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Heteroplasmy in Chronic External Ophthalmoplegia: Clinical and Molecular Observations

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ABSTRACT. Chronic progressive external ophthalmoplegia (CPEO) describes a recognizable clinical syndrome frequently associated with variable dysfunction in other organ systems. Histochemical and biochemical studies suggested primary dysfunction of oxidative phosphorylation. This has recently been confirmed by demonstration of partially deleted as well as normal mitochondrial DNA—heteroplasmy—in some of these patients, most of them sporadic. In the six heteroplasmic CPEO patients that we have examined to date, the partially deleted species has been detected in all tissues tested, albeit in vastly different proportions. We report here detection of physiologically significant proportions of partially deleted mitochondrial DNA in several organs taken at autopsy from a CPEO patient with severe multisystem disease. We discuss the relationship of CPEO to several other clinical phenotypes associated with mitochondrial dysfunction, and discuss the possible implications of heteroplasmy for the development of variable phenotypes. (*Pediatr Res* 28: 542–548, 1990)

Abbreviations

CPEO, chronic progressive external ophthalmoplegia
MELAS, mitochondrial encephalopathy, lactic acidosis, and stroke-like symptoms
MERRF, myoclonic epilepsy with ragged red fibers
LHOA, Leber's hereditary optic atrophy

CLINICAL FEATURES OF CPEO

In strict usage, the term CPEO describes only a clinical sign—paresis of the muscles that rotate the eyes in the orbit and elevate the eyelids, with sparing of internal ocular muscles that control the lens and the iris. However, it has also come to connote a specific disease in which such paresis is the predominant clinical finding. This latter meaning was used interchangeably with the terms "mitochondrial myopathy" or "ragged-red fiber disease," until recent descriptions of other clinical phenotypes associated with similar pathologic and biochemical changes.

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Although this term has proven useful, definition of a specific disease state on the basis of a single clinical sign is fraught with hazard. Frequent but variable involvement of nonocular tissues has added to the complexity of a strictly clinical diagnosis of CPEO. Kiloh and Nevin (1) considered CPEO to be a distinctive myopathic syndrome, with relatively isolated myopathy of the extraocular muscles, and only occasional mild weakness in the extremities. However, it became apparent that many CPEO patients had evidence of multisystem disease (2–4). There can be pigmentary degeneration of the retina, optic atrophy, cerebellar ataxia, sensorineural hearing loss, peripheral neuropathy, electroencephalographic abnormalities, and/or elevation of protein content of the cerebrospinal fluid. Less frequently, there are pyramidal or extrapyramidal signs, seizures, or frank cognitive impairment. A few pathologic studies of the nervous system have demonstrated spongiform poliodystrophy.

Although the paresis of the extraocular muscles in CPEO is assumed to be a myopathy, their dysfunction may result from primary failure of the nervous structures that control them (5). In CPEO, frank ophthalmoparesis is preceded by profound slowing of saccadic eye movements, with relative preservation of pursuit and vestibular eye movements, suggesting involvement of excitatory medium-lead "burst" neurons (6). These neurons likely have unusually high energetic demands, inasmuch as they selectively initiate saccadic eye movements with an extremely rapid volley of electrical discharge.

Cardiac pathology most frequently takes the form of conduction defects, typically intraventricular branch blocks or interventricular block, which, curiously, can be heralded by a period of enhanced A-V nodal conduction (7, 8). We have also observed supraventricular tachycardias and congestive heart failure. Pathologic studies have demonstrated fatty infiltration, fibrosis, and occasional giant mitochondria, both in conducting fibers and elsewhere in the myocardium (9).

Pathology is not limited to electrically excitable tissues. Renal dysfunction most often involves a proximal tubular defect, with aminoaciduria and phosphaturia—the De Toni-Fanconi-Debre syndrome (3)—but we have also observed glomerulopathy and renal failure. Proportionate short stature, scoliosis, and a high-arched palate have in some instances been associated with defective release of growth hormone; in others, they have not (3). Other endocrine dysfunction includes diabetes mellitus, sometimes latent until the patient is stressed by glucocorticoids (10), which had been used by some clinicians to treat these patients. Parathyroid dysfunction has also been described (3).

In summary, CPEO can present with clinical dysfunction of variable severity in a number of highly oxidative organs, reflecting primary pathology of tissues derived from many cell lineages.

Furthermore, the pattern of organ involvement and severity is notoriously variable. Understandably, this extreme pleiotropy gave rise to a plethora of diagnostic labels: in McKusick's catalogue, *Mendelian Inheritance in Man* (11), there are over a dozen listings for CPEO with different patterns of organ involvement. Of these, the most enduring has been that of Kearns and Sayre, who proposed that the triad of CPEO, retinal pigmentary degeneration, and heart block constitutes a distinctive syndrome (12). Several years later, Rowland and his colleagues (5) proposed that such a syndrome is indeed distinctive and that the definition be modified to include ophthalmoplegia, pigmentary retinopathy, onset before age 20, and at least one of three other features: either high cerebrospinal fluid protein, heart block, or ataxia. A commonly held view is that this expanded definition will distinguish those patients with systemic disease from those who have isolated involvement of the extraocular muscles.

In an earlier clinical review (13, 14), Drachman proposed a unified concept—ophthalmoplegia plus—lumping together all CPEO patients, whatever the pattern of nonocular disease. Some authors have even included patients without CPEO but with a typical constellation of neural and systemic involvement (3). Although this view may seem intellectually unsatisfactory—and indeed is demonstrably too global—recent genetic and laboratory studies recommend that aspects of this unitary view be reexamined.

BIOCHEMICAL AND MORPHOLOGIC FEATURES OF CPEO

The idea that many CPEO syndromes might be variations on the same fundamental theme finds support from laboratory findings indicating primary dysfunction of mitochondrial oxidative phosphorylation. Most studies have been performed on skeletal muscle, because of both historical tradition and practical considerations. However, similar changes have occasionally been observed in other tissues (15).

Histochemical and electron microscopic observation of skeletal muscle demonstrates that some, but never all, of type I fibers contain abnormally large collections of mitochondria, the morphology of which may be altered (16–18). Similar mitochondrial alterations have been induced in experimental animals that were exercised excessively or treated with toxins that interfere with oxidative phosphorylation at any of several biochemical sites. These changes can be prevented with chloramphenicol, a specific inhibitor of mitochondrial, but not nucleocytoplasmic, protein synthesis (19, 20). This suggests that these cellular changes are a nonspecific proliferative response to a mismatch between demands for oxidative phosphorylation and the ability to meet these demands.

Some of these mitochondria may contain as yet biochemically uncharacterized inclusions, often with a characteristic paracrystalline "parking-lot" morphology (16, 17). Curiously, the majority of fibers, including those immediately adjacent, have no morphologic alterations of mitochondria. This indicates heterogenous involvement even within the class of type I fibers, all of which are likely expressing identical nuclear-encoded isozymes. An even more telling observation was reported by John Morgan-Hughes and others (21–23): serial cross-sections revealed that most ragged-red fibers contain excessive activity of all mitochondrial enzymes, as would be expected. However, an occasional fiber showed increased numbers of ragged-red mitochondrial membranes by the Gomori trichrome stain and the expected increase of succinate dehydrogenase activity, but complete absence of cytochrome oxidase activity.

Biochemical observations give further support to the hypothesis of defective oxidative phosphorylation. Elevations of lactic acid, although mild and variable within the same patient, are observed frequently. In several of our CPEO patients, venous levels of lactic acid are normal at rest, but rise abnormally on graded exercise. Such tests are ideally performed with concomitant measurement of cardiac output, oxygen extraction, and consumption, with precise delineation of an anaerobic threshold (24–26). However, such tests are laborious and commonly require invasive catheterization.

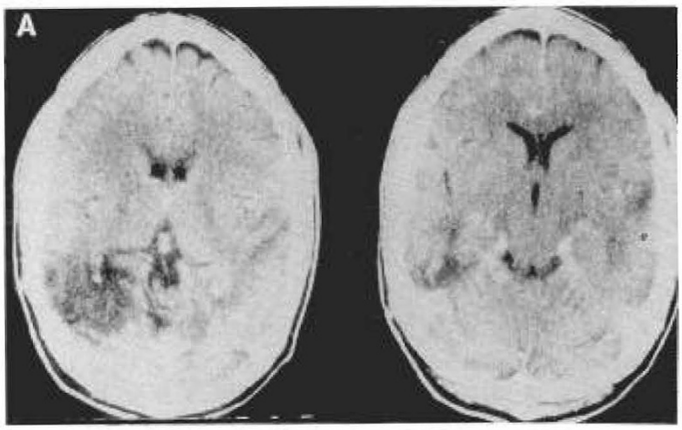
A more convenient indirect measure of oxidative phosphorylation, nuclear magnetic resonance spectroscopy of high energy phosphates before and after exercise, has indicated abnormalities in skeletal muscle of many CPEO patients (27). Recently, we have used a similar procedure to detect abnormalities of high energy phosphates in the frontal lobes of five patients with dissimilar mitochondrial encephalomyopathy phenotypes (28), among them two patients with different CPEO syndromes with no clinically evident dysfunction of the telencephalon.

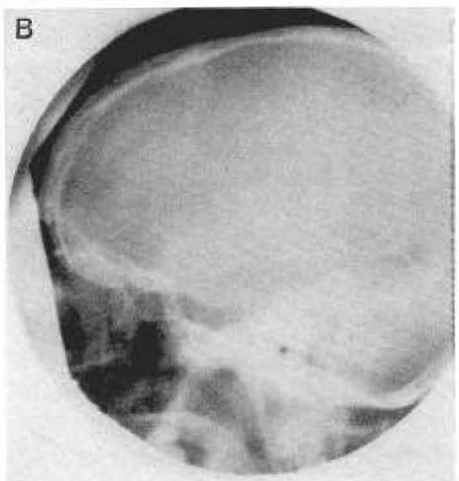
Further indirect evidence implicating mitochondrial dysfunction in CPEO and other ragged-red syndromes comes from reports of improvement after treatment with agents that might correct defective oxidative phosphorylation or its immediate consequences (29–32): cofactors for the protein components of the electron transport chain; "electron shuttles," substituting for a defective respiratory chain component, or scavengers of toxic free radicals that could accumulate in high ambient oxygen concentrations. Although isolated reports of clinical improvement have been impressive and, in some cases supported by spectroscopic, electrophysiologic, and/or lactate measurements, some of us have been disappointed in our ability to intervene successfully in the majority of our patients.

More direct assessments of mitochondrial function support further the idea of defective oxidative phosphorylation in CPEO, but even these assays can be problematic. Our current preference is for polarographic analysis of intact fresh mitochondria harvested from biopsied muscle (33, 34). Unfortunately, this requires a large biopsy specimen and, ideally, assay of nonfrozen material. The isolation of intact mitochondria from skeletal muscle—a tough, fibrillar tissue—is a delicate procedure: artifactual uncoupling (*i.e.* mimicking the biochemical phenotype of Luft's disease) can occur with minor variations in the homogenization procedure. Furthermore, polarography is routinely performed at ambient atmospheric oxygen concentrations, considerably higher than those encountered by most mitochondria *in vivo*. Finally, there is the problem of studying a mixture of normal and abnormal mitochondria.

Given these considerations, only equivocal alterations in oxidative phosphorylation have been found in many CPEO patients, even those in whom there is other indirect evidence of mitochondrial dysfunction. However, certain CPEO patients have demonstrated dysfunction in many sites along the electron transport chain. Complex I (NADH-coenzyme Q reductase) dysfunction has been found in a typical CPEO syndrome (35, 36) as well as a case in which paresis of vergence and upgaze only were minor features of a moderately severe, generalized myopathy (37); other CPEO patients had partial deficiency of complex IV (cytochrome oxidase) (21–23), sometimes with increased levels of cytochrome c (34). Complex III deficiency was observed in a father and son, only the latter having CPEO (38); a child with myopathy and ptosis but no other ophthalmoparesis (39) was reported to have severe deficiency of complex II (succinate dehydrogenase), the only respiratory complex that does not contain subunits encoded by mitochondrial DNA.

Conversely, dysfunction of a given site along the electron transport chain has been associated with many seemingly disparate clinical phenotypes (40, 41): some cases of normoglycemic congenital lactic acidosis (42–45); individual cases with Leigh's polioencephalopathy (46–48); MELAS syndrome (49–51); MERRF syndrome (26, 52–55); and proximal myopathy with easy fatiguability (2). Despite their biochemical and histologic similarities, they have been described as distinct clinical syndromes. However, upon following patients for several years, we have observed several instances of individuals progressing from one syndrome to another: a 34-y-old patient with MELAS who later developed typical CPEO and retinal pigmentary degeneration (Fig. 1); another 34-y-old in whom CPEO preceded the





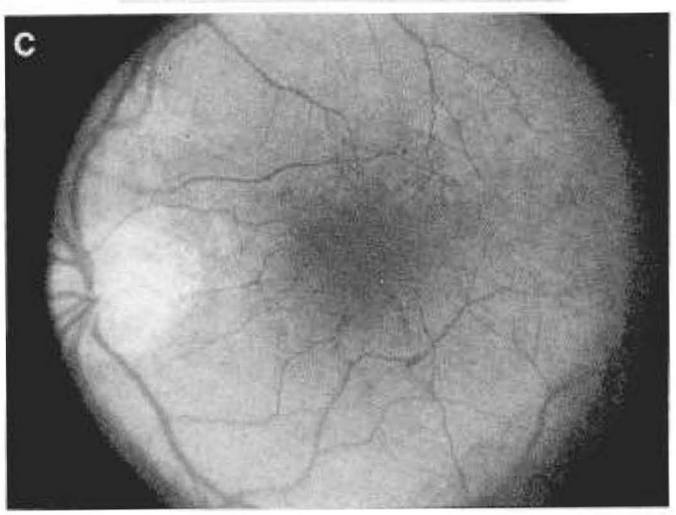


Fig. 1. Studies of a middle-aged man with MELAS and CPEO. *a,* Computerized tomogram of brain after his first stroke-like episode that left him with a permanent left homonymous hemianopia. *b,* Capillary phase of carotid arteriogram with early-draining vein, taken 2 y later when he developed pure word deafness and a lucency of his superior temporal lobe. *c,* Retinogram showing atypical pigmentary retinal degeneration and mild pallor of the optic nerve.

development of the MERRF syndrome by 6 y; and a patient who succumbed at age 23 after developing sequentially CPEO and pigmentary retinopathy, MELAS, and terminally, the MERRF syndrome.

These observations suggest that the clinical phenotype reflects more accurately the degree of impairment of flux through the entire electron transport chain and the anatomic distribution of abnormal mitochondria, rather than the precise biochemical site at which such flux is impaired.

GENETIC AND MOLECULAR OBSERVATIONS ON CPEO

The majority of CPEO patients are sporadic. Many CPEO patients with severe multisystem involvement are too ill to have children. However, one of our CPEO patients [a 52-y-old woman with partial deletions of mitochondrial DNA detected in skeletal muscle and peripheral blood (56)] has three healthy children in college. Small pedigrees with multiple individuals affected with CPEO have also been encountered (20, 57–59). In most of these pedigrees, including our own, CPEO has been transmitted along the maternal lineage. There have also been cases of paternal transmission of mitochondrial CPEO with systemic involvement to a son (3, 38, 59), suggesting involvement of an autosomal nuclear gene in those kindreds.

Speculation about the possible role of mitochondrial DNA in the pathogenesis of some ragged red syndromes was initially based on consideration of biochemical and morphologic abnormalities (60). More compelling, albeit inconclusive, evidence came from transmission genetics. Small pedigrees compatible with mitochondrial inheritance were published in a seminal paper by Egger and Wilson (59). In several of these pedigrees some affected individuals had ophthalmoparesis, but others—presumably affected by the same mutation—only had clinically apparent disease in other organ systems. Egger and Wilson presciently speculated that such variable organ involvement within a given family might reflect random segregation of normal and mutant mitochondria within a given individual. We have encountered such dramatic pleiotropy in one kindred that is segregating cytochrome oxidase deficiency (49).

Although mutations of either mitochondrial or nuclear genes may result in a CPEO or related phenotype, until recently the evidence for involvement of mitochondrial DNA in CPEO was inconclusive. In small pedigrees, one would expect occasional chance occurrence of strictly maternal transmission; many heritable diseases that exhibit pleiotropy and phenotypic variability within the same pedigree have impeccable credentials as autosomal dominant traits. The earliest direct evidence that a mitochondrial mutation was responsible for a case of CPEO came from Byrne *et al.* (61), who observed an altered electrophoretic mobility of a mitochondrial translation product in skeletal muscle. More direct evidence for mitochondrial DNA mutations in some instances of CPEO was provided by Holt, Harding, and Morgan-Hughes (62, 63), who demonstrated partial deletions in a subpopulation of mitochondrial DNA extracted from skeletal muscle. To their initial reports have been added others, including six of our own (56, 64–70). Most reported cases of mitochondrial DNA deletions, including all of ours, have come from patients with a sporadic CPEO syndrome, some of them fulfilling diagnostic criteria for the Kearns-Sayre syndrome, others not. No correlation between the size or molecular location of the deletion and the pattern of organ involvement is apparent. Indeed, this is not unexpected as most reported deletions associated with CPEO remove genes encoding components of the electron transport chain and also several transfer RNA genes. All but two of our patients have different deletions and all but one involve directly repeated sequences at the deletion junctions, the consensus of which resembles putative recombination signals (69, 70).

In all reported CPEO patients with partial deletions of mitochondrial DNA detected by Southern analysis, the partially deleted species were not found in blood cells. However, using a more sensitive assay (58, 68, 69) we and others have observed some partially deleted mitochondrial DNA molecules in all tissues tested from each such patient, albeit in vastly different proportions (56, 69, 70). This procedure is a modification of the standard polymerase chain reaction, using oligonucleotide primers with recognition sites widely spaced on normal mitochondrial DNA but closely bracketing the deleted region. It selectively amplifies deletion junctions even when normal, undeleted mitochondrial DNA is present in 1000-fold excess (69). As currently performed, it does not permit precise quantitation;

we can only infer that partially deleted mitochondrial DNA is a very minor species in the blood and urinary epithelial cells of those CPEO patients tested to date, because we can detect it by the polymerase chain reaction but not by Southern analysis. Although the small amounts of partially deleted species in blood and epithelium seem well below the threshold necessary for clinically significant disruption of oxidative phosphorylation in those cells, their presence clearly indicates that the origin of the mutation in these patients anteceded divergence of the muscle lineage. Thus, physiologically significant replacement of normal by mutant mitochondrial DNA is possible in tissues derived from any of the three primary cell layers.

Our only direct evidence on this point comes from Southern analysis on multiple tissues from an autopsied case, whose clinical and biochemical features were reported elsewhere (34, case 1). We report here that different proportions of partially deleted and normal mitochondrial DNA were readily detectable by Southern analysis of skeletal muscle, brain, heart, liver, and kidney (Fig. 2). The decrease in oxidative activity measured polarographically in these tissues paralleled the rank order of increasing proportion of partially deleted mitochondrial DNA in all tissues but the liver, which was presumed to be autolyzed by the time of polarography. There was clinically evident dysfunction of skeletal muscle, heart, and brain, but liver and kidney were judged normal *in vivo*. In all tissues, the partially deleted species were identical, indicating common descent from a single mutational event. These observations, albeit limited, suggest that different patterns of organ involvement in CPEO patients may be, in part, quantitative rather than qualitative. What may matter most in these patients is not which region of the mitochondrial DNA is deleted, but how many normal mitochondria remain in a given cell. Indeed, several patients with Pearson's syndrome (infantile aplastic anemia and pancreatic failure) have a subpopulation of mitochondrial DNA with deletions identical at the molecular level to three of our adult CPEO patients, but with differing tissue distributions (71, 72).

An interesting contrast can be made between the ragged-red fiber syndromes described above, and another heritable disorder affecting oxidative phosphorylation, LHOA, discussed earlier in this symposium by Dr. Douglas Wallace. Although biochemical and histologic evidence of mitochondrial dysfunction in the ragged-red syndromes is strong, evidence from transmission genetics is indirect and inconclusive. In contrast, the major evidence of mitochondrial involvement in LHOA comes from compelling data in transmission genetics (73, 74), whereas biochemical and histochemical evidence of mitochondrial dysfunction is weak or lacking: lactate levels are normal, as are most polarographic and enzymatic studies of isolated mitochondria, and equivocal alterations of mitochondrial morphology are only evident in some electron microscopic studies (75). Definite proof of a minor oxidative phosphorylation defect in LHOA comes from Wallace's elegant association of a point mutation in a conserved region of a mitochondrial gene encoding a subunit of respiratory complex I (76). Furthermore, unlike those individuals with a ragged-red syndrome associated with a mutation in mitochondrial DNA, many LHOA patients appear homoplasmic for the mutant mitochondrial DNA species in all tissues tested to date.

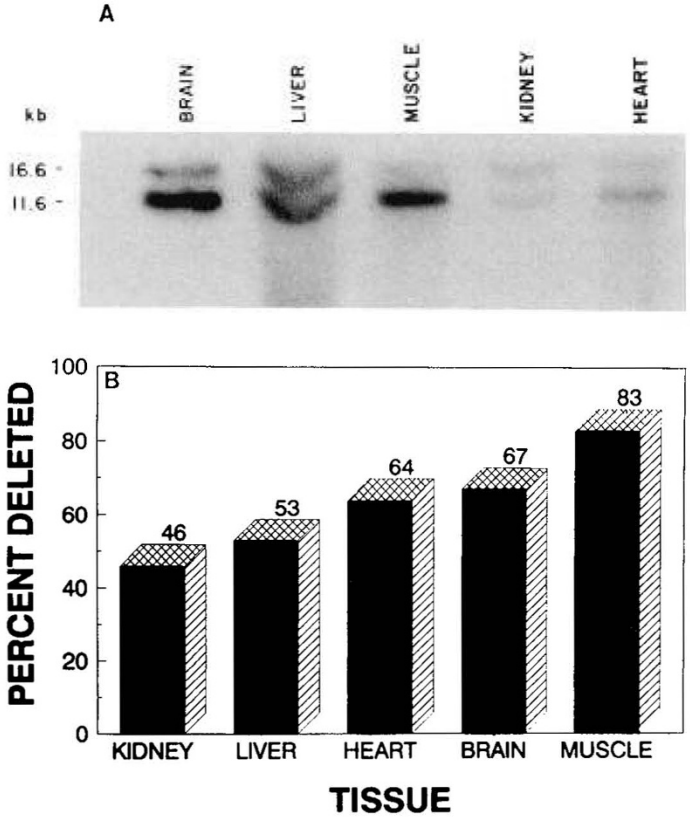


Fig. 2. Wild type and partially deleted mitochondrial DNA species in various organs harvested at autopsy from a child with CPEO and multisystem disease. *a*, Southern blot of total cellular DNA restricted with *Bam*HI, subject to electrophoresis on 1.1% agarose, transferred to nitrocellulose, hybridized with a cloned probe (prepared from HeLa mitochondrial DNA, nucleotides 16453 to 3245) that had been made radioactive by random-primer labeling, and visualized by autoradiography. *b*, Proportions of partially deleted species, as estimated by laser densitometry and expressed as a percentage of total mitochondrial circles.

THEORETICAL CONSIDERATIONS

Oxidative phosphorylation is such a fundamentally important process that it appears unlikely that a mutation completely disrupting the electron transport chain in every cell in the body will permit extrauterine life. Therefore, patients harboring mutations that affect oxidative phosphorylation must fall into one of two categories. *1*) Those patients with mutations that completely disrupt oxidative phosphorylation must also contain a complement of normally functioning mitochondria to sustain life. Tissues with a large complement of dysfunctional mitochondria would be amenable to biochemical diagnosis. This seems true in those CPEO patients with large partial deletions of mitochondrial DNA. *2*) Those mutations expressed in every mitochondrion in the body must disrupt oxidative metabolism only minimally. Therefore, direct biochemical verification of the abnormality may prove difficult. This appears true in those cases of LHOA in which a pathogenic point mutation has been determined.

In both situations, clinical involvement of some tissues but not others could result from a number of possible mechanisms. These include: *1*) differing requirements for oxidative metabolism in individual tissue types, which may change with activity and development; *2*) involvement of nuclear-encoded components of the electron transport chain or other regulatory proteins, some of which exist as tissue-specific isoforms (77); and *3*) interaction with variable, nongenetic environmental factors. Our discussion will be limited to the possible relationship of another factor—heteroplasmy, the presence of more than one type of mitochondrial DNA in an individual organism—to the clinical heterogeneity evident in CPEO and other ragged red syndromes.

In somatic cells of mammals, each mitochondrion contains about six loops of mitochondrial DNA (78), each a separate genetic unit; a mutation in one such mitochondrial genome will affect directly only itself and its daughter molecules. Because each somatic cell contains thousands of such molecules, it appears unlikely that such a mutation would significantly affect cellular metabolism unless it came to represent a major population. Such threshold effects of mitochondrial mutants conferring resistance to chloramphenicol have been demonstrated (79, 80),

and it appears likely that other mitochondrial mutants will behave similarly.

How daughters of a single mutant molecule can become a physiologically significant population in certain cell lineages may be understood by consideration of early embryonic development, during which the large cellular complement of mitochondrial DNA becomes sequestered into many smaller pools. During female gametogenesis, the ploidy of an individual mitochondrion becomes reduced to about one. After fertilization of the egg, the first few cleavage divisions occur without growth of the zygote or apparent replication of mitochondria, with the total number of mitochondria in the zygote remaining fixed (Fig. 3). Thus, a minor population of mutant mitochondria in the original ovum may, by chance, become a major population in certain cells of the blastocyst. Thus, it seems likely that stem cells of various cell lineages may, at random, receive different proportions of normal and mutant mitochondria. The relative proportions of normal and mutant mitochondria in adult tissues, may, in part, reflect the proportions contained in their progenitor cells.

In mammals, only a few cells in the blastocyst give rise to the embryo; most others are trophoblastic, and are thus eventually discarded. It is by this mechanism that homoplasmy is usually maintained (81). In studies of cows with nonpathogenic polymorphisms of mitochondrial DNA, Laipis, Hauswirth, and colleagues (82–85) documented occasional transitions to heteroplasmy, as well as rapid shifts between one apparently homoplasmic state and another. Because these shifts occur in the absence of any obvious selective pressure, they presumably result from random mitotic drift and embryonic sequestration.

Similar processes may occur in patients with pathogenic mutations of mitochondrial DNA, with added complexity superimposed by various selective pressures. In the specific case of partially deleted mitochondrial DNA, different selective pressures may be exerted at different levels of organization. At the level of an individual molecule with intact origins of replication, the deleted species, being smaller, may enjoy a replicative advantage over its intact, wild type counterparts. This effect would be most evident in postmitotic tissues, allowing gradual selective accumulation throughout life. At the level of the cell, selection may be a function of the proportion of normal mitochondria and the oxidative demands on a particular cell. Early mammalian embryogenesis in the fallopian tubes and shortly after implantation occurs at low oxygen concentrations. Thus, mutations affecting oxidative phosphorylation may be selectively neutral during the early sequestration and sorting of mitochondria described above. However, at later stages, whenever a particular cell's capacity for oxidative metabolism drops below a critical threshold, one may expect negative selection, poor growth or possibly death of the cell. Cells that remain mitotically active throughout life, such as blood and epithelium, are more likely to generate, through random drift, populations with skewed representation of either normal or mutant species, and on whom such selection may be exaggerated. However, above a critical threshold of oxidative capacity, one might expect positive selective pressure—preferential proliferation of all mitochondria in those cells with some defective organelles—because cells can respond to a mismatch between oxidative capacity and demand by inducing mitochondrial proliferation and increasing cellular ploidy of mitochondrial DNA (86, 87). We speculate that such selective mitochondrial proliferation may account for the formation of ragged red fibers. Furthermore, rare deletion events in mitochondrial DNA of postmitotic tissues such as heart or brain may become selectively amplified in the course of normal aging.

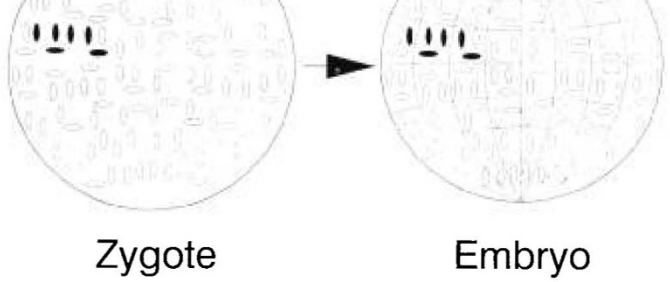


Fig. 3. During early embryogenesis, the total number of mitochondria does not increase, but the subdivision of the fertilized zygote into a large number of cells sequesters small groups of adjacent mitochondria. Only a few of these cells give rise to the final embryo. By chance, certain cells acquire mostly mutant mitochondria (*darkened ovals*). Thus, a previously minor population of mutant species may become predominant in certain cell lineages.

Evaluation of the relative roles of random drift and the effects of such proposed selective pressures (80) in the production of the final phenotype associated with heteroplasmic mitochondrial DNA mutations is an intriguing and difficult problem. Is there something peculiar to skeletal muscle and brain that invites selective proliferation of mutant mitochondria or is this simply ascertainment bias—patients with mitochondrial dysfunction predominating in other organs either not surviving or not being recognized? Excellent mathematical paradigms for analyzing such problems have already been developed by population biologists (88); biochemical and molecular measures such as the ones discussed in this symposium will be invaluable in testing such models.

"One cannot separate theory from observation, or perhaps more precisely, one must have a theory, however vague it may be."—O. Kempthorne.

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