxidative metabolism, is achieved by the five multisubunit protein complexes of the mitochondrial respiratory chain. The first four of these (complexes I–IV) are responsible for the reoxidation of reduced cofactors generated by oxidation of foodstuffs. An electrochemical gradient generated across the inner mitochondrial membrane is then harnessed by the fifth complex, the ATP synthetase, to drive the formation of ATP. While the vast majority of protein subunits comprising the respiratory complexes of oxidative phosphorylation are encoded by the nuclear DNA, some thirteen essential polypeptide subunits (seven of complex I, one of complex III, three of complex IV, and two of complex V) are encoded by mitochondrial DNA (mtDNA). In addition, mtDNA encodes two rRNAs and twenty-two tRNAs (Anderson *et al.*, 1981), the full complement required for intramitochondrial protein synthesis. Therefore, while the nuclear DNA is responsible for encoding the majority of intramitochondrial proteins, both normal mitochondrial and cellular function are wholly dependent upon the concerted action of both genomes. As a consequence, mutations within any of the mtDNA-encoded respiratory chain subunits, mitochondrial rRNAs or tRNAs, would impair respiratory chain function, therefore compromising the amount of available ATP for normal cellular function.

Mitochondrial DNA and Disease

Defects of the mitochondrial genome are important causes of disease in man. They may take

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the form of either point mutations or rearrangements, and are associated with a broad spectrum of clinical manifestations (as recently reviewed by Johns, 1995). The first described human mtDNA mutation was a deletion (Holt *et al.*, 1988), and subsequently the majority of patients with the Kearns–Sayre Syndrome (KSS) or chronic progressive external ophthalmoplegia (CPEO) have been shown to exhibit major rearrangements of mtDNA (Schon *et al.*, 1994). Point mutations within the mitochondrial genome may affect protein-encoding genes, such as those described in Lebers hereditary optic neuropathy (LHON) (Wallace *et al.*, 1988; Howell *et al.*, 1991; Johns *et al.*, 1993) or the T8993G mutation in the ATPase 6 gene associated with neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) (Holt *et al.*, 1990), or more commonly one of the tRNA genes, for example the A3243G mutation associated with mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Goto *et al.*, 1990) or the A8344G mutation associated with myoclonus, epilepsy, and ragged-red fibers (MERRF) (Shoffner *et al.*, 1990); some 30 tRNA mutations have now been described (Wallace, 1995). Irrespective of the genetic defect, patients with mitochondrial disorders may present at any age, and there may be immense variation in both the severity and nature of the clinical presentation associated with a single genetic abnormality. For example, the A3243G $tRNA^{Leu(UUR)}$ mutation, predominantly associated with the neurological syndrome of MELAS, may also manifest clinically as CPEO, myopathy, diabetes, and deafness (Moraes *et al.*, 1993; van den Ouweland *et al.*, 1992). In addition, deletions of mtDNA have been shown to occur in normal individuals, albeit at very low levels (<0.1% of total mtDNA), and the incidence of mutations increases with age. As such, mtDNA mutations have been implicated in the etiology of several common neurodegenerative disorders and in the aging process (Wallace, 1992a, b; Brierley *et al.*, manuscript submitted).

A number of features explain the frequent association of mtDNA with human disease. The mitochondrial genome has a mutation rate some 10-fold greater than that of nuclear DNA, and with no protective histone proteins or effective DNA repair mechanism it is highly vulnerable to nucleolytic attack by free radicals generated by oxidative phosphorylation. On account of mtDNA having no introns and little redundancy, any mutation event in the genome is likely to affect a coding sequence. Furthermore, there are several key factors that must be considered relating to the behavior of pathological mtDNA mutations. First, the mitochondrial genome is maternally inherited (Giles *et al.*, 1980), and is present in multiple copies in individual organelles. Therefore, depending upon the oxidative demand of the tissue in question, there may be up to several thousand genomes within individual cells. Second, in the majority of patients with mtDNA defects, both mutant and wild type genomes are found within the same mitochondria, and consequently the same cell and tissue. This is the phenomenon of heteroplasmy, whereby an otherwise lethal mtDNA mutation may be maintained and subsequently inherited. The proportion of mutant to wild type genomes can vary between tissues and during development, with high levels of mutation often found in postmitotic tissues. Furthermore, the levels of mutant mtDNA often increase with time in these postmitotic tissues (Weber *et al.*, 1997). Third, in the presence of heteroplasmy there is a critical threshold whereby a certain level of mutant mitochondrial genomes is necessary before the disease becomes both biochemically and clinically apparent, the so-called "threshold effect." While this threshold level is dependent upon both the nature of the mutation and the tissue affected, every pathological mtDNA mutation investigated has been shown to be extremely recessive with levels of >85% of mutant mtDNA often required before any biochemical dysfunction is noticeable (Boulet *et al.*, 1992). These observations have been further confirmed by studies of cybrid fusions, in which pathological mtDNA mutations have been transferred into cell lines depleted of mtDNA (rho°) by exposure to ethidium bromide (King and Attardi, 1989). Under such conditions, transformants exhibit the same biochemical abnormality (as measured by respiratory chain activity or *in vitro* mitochondrial protein synthesis) as expressed by the donor cells (Chomyn *et al.*, 1992; King *et al.*, 1992).

TREATMENT OF MITOCHONDRIAL DNA DISORDERS

Current Management of Mitochondrial Disease

Despite major advances in our understanding of mitochondrial disease, treatment options remain severely limited. Pharmacological therapies have been reported to be of some benefit in isolated cases, but for the vast majority of patients, the current emphasis

is primarily supportive. In the absence of effective therapy, it is important that we consider all possible modes of treatment. Ideally, patients with mitochondrial disease should be managed by an integrated team which should include a coordinating physician (usually a neurologist), other specialist medical staff (including a diabetologist and ophthalmologist), who work closely with a specialist nurse, physiotherapist, dietitian, and speech therapist. Social support should be readily available, and patients should be encouraged to interact with other individuals through active patient support groups.

Genetic Advice

After establishing the diagnosis, key issues such as prognostic advice and genetic counselling need to be considered. The extreme phenotypic diversity of mitochondrial disease has made it very difficult to give a realistic prognostic guide to individuals harboring mtDNA defects. However, we have studied 265 cases of mtDNA defects and we have shown that the level of mutant mtDNA in muscle can be used to predict likely complications associated with the more common point mutations (Chinnery *et al.*, 1997).

Until recently, genetic counselling for patients with mitochondrial disease has been limited to information about maternal inheritance, and the male predominance in LHON. It was generally accepted that the majority of mtDNA rearrangements occur by somatic mutation and are not transmitted to the offspring (Moraes *et al.*, 1989). However, although in many cases mtDNA rearrangements cannot be detected in the mother's blood or muscle, mtDNA deletions have been identified in oocytes alone (Chen *et al.*, 1995), and maternally transmitted mtDNA duplications are now well recognized (Brockington *et al.*, 1993). In contrast, although the majority of mtDNA point mutations are inherited down the maternal line, genetic heterogeneity has prevented the identification of a clear inheritance pattern based upon the level of maternal mutant load. We believe that it is possible to predict the outcome based on our study of 265 cases of transmitted mtDNA point mutations (Chinnery *et al.*, manuscript submitted). Using these data it is possible to give broad prognostic guidelines to individuals harboring mtDNA mutations, and also to provide prospective mothers with an estimate of the risks that an affected offspring may inherit the disorder.

Supportive Therapy

A detailed understanding of the potential systemic features is essential for any clinician managing patients with mitochondrial disease. The early detection and appropriate treatment of associated features may significantly improve an individual's quality of life (such as ptosis surgery in CPEO, or intraocular lens replacements in an individual with cataracts), and in some cases it may also prevent premature death (such as cardiac transplantation in "fatal" infantile cardiomyopathy, or endocardial pacing for cardiac conduction defects in Kearns–Sayre syndrome). In many situations, supportive care for mitochondrial disease is no different from any other progressive illness. There are, however, a few notable exceptions which are based principally on theoretical grounds. The anticonvulsant sodium valproate inhibits oxidative phosphorylation (Haas *et al.*, 1981), and if possible should not be used to treat patients with mitochondrial disease. Infection should be treated vigorously in patients with mitochondrial disease but antibiotics should be chosen carefully since a number of antibiotics may have adverse effects on mitochondrial function. The majority of antiviral agents inhibit mitochondrial DNA polymerase γ; azidothymidine (AZT) may induce a myopathy and mtDNA depletion, and fialuridine can induce a fatal encephalopathy with hepatic failure (Lewis and Dalakas, 1995). To date, mitochondrial toxicity with acyclovir has not been reported. Patients with mitochondrial disease are more susceptible to complications from general anaesthesia, and as such anaesthetists should be aware of potential cardiac conduction defects. Furthermore, an increased sensitivity to etomidate and thiopentone has been documented in the Kearns–Sayre syndrome (Lauwers *et al.*, 1994). Chronic hypoxia and CO_2 insensitivity may also cause problems on reversal of anaesthesia (Barohn *et al.*, 1990) or in the intensive care unit (Howard *et al.*, 1995).

Specific Pharmacological Therapy

A variety of pharmacological agents been used in the treatment of patients with mitochondrial disease by intervening at each level of the pathological cascade thought to be responsible for cellular dysfunction and death. Evidence to support their efficacy is primarily anecdotal or is based upon small clinical trials using surrogate endpoints. Clinical trials involving mito-

chondrial disorders are fraught with difficulties: patients with identical genetic defects may present in a different way, each individual will progress with a different clinical course, and recruitment into trials is difficult with each center having only a relatively small number of patients. Designing a reliable but sensitive disability assessment rating scale to cover such a broad range of phenotypes is a major challenge for the future.

Modifying Respiratory Chain Function. In a few well documented cases of isolated coenzyme Q_{10} (CoQ_{10}) deficiency, treatment with CoQ_{10} has been highly effective (Ogasahara *et al.*, 1989, Servidei *et al.*, 1996). CoQ_{10} is an essential component of the respiratory chain that functions as a mobile electron transfer component between membranous flavoprotein dehydrogenases and complex III in the inner mitochondrial membrane. However, the benefits of CoQ_{10} in the treatment of other respiratory chain complex deficiencies have yet to be established. In isolated cases, CoQ_{10} improved the symptoms (Ihara *et al.*, 1989), CSF lactate levels (Abe *et al.*, 1991), and the abnormal MRS findings associated with mitochondrial myopathies (Nishikawa *et al.*, 1989; Bendahan *et al.*, 1992; Gold *et al.*, 1996). However, it has not always been possible to document an objective improvement (Zierz *et al.*, 1989; Matthews *et al.*, 1993), and in the only double-blind multi-center study using CoQ_{10}, the results were inconclusive (Bresolin *et al.*, 1990). In our experience, many patients report an improvement in muscle symptoms (fatigue, aches, or cramps) on starting CoQ_{10} (30 mg t.d.s. increasing to 60 mg t.d.s.). This effect is often short lived and suggests a strong placebo component to the therapeutic response. Adverse effects from CoQ_{10} are rare, so despite the controversy about efficacy, most clinicians will attempt a therapeutic trial of CoQ_{10} in the hope of some improvement. An improvement in cardiac conduction with CoQ_{10} has been documented in one patient with the Kearns–Sayre syndrome (Ogasahara *et al.*, 1986) and in a recent report, CoQ_{10} improved calcium homeostasis in two patients with hypoparathyroidism and mtDNA deletions (Papadimitriou *et al.*, 1996). These cases highlight the potential benefits from CoQ_{10} for non-neurological features of mitochondrial disease.

In theory, artificial electron acceptors could lead to symptomatic improvement in patients with respiratory chain disease. Ascorbate (vitamin C, 1 g o.d.) and menadione (vitamin K_3, 10 mg o.d.) may accept electrons from reduced CoQ_{10} and deliver them to cytochrome *c*, effectively by-passing complex III. Although these agents improved the symptoms and oxidative muscle metabolism in one patient with complex III deficiency (Eleff *et al.*, 1984), objective evidence of benefit in other patients has yet to be demonstrated (Morris *et al.*, 1995).

Riboflavin indirectly acts as a cofactor for respiratory chain complexes I and II. In a thirteen-year-old boy with complex I deficiency and myopathy, 100 mg daily led to an improvement in symptoms and his lactic acidosis (Arts *et al.*, 1983), and in one encephalopathic patient with MELAS, 100 mg t.d.s. led to an improvement in conscious level paralleled by enhanced peripheral nerve conduction and MR spectroscopy results (Penn *et al.*, 1992). However, in a larger study of six cases, only one showed a clinical improvement (Bernsen *et al.*, 1993), and although increased complex I activity has been noted in patients taking riboflavin, again the results did not correlate with a useful clinical response (Bernsen *et al.*, 1993). Thiamine (300 mg o.d.) is a cofactor for pyruvate dehydrogenase and may stimulate the respiratory chain through the production of NADH. However, although thiamine may reduce blood lactate levels (Lou, 1981), its clinical effects are minimal (Mathews *et al.*, 1993). L-Carnitine (1–3 g/day) is often used in conjunction with CoQ_{10} in patients with respiratory chain disease (DiMauro, 1996). Although only a minority of patients with respiratory chain defects have a secondary carnitine deficiency, due to the inhibition of mitochondrial fatty acid oxidation (Hsu *et al.*, 1995), many individuals report an improvement in symptoms on taking L-carnitine although the precise mechanism remains obscure.

Reducing Cytotoxic Metabolites and Free Radicals. MRI spectroscopy has confirmed diffuse intracerebral lactic acid accumulation in patients with mitochondrial disease. It is possible that high levels contribute to neural dysfunction and death and attempts to reduce lactic acidosis could theoretically reduce the rate of disease progression. Oral sodium bicarbonate may improve the breathlessness associated with lactic acidosis, and intravenous sodium bicarbonate is useful for the rapid correction of severe intravascular acidosis. However, the potential for fluid overload is significant in patients who often have major intercurrent illness. Dichloracetate (DCA) is thought to reduce serum lactate levels through activation of the pyruvate dehydrogenase complex, and a recent MRI spectroscopy study has confirmed a reduction in cerebral lactic

acidosis in mitochondrial patients treated with this drug (25 mg/kg/day, De Stefano *et al.*, 1995). Significant clinical improvement has yet to be documented.

Antioxidants. There is increasing evidence that oxygen free radicals and oxidative stress may play a major role in neuronal death in mitochondrial and neurodegenerative disease, and it may be that ascorbate, menadione, and CoQ_{10} mediate their effects, at least in part, through an antioxidant mechanism (DiMauro, 1996; Beal, 1995).

Other Agents. Corticosteroids have been used in patients with MELAS and other mitochondrial encephalomyopathies (Montagna *et al.*, 1988; Peterson, 1995), but again there have been conflicting reports, and in one case fatal metabolic acidosis was thought to have arisen as a consequence of the treatment (Curless *et al.*, 1986).

GENETIC THERAPY FOR mtDNA ABNORMALITIES

The inadequacy of these current biochemical therapeutic strategies forces us to consider the possibility of gene therapy for mtDNA disorders, although the means by which this can ultimately be achieved appears to be fraught with problems. In considering the possible approaches to this effect, we will concentrate on three main approaches (Fig. 1). The experimental work currently in progress in these areas has recently been reviewed (Chrzanowska-Lightowlers *et al.*, 1995).

Cytosolic Synthesis of Mitochondrially-Encoded Proteins

The majority of proteins involved in the structure or function of mitochondria are nuclear-encoded. They are synthesized on cytoplasmic ribosomes and subsequently imported either post- or co-translationally into the mitochondria by means of a short (N)-terminal presequence which is removed by specific proteases in the mitochondrial matrix (Bohni *et al.*, 1983). It has therefore been postulated that for some mtDNA mutations, it may be possible to express a normal copy of the defective gene in the nucleus and synthesize the protein in the cytosol, thereby complementing the genetic defect. The potential of this approach has been highlighted by the work of Nagley and colleagues in yeast, who have successfully demonstrated phenotypic rescue of a yeast mutant defective in the ATPase 8 gene. The newly cytosolically-synthesized protein was correctly imported into mitochondria and functionally assembled into the ATPase complex (Nagley *et al.*, 1988).

There are several difficulties associated with this strategy, not least being the different codon usage found in mitochondria (Anderson *et al.*, 1981) and the requirement for the addition of a mitochondrial targeting presequence. Furthermore, this approach is likely to be of major benefit only for examples of missense mutations occurring within protein-encoding genes, e.g., the 11778 LHON mutation in the ND4 gene (Wallace *et al.*, 1988) and the T8993G NARP mutation in the ATPase 6 gene (Holt *et al.*, 1990), as the application of this approach to the treatment of large-scale rearrangements would obviously require the synthesis of several mitochondrially-encoded proteins and all relevant tRNAs. This approach, however, is currently being pursued using the NARP mutation as a model. Working with murine mtDNA, Jacobs and colleagues have successfully achieved code-correction of the mouse mitochondrial genes for ATPase 6 and 8, ND4, and COXI by means of an oligonucleotide-mediated strategy, thereby allowing the correct gene product to be produced by the cytosolic translational machinery (Sutherland *et al.*, 1994, 1995). Focussing primarily on ATPase 6, constructs have been further engineered to include the N-terminal targeting presequence of the Fp subunit of succinate dehydrogenase. However, when recloned in an expression vector and transfected into NIH3T3 fibroblasts, high-level expression of these transgenes was found to be toxic to the recipient cells, on account of an impairment of ATP synthesis.

Complementation by Mitochondrial Gene Expression

Due to the recessive nature of pathogenic mtDNA mutations, the introduction of a normal copy of the mutant allele could theoretically provide a means of therapy. While such replacement therapy has found short term success in several nuclear gene therapy approaches, using various viral delivery systems or liposomal vectors to mediate nucleic acid transfer to

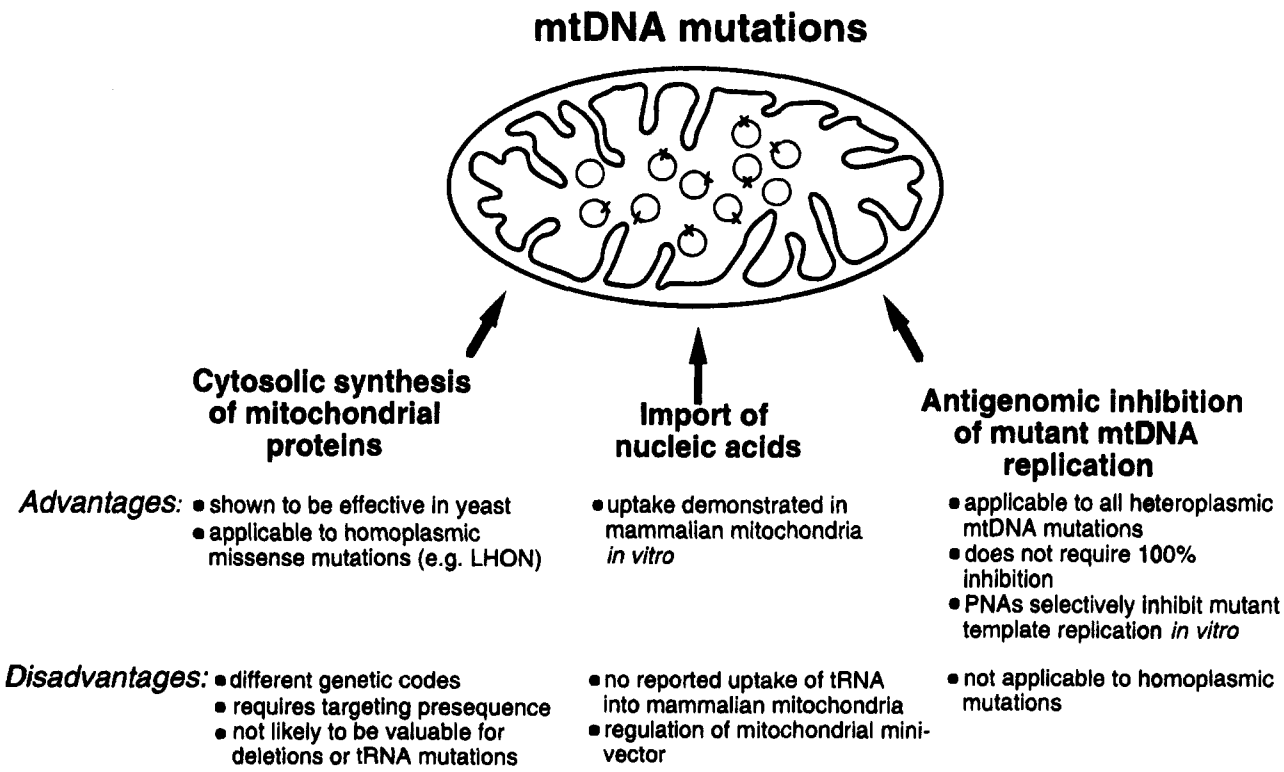

Fig. 1. Gene therapy approaches to the treatment of mtDNA mutations.

defective cells (Mulligan, 1993; Miller and Vile, 1995), the introduction of nucleic acids into mitochondria will require the negotiation of several difficulties. The mitochondrion has two, very characteristic membranes. While the outer mitochondrial membrane is highly permeable to most small (<5 kDa) molecules, entry of larger molecules (e.g., proteins) is receptor-mediated (Pfanner and Neupert, 1990). The highly lipophilic inner membrane is even more selective in respect of its permeability. However, mitochondrial import of small nucleic acid molecules, notably cytoplasmic tRNAs, has been demonstrated in the yeast *S. cerevisiae* (Tarassov and Entelis, 1992), the kinetoplastid protozoan *Leishmania* (Mahapatra *et al.*, 1994), and plants (Small *et al.*, 1992). Investigation of the mechanisms of tRNA targeting and translocation across the mitochondrial membranes have revealed alternative models of import. Using an *in vitro* system, import of RNA into *Leishmania* mitochondria has been shown in association with specific membrane-bound receptors (Mahapatra and Adhya, 1996), while the import of cytosolic $tRNA^{Lys(CUU)}$ into *S. cerevisiae* mitochondria has been shown to require additional cytoplasmic factors, recently identified as the cytoplasmic and mitochondrial lysyl-tRNA synthetases (Tarassov *et al.*, 1995). The cooperation of these two enzymes results in the aminoacylation of the $tRNA^{Lys(CUU)}$, a prerequisite for its targeting to the mitochondria. At present, there are no such reports of tRNA import occurring in mammalian cells, although one such example of nucleic acids entering the mitochondria may be the nuclear-encoded RNA component of the mitochondrial RNA processing enzyme, RNase MRP (Chang and Clayton, 1987; Li *et al.*, 1994). While it is postulated that this RNA species is taken up either complexed to cytosolic proteins or on account of a specific targeting motif located within the RNA molecule, evidence also exists against a mitochondrial location for the MRP RNA (Kiss and Filipowicz, 1992).

The most likely method to achieve uptake of large, exogenous nucleic acid molecules into mitochondria will involve the protein import machinery. The short N-terminal protein presequences which mediate transfer of nuclear-encoded proteins across the mitochondrial membranes are themselves capable of being recognized and taken up by mitochondria (Ono *et al.*, 1990), and importing proteins to the mitochondria not normally associated with the organelle (Hurt *et al.*, 1984). Using such a precursor protein (yeast cytochrome *c* oxidase subunit IV presequence fused to a

modified DHFR protein), Vestweber and Schatz (1989) have a successfully achieved the uptake of a 24-bp DNA fragment into yeast mitochondria by covalently linking the 5′ end of the oligonucleotide to the mitochondrial precursor protein. Using a similar strategy, Seibel and colleagues have obtained uptake of a DNA fragment as big as 322 bp (approximately the size of the smallest transcribable mitochondrial gene) into mammalian mitochondria (Seibel *et al.*, 1995). Studies addressing the complementation of the defective gene, and the replication and transcription of the mini-mitochondrial vector, are currently being tackled by other groups.

Sequence-Specific Inhibition of Mutant mtDNA Replication

The heteroplasmic character of mitochondrial DNA mutations naturally lends itself to an alternative gene therapy approach. Since the mutant mtDNA in heteroplasmic patients is functionally recessive, any treatment strategy that selectively inhibits the replication of the mutant genome will give the wild type genome a distinct replicative advantage. Such an approach does not require the inhibition of all mutant genome replication due to its recessive nature. By inhibiting just a subset of mutant mtDNA replication, the wild type will selectively replicate and the level of heteroplasmy will be changed. Assuming irreversible tissue damage has not occurred, it should be possible to couple reversal of both the biochemical and clinical abnormality.

Since there is extensive displacement of single-stranded mtDNA during asynchronous replication of the mitochondrial genome (Clayton, 1991), one might envisage an opportunity for binding by sequence-specific agents (e.g., oligonucleotides) which could inhibit replication if not displaced by the mtDNA replisome. However, for such a strategy to be effective, any likely therapeutic agent would have to specifically target the mutant genome, with little or no effect on the wild type genome. In this respect, peptide nucleic acids (PNAs) are very attractive candidates. PNAs are polyamide noncharged nucleic acid derivatives that obey Watson–Crick base pairing rules when forming a stable duplex with single-stranded DNA (Nielsen *et al.*, 1991; Egholm *et al.*, 1992, 1993). They bind with a greater affinity to a complementary DNA molecule than the equivalent oligonucleotide, and have been shown to be substantially more resistant to degradation than oligonucleotides in both extracellular fluid and intracellular environments (Demidov *et al.*, 1994). Furthermore, while an initial report investigating the membrane permeabilities of unmodified PNA molecules concluded that they were unlikely to be taken up into cells (Wittung *et al.*, 1995), there is now good experimental evidence demonstrating PNA uptake by different cell types in culture. Consequently, the ability of PNAs to inhibit mtDNA replication in a sequence-specific manner using an *in vitro* replication run-off assay has been investigated (Taylor *et al.*, 1997).

On the basis of published T_m for PNA:DNA duplexes (Orum *et al.*, 1993), two peptide nucleic acids were synthesized complementary to mtDNA templates containing mutant sequences. First, a 14-mer PNA was synthesized complementary to the 4977 bp "common deletion" breakpoint sequence (5′ CTGCCAATGGTGAG 3′; Schon *et al.*, 1989), of which only the first seven bases are found in the corresponding wild type template. *In vitro* replication under physiological conditions (37°C, 150 mM salt) with increasing concentrations of this PNA (up to 0.2 μM) demonstrated sequence-specific inhibition of only the mutant template by up to 80% (Taylor *et al.*, 1997). To investigate whether replication could be inhibited in a similar manner when the mutant mtDNA template contained a single point mutation, an 11-mer PNA was synthesized complementary to be mutant sequence associated with the A8344G $tRNA^{Lys}$ MERRF mutation (Shoffner *et al.*, 1990), with the mismatch base in the middle of the PNA sequence. Replication run-off from MERRF mtDNA templates was inhibited by up to 75% (Fig. 2), while identical PNA concentrations (up to 1000–fold molar excess) did not inhibit replication of the wild type template. Furthermore, this level of sequence-specific inhibition was maintained even in the presence of saturating concentrations of *E. coli* single-stranded binding protein (SSB), a protein analogous to the mitochondrial SSB whose function *in vivo* is to bind and protect single-stranded mtDNA from nucleolytic attack during replication (Curth *et al.*, 1994).

Similar to the other approaches to the gene therapy of mtDNA defects, there are many potential problems associated with using anti-genomic PNAs, not least their accurate targeting and import to the mitochondrial compartment. Nevertheless, we (Taylor *et al.*, 1997) and others have demonstrated PNA uptake into cells in culture as a first step to delivering these molecules to the mitochondrial matrix. While PNA inhibition of DNA replication in cell culture has not been reported, and it is uncertain how transient an

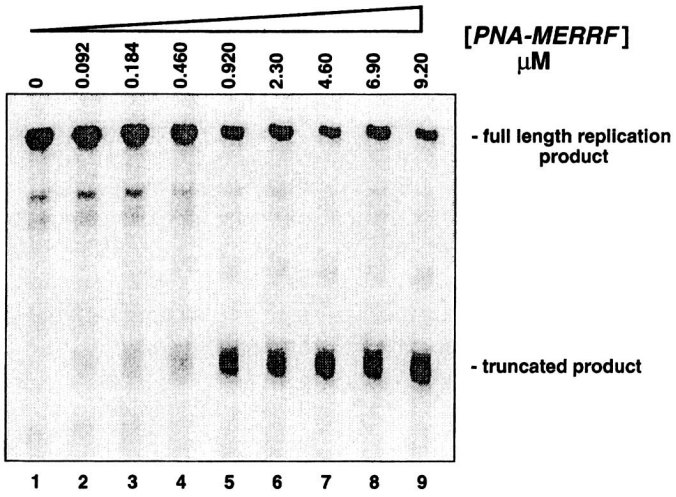

Fig. 2. Sequence-specific inhibition of A8344G MERRF template replication by PNA. *In vitro* replication run-off from single-stranded mutant templates was performed in the presence of increasing concentrations of an 11-mer PNA (*PNA-MERRF*) complementary to the light strand MERRF sequence (5′ AGAGAGCCAAC 3′; mutant base shown in bold). The inhibition of mutant mtDNA replication results in the formation of a truncated replication product. Identical concentrations of *PNA-MERRF* did not inhibit replication of a wild type template (data not shown).

effect inhibitory PNAs might have on replication *in vivo*, if at all, the clear demonstration that mutant mtDNA replication can be inhibited in a sequence-specific manner, even in the presence of a competitive DNA-binding protein, goes a very long way to establishing this strategy as a viable therapeutic regime for heteroplasmic mtDNA mutations.

FUTURE DIRECTIONS

Animal Models for Mitochondrial DNA Disease

While several strategies have been devised for the treatment of mitochondrial disease, as yet there exists no suitable animal model in which to assess their *in vivo* potentials. Mice are ideal animals for such models and have therefore been used in the investigation of several nuclear based human diseases. Recently, heteroplasmic mice have been created by the electrofusion of the cytoplast of one zygote type with a single-cell embryo of another type (Jenuth *et al.*, 1996). By this approach, in order to establish an animal model in which to analyze potential therapies, it would be necessary to obtain a zygote harboring the causative mutation. Unfortunately, mtDNA mutations have not been reported to be important causes of disease in mice, and the complexity of mitochondrial genetics places doubt on the use of genetic engineering for the creation of such a genotype. An alternative approach has therefore been implemented based on myoblast transplantation. Human myoblasts harboring mutant mtDNA have been successfully transferred to previously irradiated and barium chloride-injected tibialis anterior of SCID mice, to produce stable human fibers. Furthermore, these fibers expressed a level of the mutation similar to that of the injected myoblasts (K.M. Clark, unpublished data). This model would seem an ideal system in which to assess genetic therapies as the mtDNA defect is present in a commonly affected tissue. Theoretically, a model could be developed for any human mtDNA mutation which was expressed in cultured myoblasts and agents developed for their treatment tested directly on regenerated muscle within the SCID mouse.

Induced Muscle Regeneration as a Therapy for Mitochondrial Myopathies

Satellite cells are the myogenic precursor cells which reside in the basement membrane of skeletal

muscle. These small mononucleated cells are normally quiescent, but in response to injury become activated and proliferate to form new muscle fibers (Bischoff, 1994). Segregation of mutant mtDNA during embryogenesis and the mitotic activity of a tissue determines whether a given cell type will possess the heteroplasmic genotype. Hence, symptomatic patients harboring a high ratio of mutant to wild type mtDNA may possess satellite cells with low levels of the mutation. Injection of bupivacane causes necrosis of muscle fibers but appears to leave satellite cells intact. We have tried such a strategy in a patient whose causative $tRNA^{Leu(CUN)}$ mutation appears absent in satellite cells. Such induced muscle regeneration could be beneficial providing that newly regenerated muscle fibers, derived exclusively from the satellite cell population, contained a mutant load lower than the required threshold, or that myoblasts fusing with pre-existing fibers reduced the level of mutant mtDNA below the critical threshold level. In order for this approach to be used as a long-term therapy, a strategy to degenerate, or partially degenerate, all affected muscles would need to be established.

CONCLUDING REMARKS

While there are many studies in the literature investigating potential biochemical therapies for mitochondrial disease, there is no clear evidence that they consistently improve the symptoms of the patient or that they alter the course of the disease. If we are to offer any hope of a useful treatment, we must develop genetic strategies to treat these patients. In considering possible options, it is imperative to remember that the very characteristics of pathological mtDNA mutations (i.e., their intramitochondrial location, their heteroplasmic nature, the recessivity of these mutations) means there are fundamental differences in any approach to gene therapy for mtDNA disorders compared to nuclear gene therapy. In the case of nuclear gene therapy, the expression of nuclear transgenes is ultimately required for the lifetime of the patient in order to compensate the defective gene, and due to the transient effect of many therapies, this could require repeated treatments. In the case of mitochondrial disease, the important balance between wild type versus mutant in a heteroplasmic mutation determines clinical phenotype, and a subtle manipulation of this difference, for example by inhibiting replication of the mutant genome, has the enormous potential to reverse the genetic, and hence the biochemical and clinical, abnormality. Furthermore, once the imbalance has been addressed in this way, one might envisage that both mutant and wild type molecules may continue to replicate normally in the absence of selective mutant mtDNA propagation, thus maintaining the relative proportions of wild type and mutant mtDNA and therefore a wild type phenotype. In addition, while many of the gene-modifying drugs that have been used in nuclear gene therapy approaches have proved to have either nonspecific or toxic side effects, or be readily degraded both intra- and extracellularly, experimental evidence suggests PNAs are neither toxic nor readily degraded, and, as such we believe that they have the potential to achieve mitochondrial gene therapy.

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REFERENCES

Abe, K., Fujimura, H., Nishikawa, Y., Yorifuji, S., Mezaki, T., Hirono, N., Nishitani, N., and Kameyama, M. (1991). *Acta Neurol. Scand.* **83**, 356–369.

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R., and Young, I. G. (1981). *Nature* **290**, 457–465.

Arts, W. F., Scholte, H. R., Bogaard, J. M., Kerrebijn, K. F., and Luyt-Houwen, I. E. (1983). *Lancet* **2**, 581–582.

Barohn, R. J., Clanton, T., Sahenk, Z., and Mendell, J. R. (1990). *Neurology* **40**, 103–106.

Beal, M. F. (1995). *Ann. Neurol.* **38**, 357–366.

Bendahan, D., Desnuelle, C., Vanuxem, D., Confort-Gouny, S., Figarella-Branger, D., Pellissier, J. F., Kozak-Ribbens, G., Pouget, J., Serratrice, G., and Cozzone, P. J. (1992). *Neurology* **42**, 1203–1208.

Bernsen, P. L., Gabreels, F. J., Ruitenbeek, W., and Hamburger, H. L. (1993). *J. Neurol. Sci.* **118**, 181–187.

Bischoff, R. (1994). In *Myology* (Engel, A. G., and Fronzini-Armstrong, C., eds.), McGraw-Hill, New York, pp. 97–118.

Bohni, P. C., Damn, G., and Schatz, G. (1983). *J. Biol. Chem.* **258**, 4937–4943.

Boulet, L., Karpati, G., and Shoubridge, E. A. (1992). *Am. J. Hum. Genet.* **51**, 1187–1200.

Bresolin, N., Doriguzzi, C. Ponzetto, C., Angelini, C., Moroni, I., Castelli, E., Cossutta, E., Binda, A., Gallanti, A., Gabellini, S., Piccolo, G., Martinuzzi, A., Ciafaloni, E., Arnaudo, E., Liciardello, L., Carenzi, A., and Scarlato, G. (1990). *J. Neurol. Sci.* **100**, 70–78.

Brockington, M., Sweeney, M. G., Hammans, S. R., Morgan-Hughes, J. A., and Harding, A. E. (1993). *Nature Genet.* **4**, 67–71.

Chang, D. D., and Clayton, D. A. (1987). *Science* **235**, 1178–1184.

Chen, X., Prosser, X., Simonetti, S., Sadlock, J., Jagiello, G., and Schon, E. A. (1995). *Am. J. Hum. Genet.* **57**, 239–247.
Chinnery, P. F, Howell, N., Lightowlers, R. N., and Turnbull, D. M. (1997). *Brain*, in press.
Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S. T., Nonaka, I., Angelini, C., and Attardi, G. (1992). *Proc. Natl. Acad. Sci. USA* **89**, 4221–4225.
Chrzanowska-Lightowlers, Z. M. A., Lightowlers, R. N., and Turnbull, D. M. (1995). *Gene Ther.* **2**, 311–316.
Clayton, D. A. (1991). *Trends Biochem. Sci.* **16**, 107–111.
Curless, R. G., Flynn, J., Bachynski, B., Gregorios, J. B., Benke, P., and Cullen, R. (1986). *Neurology* **36**, 872–873.
Curth, U., Urbanke, C., Greipel, J., Gerberding, H., Tiranti, V., and Zeviani, M. (1994). *Eur. J. Biochem.* **221**, 435–443.
Demidov, V. V., Potaman, V., Frank-Kamenetskii, M. D., Egholm, M., Buchardt, O., Sonnichsen, S., and Nielsen, P. E. (1994). *Biochem. Pharmacol.* **48**, 1310–1313.
De Stefano, N., Matthews, P. M., Ford, B., Genge, A., Karpati, G., and Arnold, D. L. (1995). *Neurology* **45**, 1193–1198.
DiMauro, S. (1996). *J. Inher. Metab. Dis.* **19**, 489–503.
Egholm, M., Buchardt, O., Nielsen, P. E., and Berg, R. H. (1992). *J. Am. Chem. Soc.* **114**, 1895–1897.
Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D., Berg, R. H., Kim, S. K., Norden, B., and Nielsen, P. E. (1993). *Nature* **365**, 566–568.
Eleff, S., Kennaway, N. G., Buist, N. R., Darley-Usmar, V. M., Capaldi, R. A., Bank, W. J., and Chance, B. (1984). *Proc. Natl. Acad. Sci. USA* **81**, 3529–3533.
Giles, R. E., Blanc, H., Cann, H. M., and Wallace, D. C. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 6715–6719.
Gold, R., Seibel, P., Reinelt, G., Schindler, R., Landwehr, R., Beck, A., and Reichmann, H. (1996). *Eur. Neurol.* **36**, 191–196.
Goto, Y., Nonaka, I., and Horai, S. (1990). *Nature* **348**, 651–654.
Haas, R., Stumpf, D. A., Parks, J. K., and Eguren, L. (1981). *Neurology* **31**, 1473–1476.
Holt, I. J., Harding, A. E., and Morgan-Hughes, J. A. (1988). *Nature* **331**, 717–719.
Holt, I. J., Harding, A. E., Petty, R. K. H., and Morgan-Hughes, J. A. (1990). *Am. J. Hum. Genet.* **46**, 428–433.
Howard, R. S., Russell, S., Losseff, N., Harding, A. E., Morgan-Hughes, J., Wiles, C. M., Miller, D. H., and Hirsch, N. P. (1995). *Q. J. Med.* **88**, 197–207.
Howell, N., Bindoff, L. A., McCullough, D. A., Kubucka, I., Poulton, J., Mackey, D., Taylor, L., and Turnbull, D. M. (1991). *Am. J. Hum. Genet.* **49**, 939–950.
Hsu, C. C., Chuang, Y. H., Tsai, J. L., Jong, H. J., Shen, Y. Y., Huang, H. L., Chen, H. L., Lee, H. C., Pang, C. Y., Wei, Y. H., *et al.* (1995). *Acta Neurol. Scand.* **92**, 252–255.
Hurt, E. C., Pesold-Hurt, B., and Schatz, G. (1984). *EMBO J.* **3**, 3149–3156.
Ihara, Y., Namba, R., Kuroda, S., Sato, T., and Shirabe, T. (1989). *J. Neurol. Sci.* **90**, 263–271.
Jenuth, J. P., Peterson, A. C., Fu, K., and Shoubridge, E. A. (1996). *Nature Genet.* **14**, 146–151.
Johns, D. R. (1995). *N. Engl. J. Med.* **333**, 638–644.
Johns, D. R., Heher, K. L., Miller, N. R., and Smith, K. H. (1993). *Arch. Ophthalmol.* **111**, 495–498.
King, M. P., and Attardi, G. (1989). *Science* **246**, 500–503.
King, M. P., Koga, Y., Davidson, M., and Schon, E. A. (1992). *Mol. Cell. Biol.* **12**, 480–490.
Kiss, T., and Filipowicz, W. (1992). *Cell* **70**, 11–16.
Lauwers, M. H., Van Lersenberghe, C., and Camu, F. (1994). *Anesthesia* **49**, 876–878.
Lewis, W., and Dalakas, M. C. (1995). *Nature Med.* **1**, 417–422.
Li, K., Smagula, C. S., Parsons, W. J., Richardson, J. A., Gonzalez, M., Hagler, H. K., and Williams, R. S. (1994). *J. Cell Biol.* **124**, 871–882.
Lou, H. C. (1981). *Arch. Neurol.* **38**, 598–602.
Mahapatra, S., and Adhya, S. (1996). *J. Biol. Chem.* **271**, 20432–20437.
Mahapatra, S., Ghosh, T., and Adhya, S. (1994). *Nucleic Acids Res.* **22**, 3381–3386.
Matthews, P. M., Ford, B., Dandurand, R. J., Eidelman, D. H., Connor, D. O., Sherwin, A., Karpati, G., Andermann, F., and Arnold, D. L. (1993). *Neurology* **43**, 884–890.
Miller, N., and Vile, R. (1995). *FASEB J.* **9**, 190–199.
Montagna, P., Gallassi, R., Medori, R., Govoni, E., Zeviani, M., DiMauro, S., Lugaresi, E., and Andermann, F. (1988). *Neurology* **38**, 751–754.
Moraes, C. T., DiMauro, S., Zeviani, M., Lombes, A., Shanske, S., Miranda, A. F., Nakase, H., Bonilla, E., Werneck, L. C., Servidei, S., Nonaka, I., Koga, Y., Spiro, A., Brownell, K., Schmidt, B., Schotland, D., Zupanc, M., DeVivo, D., Schon, E., and Rowland, L. (1989). *New Engl. J. Med.* **320**, 1293–1299.
Moraes, C. T., Ciacci, F., Silvestri, G., Shanske, S., Sciacco, M., Hirano, M., Schon, E. A., Bonilla, E., and DiMauro, S. (1993). *Neuromusc. Disord.* **3**, 43–50.
Morris, A. A. M., Taylor, R. W., Birch-Machin, M. A., Jackson, M. J., Coulthard, M. G., Bindoff, L. A., Welch, R. J., Howell, N., and Turnbull, D. M. (1995). *Pediatr. Nephrol.* **9**, 547–551.
Mulligan, R. C. (1993). *Science* **260**, 926–932.
Nagley, P., Farrell, L. B., Gearing, D. P., Nero, D., Meltzer, S., and Devenish, R. J. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 2091–2095.
Nielsen, P. E., Egholm, M., Berg, R. H., and Buchardt, O. (1991). *Science* **254**, 1497–1500.
Nishikawa, Y., Takahashi, M., Yorifuji, S., Nakamura, Y., Ueno, S., Tarui, S., Kozuka, T., and Nishimura, T. (1989). *Neurology* **39**, 399–403.
Ogasahara, S., Nishikawa, Y., Yorifuji, S., Soga, F., Nakamura, Y., Takahashi, M., Hashimoto, S., Kono, N., and Tarui, S. (1986). *Neurology* **36**, 45–53.
Ogasahara, S., Engel, A. G., Frens, D., and Mack, D. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 2379–2382.
Ono, H., and Tuboi, S. (1990). *Arch. Biochem. Biophys.* **280**, 299–304.
Orum, H., Nielsen, P. E., Egholm, M., Berg, R. H., Buchardt, O., and Stanley, C. (1993). *Nucleic Acids Res.* **21**, 5332–5336.
Papadimitriou, A., Hadjigeorgiou, G. M., Divari, R., Papagalanis, N., Comi, G., and Bresolin, N. (1996). *Neuromusc. Disord.* **6**, 49–53.
Penn, A. M., Lee, J. W., Thuillier, P., Wagner, M., Maclure, K. M., Menard, M. R., Hall, L. D., and Kenaway, N. G. (1992). *Neurology* **42**, 2147–2152.
Peterson, P. L. (1995). *Biochim. Biophys. Acta* **1271**, 275–280.
Pfanner, N., and Neupert, W. (1990). *Biochem. Soc. Trans.* **18**, 513–515.
Schon, E. A., Rizzuto, R., Moraes, C. T., Nakase, H., Zeviani, M., and DiMauro, S. (1989). *Science* **244**, 346–349.
Schon, E. A., Hirano, M., and DiMauro, S. (1994). *J. Bioenerg. Biomembr.* **26**, 291–299.
Seibel, P., Trappe, J., Villani, G., Klopstock, T., Papa, S., and Reichmann, H. (1995). *Nucleic Acids Res.* **23**, 10–17.
Servidei, S., Spinazzola, A., and Crociani, P. (1996). *Neurology* **46**, A420.
Shoffner, J. M., Lott, M. T., Lezza, A. M., Seibel, P., Ballinger, S. W., and Wallace, D. C. (1990). *Cell* **61**, 931–937.
Small, I., Marechal-Drouard, L., Masson, J., Pelletier, G., Cossat, A., Weil, J.-H., and Dietrich, A. (1992). *EMBO J.* **11**, 1291–1296.
Sutherland, L., Davidson, J., and Jacobs, H. T. (1994). *Biochem. Soc. Trans.* **22**, 413S.
Sutherland, L., Davidson, J., Glass, L. L., and Jacobs, H. T. (1995). *Biotechniques* **18**, 458–464.

Tarassov, I. A., and Entelis, N. S. (1992). *Nucleic Acids Res.* **20**, 1277–1281.
Tarassov, I. A., Entelis, N. S., and Martin, R. P. (1995). *EMBO J.* **14**, 3461–3471.
Taylor, R. W., Chinnery, P. F., Turnbull, D. M., and Lightowlers, R. N. (1997). *Nature Genet.* **15**, 212–215.
van den Ouweland, J. M. W., Lemkes, H. H. P. J., Ruitenbeek, W., Sandkuijl, L. A., de Vijlder, M. F., Struyvenberg, P. A. A., van de Kamp, J. J. P., and Maassen, J. A. (1992). *Nature Genet.* **1**, 368–371.
Vestweber, D., and Schatz, G. (1989). *Nature* **338**, 170–172.
Wallace, D. C. (1992a). *Ann. Rev. Biochem.* **61**, 1175–1212.
Wallace, D. C. (1992b). *Science* **256**, 628–632.
Wallace, D. C. (1995). *Am. J. Hum. Genet.* **57**, 201–223.
Wallace, D. C., Singh, G., Lott, M. T., Hodge, J. A., Schurr, T. G., Lezza, A. M. S., Elsas II, L. J., and Nikoskelainen, E. K. (1988). *Science* **242**, 1427–1430.
Weber, K., Wilson, J. N., Taylor, L., Brierley, E., Johnson, M. A., Turnbull, D. M., and Bindoff, L. A. (1997). *Am. J. Hum. Genet.* **60**, 373–380.
Wittung, P., Kajanus, J., Edwards, K., Nielsen, P., Norden, B., and Malmstrom, B. G. (1995). *FEBS Lett.* **365**, 27–29.
Zierz, S., Jahns, G., and Jerusalem, F. (1989). *J. Neurol.* **236**, 97–101.