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Disorders of Nuclear-Mitochondrial Intergenomic Signalling

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In addition to sporadic or maternally-inherited mutations of the mitochondrial genome, abnormalities of mtDNA can be transmitted as mendelian traits. The latter are believed to be caused by mutations in still unknown nuclear genes, which deleteriously interact with the mitochondrial genome. Two groups of mtDNA-related mendelian disorders are known: those associated with mtDNA large-scale rearrangements and those characterized by severe reduction of the mtDNA copy number. The most frequent presentation of the first group of disorders is an adult-onset encephalomyopathy, defined clinically by the syndrome of progressive external ophthalmoplegia "plus," genetically by autosomal dominant transmission of the trait, and molecularly by the presence of multiple deletions of mtDNA. The second group of disorders comprises early-onset, organ-specific syndromes, associated with mtDNA depletion, that are presumably transmitted as autosomal recessive traits. Linkage analysis and search for candidate genes are two complementary strategies to clarify the molecular basis of these disorders of the nuclear-mitochondrial intergenomic signalling.

KEY WORDS: mtDNA; multiple mtDNA deletions; mtDNA depletion; PEO; oxidative phosphorylation; respiratory chain; mitochondrial disorders; mitochondrial replication; maternal inheritance.

INTRODUCTION

Mutations of human mtDNA can be divided into (i) single sporadic deletions or insertions; (ii) maternally-inherited point mutations, and (iii) nucleus-driven mtDNA mutations (reviewed in Larsson and Clayton, 1995). The latter category is characterized by the presence of mtDNA abnormalities associated with disorders inherited as mendelian genetic traits. Mendelian inheritance rules out the possibility that the primary defect may be a transmissible mutation of mtDNA, and suggests that the defect must rather be present in a trans-acting nucleus-coded factor, which can ultimately damage the mtDNA molecule.

Two groups of mendelian mtDNA defects have been identified. The first group comprises several different clinical phenotypes, all characterized by the presence of *multiple large-scale deletions of mtDNA* in stable tissues. The second group comprises a number of paediatric syndromes due to *tissue-specific depletion of mtDNA*.

MULTIPLE FAMILIAL mtDNA DELETIONS

Clinical Features

Autosomal Dominant PEO

In 1989 we found an Italian family in which an adult-onset mitochondrial encephalomyopathy characterized by the presence of progressive external ophthalmoplegia (PEO) was inherited as a mendelian,

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autosomal dominant (ad) trait (Zeviani *et al.*, 1989). Southern-blot analysis of muscle mtDNA of the probands revealed the presence of a collection of mtDNA species harboring different deletions (Zeviani *et al.*, 1989, 1990). Maternal inheritance was excluded because individuals of both sexes belonging to both paternal and maternal lineages were affected in subsequent generations. Numerous adPEO families have later been reported by different groups, contributing to the delineation of a well-characterized clinical phenotype (Ciafaloni *et al.*, 1991; Suomalainen *et al.*, 1992a; Moslemi *et al.*, 1996). An example of an adPEO family is shown in Fig. 1.

Subjects of each family can be divided into three groups: symptomatic patients, presymptomatic relatives, and normal relatives (Servidei *et al.*, 1991; Haltia *et al.*, 1992).

Clinically affected patients are characterized by adult-onset PEO, proximal weakness and wasting, and sensorineural hearing loss. Retinal degeneration is absent. Less consistent features are bilateral cataract, tremor, ataxia, extrapyramidal signs, and chronic sensorimotor peripheral neuropathy. In some families endogenous depression and psychotic traits were observed in several affected individuals (Ciafaloni *et al.*, 1991; Haltia *et al.*, 1992; MZ, unpublished observations). In other families endocrine manifestations were reported, including diabetes mellitus and hypogonadism (Melberg *et al.*, 1996). Serum lactate at rest may be moderately elevated in some cases. Examination of skeletal muscle typically shows the presence of ragged-red fibers, and a localized absence (or decrease) of the histochemical reaction to cytochrome *c* oxidase (COX). Biochemically, the activities of both respiratory complexes I and IV in muscle homogenates range from normal values to about 50% of the normal mean.

The penetrance of the disease is high, although not complete: in all series of adPEO families that were reported in the literature approximately 50% of isogenerational adult individuals were affected (Fig. 1). However, some individuals can appear clinically normal, but in most cases the presence of a subclinical mitochondrial disorder can be documented on the basis of laboratory (e.g., elevated blood lactate levels at rest), electrophysiological (e.g., abnormal EMG), morphological, or biochemical findings. Interestingly, the subclinically affected patients have lesser amounts of deleted mtDNAs (ΔmtDNA) in their muscle biopsies, compared to clinically affected patients, further indicating a quantitative relationship between mtDNA damage and phenotypic expression (Moslemi *et al.*, 1996). Moreover, the subclinically affected individuals are usually younger than the symptomatic patients, suggesting an age-related accumulation of the mtDNA lesions in muscle.

Autosomal Recessive PEO

Multiple ΔmtDNAs were reported by Yuzaki *et al.* (1989) in muscle specimens from two siblings with PEO, optic atrophy, muscle weakness, and peripheral neuropathy. However, in contrast to the adPEO families, the two siblings were the only affected members

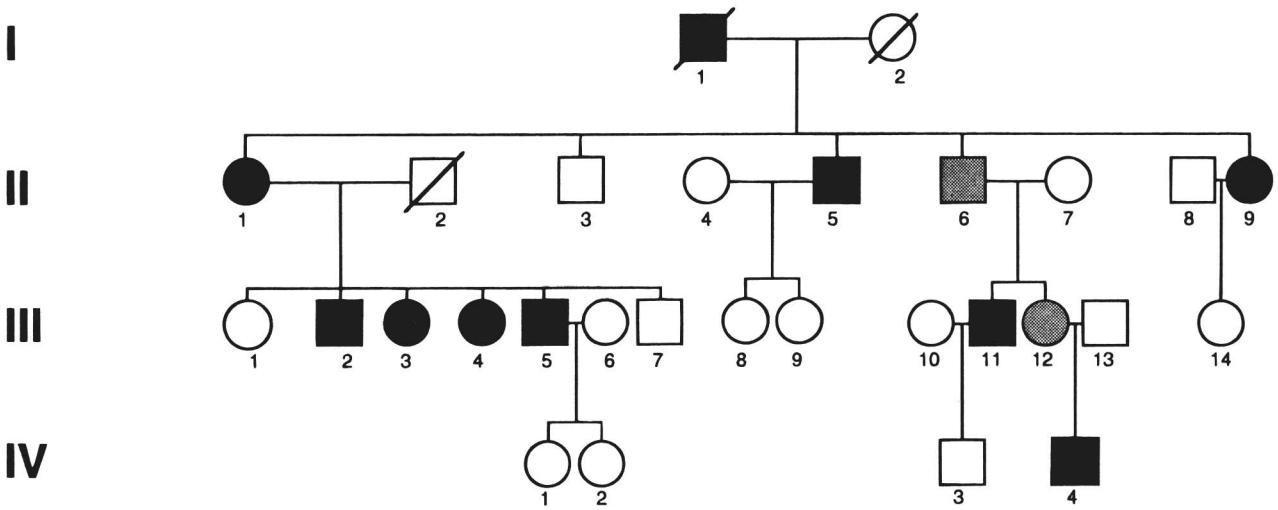

Fig. 1. Pedigree tree of an adPEO family. Black symbols indicate clinically affected patients; shaded symbols and open symbols indicate carriers (or subclinically affected patients) and normal individuals, respectively.

of the pedigree, and were born from consanguineous, apparently healthy parents. The suggested mode of transmission was autosomal recessive (Mizusawa *et al.*, 1988).

An autosomal recessive syndrome associated with multiple mtDNA deletions in muscle was later found in six patients from two unrelated families from eastern Arabia. The patients presented with childhood-onset, autosomal recessive PEO (arPEO), mild facial and proximal limb weakness, and severe cardiomyopathy, requiring cardiac transplantation (Bohlega *et al.*, 1996).

Clinical Variants

Multiple mtDNA deletions were found in one family in which the proband suffered from ataxia and ketoacidotic comas (Cormier *et al.*, 1991). A progressive multisystem involvement appeared in the course of the disease, and histopathological investigation demonstrated a mitochondrial myopathy with ragged-red fibers. Deletions were found in the healthy mother and maternal aunt as well, but neither in other maternal nor in the paternal members of the family. Deleted mtDNAs were much less numerous than in the other reported cases, and were clearly detectable in both muscle and peripheral lymphocytes. These unusual features suggest that this disease should be considered as a distinct nosological entity.

A further phenotype was described by Ohno *et al.* (1991) in two brothers with inherited recurrent exertional myoglobinuria and alcohol intolerance. Distinct morphological abnormalities were observed in muscle mitochondria, including ragged-red fibers, partial depletion of COX, and intramitochondrial paracrystalline inclusions. Neither patient showed external ophthalmoplegia, pigmentary retinopathy, cardiac conduction block, ataxia, myoclonic epilepsy, or stroke-like episodes. Several bands corresponding to deleted mtDNA were found in muscle specimens from both siblings, although in different quantitative proportion. PCR amplification failed to detect deletions in platelet mtDNA of the two patients, their mother, and a healthy brother. Genetic analysis suggested an autosomal recessive trait. Similar clinical and molecular features were found in two Italian brothers (F. Dworzak and M. Z., unpublished observation).

Multiple deletions of mtDNA have also been reported in yet other familial or sporadic phenotypes, including sporadic PEO cases (up to 11% in our series) (Zeviani *et al.*, 1996), Mitochondrial Neuro-gastro-intestinal encephalomyopathy (MNGIE syndrome) (Hirano *et al.*, 1994), sideroblastic anemia and mitochondrial myopathy (Casademont *et al.*, 1994), periodic paralysis (Prelle *et al.*, 1993), dilated cardiomyopathy (Suomalainen *et al.*, 1992b), Wolfram syndrome (Barrientos *et al.*, 1996), and mitochondrial myopathy and hypertrophic cardiomyopathy (Takei *et al.*, 1995).

Finally, the discovery of multiple mtDNA deletions in inclusion body myositis (IBM) (Oldfors *et al.*, 1993) deserves special mention. In a study on 56 IBM patients, Santorelli *et al.* (1996) found multiple deletions detectable by Southern blotting in 26 cases (46%), while PCR revealed deleted mitochondrial genomes in an additional 15 patients, in whom Southern blotting results were negative. Although a primary pathogenic role of the accumulation of mtDNA deletions in IBM appears unlikely, it is tempting to speculate that the intranuclear alterations typical of IBM may damage the same nuclear gene(s) involved in adPEO or arPEO.

Molecular Features

Southern-blot analysis of mtDNA from symptomatic and presymptomatic patients, as well as normal individuals, is usually performed on "linearized" mtDNA obtained by digesting total muscle DNA with suitable restriction endonucleases. In affected individuals results show the presence of numerous hybridization bands of different size, including a major 16.5-kb band, corresponding to wild-type mtDNA, as well as several smaller bands, corresponding to deleted mtDNAs (Fig. 2). "Pleioplasmy" is the term proposed by Yuzaki *et al.* (1989) to indicate the coexistence of multiple forms of mtDNA. No deletions are detected in normal individuals, while young presymptomatic individuals may show fewer or hardly any abnormal bands.

The physical mapping of the deletions has been worked out by both Southern-blot and PCR-based strategies, and by sequence analysis of the abnormal mtDNAs around the deletion breakpoints (Zeviani *et al.*, 1989, 1990; Ville-Ferlin *et al.*, 1995; Moslemi *et al.*, 1996).

The main results of these studies can be summarized as follows (Fig. 2):

1. Most, albeit not all, deletions were contained within the major arc spanning from the origin of H-strand replication (O_H) to the origin of L-strand replica-

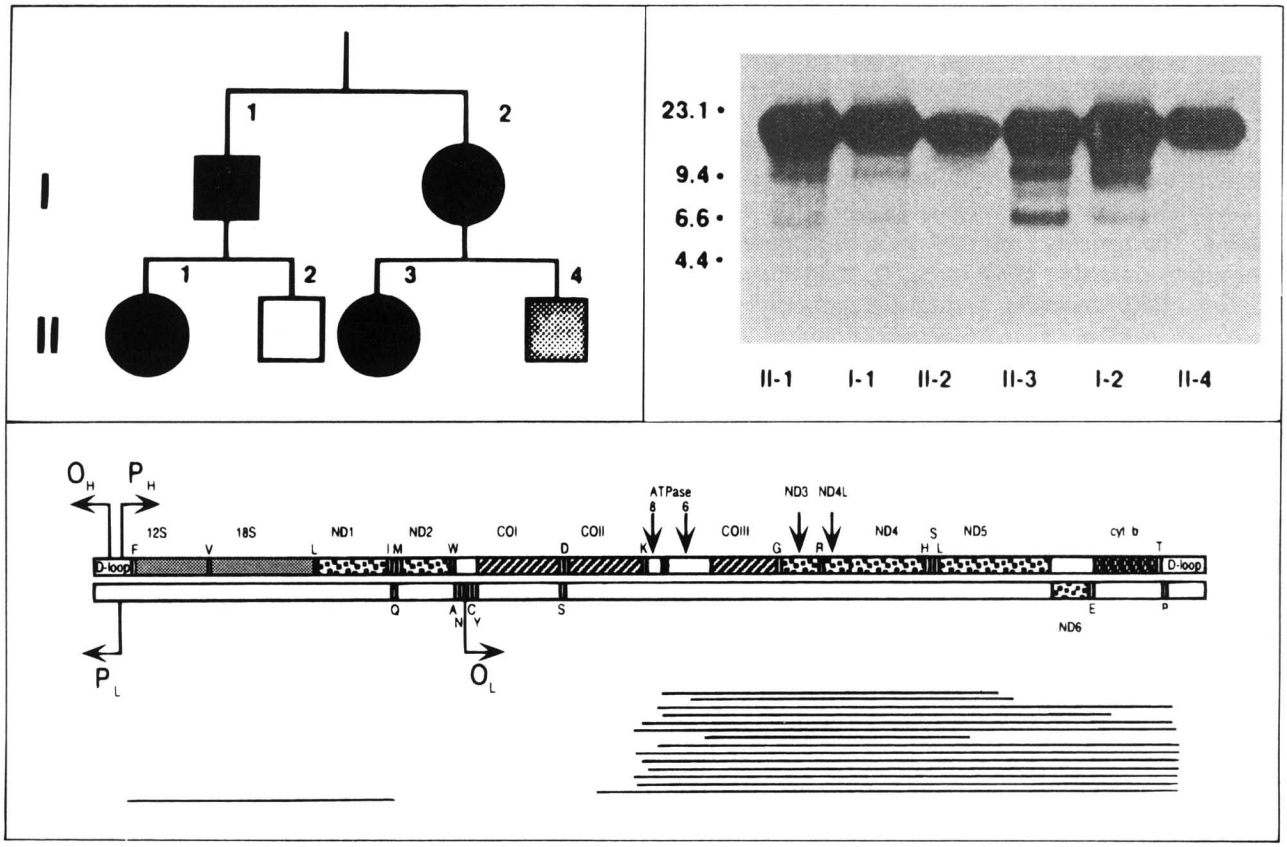

Fig. 2. adPEO: molecular features. Southern-blot analysis of *Pvu* II-digested muscle DNA extracted from individuals of an adPEO family (right upper panel). Samples of DNA from each propositus are indicated according to the nomenclature of the pedigree (left upper panel). Sizes of the markers are in kilobases. The solid bars in the bottom panel represent a collection of deletions positioned against a linearized map of human mtDNA. O_H and O_L indicate the origins of replication of the H-strand and L-strand; P_H and P_L denote the heavy-strand and light-strand transcriptional promoters; ND1-6 are the genes encoding the subunits of complex I; COI–III those for complex IV; ATPase 6 and 8 are the genes encoding subunits 6 and 8 of complex V; cyt *b* is the gene encoding cytochrome *b* (complex III); the tRNA genes are indicated by the single-letter code of the corresponding amino acids; 12S and 16S denote the genes encoding the small and large rRNAs.

tion (O_L). By contrast, only a minority of ΔmtDNA molecules did show deletions in the minor arc region spanning from O_L to O_H. Detailed PCR-based analysis of deletion breakpoints strongly suggested the existence of a molecular mechanism acting on "hot-spot" sequences scattered throughout a wide area of mtDNA. In particular, a hot-spot for mutations was localized at the end of the D-loop region, between nt 16060 and nt 16080 (Anderson *et al.*, 1981). However, in contrast to some previous indications (Zeviani *et al.*, 1989), this was not the only site where deletions occurred, because breakpoints were also detected in regions well downstream from the end of the D-loop (Zeviani *et al.*, 1990; Ville-Ferlin *et al.*, 1995; Moslemi *et al.*, 1996).

2. The D-loop, a crucial region containing both the O_H and the transcriptional promoters, was conserved in all of the mutant species. However, deletions encompassing the entire D loop region, including O_H, or the region containing O_L, were described in one sporadic case (Ville-Ferlin *et al.*, 1995). This finding raises the interesting hypothesis that alternative replication initiation sites may be present in non-D loop areas or outside O_L, allowing the maintenance and perpetuation of D loop-less or O_L-less mitochondrial genomes.

3. The molecular rearrangements occurred across flanking direct repeats of variable length. However, in contrast to single, sporadic deletions (Schon *et al.*, 1989; Mita *et al.*, 1990), repeats were often very short, and/or were present in the vicinity of, but not exactly at, the breakpoints (Zeviani *et al.*, 1989, 1990; Moslemi *et al.*, 1996).

Tissue Distribution

In adPEO, multiple ΔmtDNA species were first detected in skeletal muscle biopsies, the only affected

tissue available for molecular studies. Cultured fibroblasts, peripheral blood cells, and cultured myoblasts from our patients consistently failed to show any ΔmtDNA (Servidei *et al.*, 1991). Moreover, ΔmtDNAs were absent in cultured clones of both postmitotic muscle cell lines (myotubes), and *in vitro* innervated muscle cells, which were cultured for more than two months (G. Meola, unpublished results). However, the only postmortem study performed to date showed that one patient belonging to a Finnish adPEO family suffered from a multisystem disorder with widespread distribution of ΔmtDNAs in stable tissues. The autopsy of the patient's sister, who also had PEO, confirmed these results: the basal ganglia and the cerebral cortex harbored the highest proportion of mutant mtDNA, followed by skeletal muscle, extraocular muscles, and heart. By contrast, the viscera (kidney, liver) and some brain areas, such as the cerebellum, contained hardly any deleted genomes (Suomalainen *et al.*, 1992a).

In a recent study on a large Swedish adPEO family, Moslemi *et al.* (1996) have demonstrated that ΔmtDNAs are not randomly distributed in muscle fibers of affected individuals. Experiments based on *in situ* hybridization showed that the highest accumulation of transcripts from ΔmtDNA species was confined to the COX-deficient, ragged-red fibers. In these fibers, low levels of transcripts of wild-type mtDNA were also observed. This is similar to what has been reported in sporadic PEO or Kearns–Sayre syndrome due to single mtDNA deletions (Shoubridge *et al.*, 1990; Moraes *et al.*, 1992; Hammans *et al.*, 1992).

Molecular Pathogenesis

An etiological role of the ΔmtDNA pleioplasmy in adPEO is indicated by the following lines of evidence:

(1) A tight segregation of the molecular lesions with the disease was found in all of the adPEO families so far reported.

(2) The PEO phenotype, frequently complicated by proximal muscle weakness and progressive signs of CNS involvement, is commonly associated with mtDNA deletions in sporadic patients (Zeviani *et al.*, 1988; Moraes *et al.*, 1989; Holt *et al.*, 1989).

(3) A close correlation between accumulation of deletions and the segmental ragged-red transformation of muscle fibers, accompanied by the localized absence of COX activity, has recently been demonstrated by Moslemi *et al.* (1996) by *in situ* detection of ΔmtDNA transcripts in muscle biopsies of adPEO patients.

The pedigree structure of adPEO families (Fig. 1) indicates that the mitochondrial genome is damaged by dominantly inherited mutation(s) of still unknown nuclear gene(s).

Although the molecular mechanism leading to deletions is still obscure, one possibility is that the abnormal product(s) of the nuclear gene(s) act(s) by facilitating and amplifying an intrinsic propensity of mtDNA to undergo rearrangements. This view is supported by the observation that direct repeats and palindromic sequences have been implicated in the excision and recombination of long stretches of mtDNA in lower eukaryotes (de Zamaroczy *et al.*, 1983, Gross *et al.*, 1984; Turker *et al.*, 1987) and in several metazoan species (Powers *et al.*, 1986; Moritz and Brown, 1987; Buroker *et al.*, 1990). Furthermore, the identification of single mtDNA deletions or duplications in humans, produced by the clonal amplification of an apparently sporadic event, indicates that large-scale rearrangements of mtDNA can spontaneously, though rarely, occur. Likewise, ΔmtDNAs are detected in low amounts in tissues of normal elder humans (Cortopassi and Arnheim, 1990), and sequence heterogeneity may occur in mtDNA of humans (Sato *et al.*, 1989) and rodents (Pikò *et al.*, 1988) as an age-related phenomenon.

Similar to single sporadic mtDNA deletions, multiple familial deletions occur across flanking direct repeats. In contrast to sporadic cases, however, in familial cases the repeats are frequently nonperfect, usually very short (triplets or quadruplets), sometimes slipped and occasionally interrupted or inverted. Furthermore, deletions take place at hot-spot regions which have never been described in single, sporadic deletions (Mita *et al.*, 1990). These observations do suggest that the molecular mechanism by which multiple mtDNA deletions are produced is probably identical, or very similar, to that causing single deletions, but both the frequency of, and the propensity for, deletional events are extremely potentiated in the familial cases. Since homologous recombination seems to be absent or rare in the mitochondrial system of multicellular animals (Wallace, 1987), the most likely mechanism could be the slippage and mismatching of single strands during replication (Shoffner *et al.*, 1989). This hypothesis strongly suggested by the observation that in both single and multiple deletions the molecular rearrangements predominantly affect the portion of the mtDNA molecule which is exposed as single strands for most of the replication process (Clayton, 1982). Base-mispairing could be facilitated by the extremely slow replication of mtDNA in tissue cells, the presence

of long single-stranded stretches in replicating molecules, due to the asymmetry in the replication of the two strands, and the potential for secondary structure formation in the displaced mtDNA strand during replication (Clayton, 1982; Shoffner *et al.*, 1989; Mita *et al.*, 1990).

A second possibility to explain the generation of multiple mtDNA deletions is the malfunctioning of a gene encoding a hypothetical factor devoted to selecting and eliminating abnormal mtDNAs. This possibility is supported by the observation that in normal mammalian tissues mtDNA heterogeneity is rare and usually confined to small length variations in the D-loop region (Greenberg *et al.*, 1983; Hauswirth *et al.*, 1984), suggesting that extensive intraindividual mtDNA heterogeneity may not be tolerated and that heteroplasmic genomes may be rapidly sorted out by either cellular or molecular selection (Upholt and Dawid, 1977; Takahata and Maruyama, 1981; Chapman *et al.*, 1982).

A third hypothesis is that accumulation of multiple deletions occurs as a result of malfunctioning of the scavenging systems that protect the mitochondrial environment from the action of oxygen free radicals. It has been suggested that the formation of free radicals results in mtDNA mutations in normal subjects. Free oxygen radicals, particularly the hydroxyl radical, arise as by-products of the oxidative metabolism, and as compounds of high chemical reactivity causing peroxidation of membrane lipids, accumulation of age-related pigments, and DNA fragmentation (reviewed by Harman, 1981). In the brain, the formation of free radicals could be associated especially with the high glucose uptake and the oxidative metabolism of the cerebral cortex, or with the dopaminergic metabolism of basal ganglia (Soong *et al.*, 1992; Corral-Debrinski *et al.*, 1992). The cerebellum, not showing as much deleted mtDNA as the other brain regions, is not as active in these metabolic functions (Soong *et al.*, 1992; Corral-Debrinski *et al.*, 1992). If the free radicals accounted for the multiple deletions of mtDNA in familial CPEO, the defective nuclear protein that predisposes mtDNA to deletions could be a protein normally eliminating the free radicals or protecting mtDNA from them, or even a protein that, when defective, could create them.

Finally, in experiments based on PCR amplification of mtDNA from sections of single muscle fibers, Moslemi *et al.* (1996) showed that within a single COX-deficient muscle fiber segment, only one single deletion could be detected. However, different deletions were identified in different segments. These results indicate clonal expansion of a single ΔmtDNA in each COX-deficient muscle fiber segment. A two-hit mechanism can therefore be hypothesized, consisting of the combination of a nuclear factor, which is somehow predisposed to mtDNA deletions, with the subsequent clonal expansion of each ΔmtDNA in muscle and other stable tissues. According to this model, the number of somatic mutational events leading to mtDNA deletions should be relatively low. A combination of low mutational rate and active selection against deletion-containing cells could explain the absence of ΔmtDNAs in *in vitro* systems such as fibroblast- and muscle-cell cultures, or in rapid-turnover cells such as lymphocytes. The functional status of any given tissue will ultimately depend upon the accumulation of deleted mtDNA species. The mechanism leading to such accumulation is presently unknown. However, it seems reasonable that it can occur independently from the "first hit" due to the primary nuclear mutation, and be influenced by local conditions within individual cells or areas in a tissue. This could explain the regional variation of detectable deletions in muscle fibers and brain, as well as the variability of neurological symptoms in different individuals.

Identification of adPEO Loci

The availability of numerous adPEO families has made it possible to adopt a strategy based on primary linkage analysis, to localize the adPEO disease locus (or loci), a crucial step to eventually identify the responsible gene(s). Linkage analysis on large selected families with microsatellite markers regularly spaced throughout the human autosome has been effective in finding two adPEO loci in selected families. As in other autosomal dominant traits, the major obstacles encountered in this work have been the presence of genetic heterogeneity, in spite of a substantially phenotypic homogeneity, and the uncertainty in the attribution of the affection status to apparently healthy individuals that could not undergo a muscle biopsy examination. A first locus was found on chromosome 10q23.3–24.3 in a single large Finnish family (Suomalainen *et al.*, 1995). This locus was excluded in two Italian families, indicating that the disease was genetically heterogeneous. A second adPEO locus was later found in three Italian families on chromosome 3p14.1–21.2 (Kaukonen *et al.*, 1996). Evidence indicating the existence of at least a third locus was provided in the same study by exclusion data of additional adPEO

families. The presence of genetic heterogeneity is not unexpected, considering the extraordinary complexity of the nuclear control over mitochondrial biogenesis.

The physical cartography of the two identified adPEO loci, the genetic characterization of new families, and the search for gene candidates by position and function, are complementary strategies aimed at the elucidation of the genetic basis of this disease.

QUANTITATIVE ABNORMALITIES: mtDNA DEPLETION

Depletion of mtDNA is a tissue-specific mitochondrial disorder whose clinical manifestations fall into three groups: (1) a fatal infantile congenital myopathy with or without a DeToni–Fanconi renal syndrome; (2) a fatal infantile epatopathy leading to rapidly progressive liver failure; and (3) a late infantile or childhood myopathy characterized by progressive myopathy causing respiratory failure and death by 3 years of age. In a few cases, the presence of affected siblings born from healthy parents suggested an autosomal recessive mode of inheritance, possibly involving a nuclear gene controlling the mtDNA copy number. Southern blot analysis is diagnostic (Fig. 3) in demonstrating the severe reduction of mtDNA in affected tissues (up to 98% in the most severe forms) (Moraes *et al.*, 1991; Tritschler *et al.*, 1992; Mazziotta *et al.*, 1992; Mariotti *et al.*, 1995).

Etiological Considerations

The etiology of mtDNA depletion in humans remains obscure.

Three hypotheses have been proposed to explain the pathogenesis of this disease.

According to the first hypothesis (Moraes *et al.*, 1991), depletion of mtDNA could be the result of a nuclear DNA mutation causing an alteration in mtDNA that impairs replication. However, no evidence of mtDNA mutations in regions responsible for the replication of the mitochondrial genome (e.g., D-loop, O_H, O_L) was obtained in two siblings affected by early-onset mitochondrial myopathy (Moraes *et al.*, 1991), and in a third patient with a similar phenotype studied by us (Mariotti *et al.*, 1995). In addition, no clearcut evidence of maternal inheritance was obtained in any of the reported families.

The nonmaternal mode of inheritance suggests a mutation in a nuclear gene. Accordingly, a defective mutation in any of the (nuclear-encoded) proteins which are known to participate in mtDNA replication could also result in mtDNA depletion. This is supported by the demonstration that AIDS patients under long-term treatment with Zidovudine (AZT) can develop a destructive mitochondrial myopathy with ragged-red fibers (Dalakas *et al.*, 1990; Arnaudo *et al.*, 1991). This phenotype resembles the infantile myopathy described by Moraes *et al.*, (1991) and it is likewise associated with severe depletion of mtDNA in muscle. The proposed pathogenetic mechanism is the pharmacological block of γ-DNA polymerase, whose enzymatic activity is profoundly inhibited by AZT (Izuta *et al.*, 1991). Decreased mtTFA cross-reacting material has indeed been reported in one case of mtDNA depletion in muscle, but this finding is probably due to rapid degradation of the unbound protein, rather than to mutations or reduced expression of the mtTFA gene (Larsson *et al.*, 1994).

A third possibility (Moraes *et al.*, 1991) is that the genetic defect causes an error in the resumption of mtDNA replication after the blastocyst stage during early embryogenesis. The number of mitochondria and mtDNAs does not change appreciably until the blastocyst stage (Pikò and Taylor, 1987). A delay of mtDNA replication beyond the blastocyst stage, at the time of germ-layer differentiation, could produce a permanent tissue-specific depletion of mtDNA.

Recently, Bodnar *et al.*, (1993) observed the repopulation to normal mtDNA levels of transmitochondrial cybrids obtained by fusing cytoplasts derived from fibroblasts affected by mtDNA depletion with a mtDNA-less human tumoral cell line. This result suggests the nuclear origin of the defect in their case. However, the case of Bodnar *et al.* is the only example so far reported of mtDNA depletion found in cultured fibroblasts; in all of the remaining cases, the amount of mtDNA in rapid-turnover cells turns out to be normal. In one early-onset myopathic case studied by us (Mariotti *et al.*, 1995), fibroblasts, myoblasts, and lymphocytes all showed normal amounts of mtDNA, in spite of a reduction to 2–10% of the normal content of mitochondrial genomes in mature skeletal muscle. Likewise, myoblast-derived myotubes were unaffected, even after prolonged innervation. In addition, a complete and rapid recovery of mtDNA content was observed after pharmacological mtDNA depletion in the patient's myoblasts produced by exposure to ethidium bromide. These considerations, and the observa-

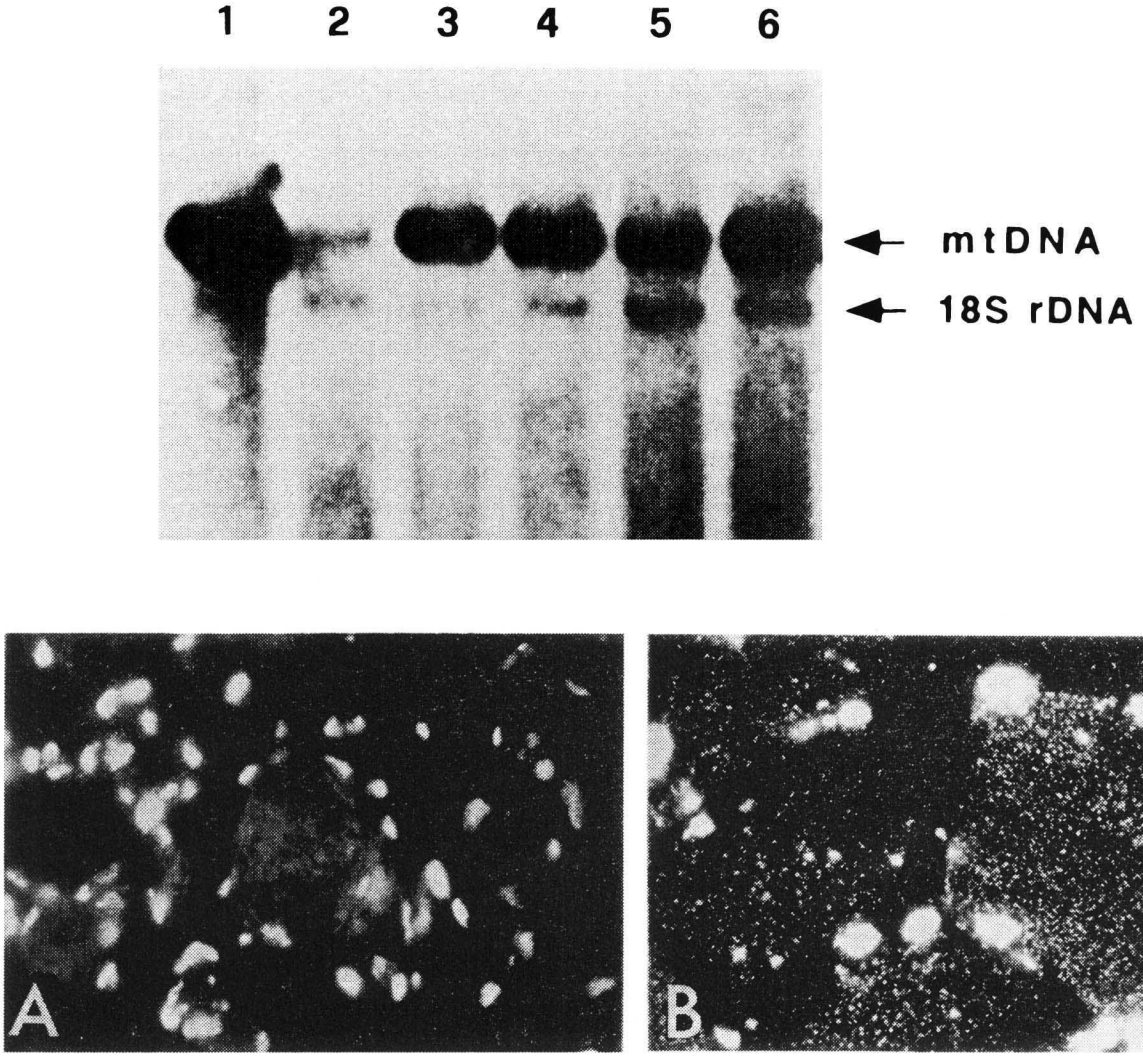

Fig. 3. MtDNA depletion. Upper panel: Autoradiography of Southern-blot hybridization of *Pvu* II-digested genomic DNA samples probed with chemiluminescent mtDNA and 18S (nuclear) rDNA probes. Lane 1: muscle DNA sample from the mother of a patient affected by early-onset mitochondrial encephalomyopathy. Lanes 2–6: DNA samples from different tissues of the propositus. Lane 2. Muscle. Lanes 3. Cultured myotubes. Lane 4: Cultured myoblasts. Lane 5: Cultured skin fibroblasts. Lane 6: Blood lymphocytes. Lower panel: Anti-DNA immunostaining of a muscle biopsy of the propositus (A) and of a control subject (B). A strong signal is detected in the nuclei from both the patient's and a normal human muscle. A fine "starry sky-like" pattern, corresponding to mtDNA, is present in the cytoplasm of the control muscle (B), but not in the cytoplasm of the patient's muscle (A). Magnification × 250.

tion that mtDNA depletion can be associated with a wide spectrum of clinical presentations, suggest that this condition is probably heterogeneous.

CONCLUSIONS

The existence of abnormalities in the nuclear gene repertoire controlling mitochondrial biogenesis has been proposed as the cause of clinical phenotypes characterized by the presence of mtDNA abnormalities associated with disease and transmitted as Mendelian traits. Genes involved in the control of mtDNA replication and expression can be considered as attractive candidates. Thus, characterization of the human mitochondrial proteome is a matter of convergent interest between basic scientists involved in the elucidation of the fundamental mechanisms of nucleus–mitochondrion intergenomic

signalling, and clinically-oriented researchers interested in mitochondrial disorders. Intensive investigation in recent years, based on the information obtained from the purification of several mtDNA-related activities, has allowed the identification of important human genes, including those encoding the mitochondrial transcription factor A (Parisi and Clayton, 1991), the mitochondrial single-stranded DNA binding protein (Tiranti *et al.*, 1993), the gamma DNA polymerase (Ropp and Copeland, 1996), etc. However, this work has been complicated by protein lability, low amount, or difficulty of separating mitochondrial activities from analogous activities present in the nucleus or in other cellular compartments. A complementary approach relies upon the remarkable wealth of information on mitochondrial housekeeping genes that has been accumulated over the last two decades in lower eukaryotes, particularly in the yeast *Saccharomyces cerevisiae*. Cross-species hybridization between yeast and human genes has allowed the identification of additional mitochondrial genes such as OXA1 (Bonnefoy *et al.*, 1994), which encodes an important protein controlling the assembly of respiratory complexes IV and V. A further potentiation of this approach has recently been proposed, namely the computer-based screening of the human Expressed Sequence Tags database (dbEST) using sequences of mtDNA-related proteins available in yeast. Preliminary results from our laboratory and elsewhere have confirmed the power of this strategy, which can usefully complement traditional methods in working out the molecular basis of nuclear-mitochondrial communication.

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