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A novel heteroplasmic tRNA$^{leu(CUN)}$ mtDNA point mutation in a sporadic patient with mitochondrial encephalomyopathy segregates rapidly in skeletal muscle and suggests an approach to therapy

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A novel mtDNA point mutation was detected in the tRNA$^{leu(CUN)}$ gene (G to A at position 12315) in a sporadic patient with chronic progressive external ophthalmoplegia, ptosis, limb weakness, sensorineural hearing loss and a pigmentary retinopathy. The mutation disrupts base pairing in the TΨC stem at a site which has been conserved throughout evolution. Although the other mtDNA tRNA^{leu} gene (UUR) is a hotspot for mutation, this is the first pathogenic mutation to be reported in the gene coding for tRNA$^{leu(CUN)}$. MtDNAs carrying the mutation constituted 94% of total mtDNAs in two separate muscle biopsies. Single fibre analysis showed that skeletal muscle fibres without detectable cytochrome *c* oxidase activity (COX–ve fibres) contained predominantly mutant mtDNAs (93–98%) while fibres with apparently normal COX activity had up to 90% mutant mtDNAs, demonstrating that the G12315A mutation is functionally recessive. Immunofluorescence studies with specific antibodies to mtDNA- or nuclear-encoded subunits of COX were consistent with a defect in mitochondrial protein translation. The mutation was not present in blood cells or cultured fibroblasts and surprisingly, it could not be detected in satellite cells cultured from the patient's muscle. This pattern, which may be typical of patients who have inherited new germline pathogenic mtDNA mutations, possibly reflects loss of the mutation by random genetic drift in mitotic tissues and proliferation of mitochondria containing the mutant mtDNA in post-mitotic cells. The absence of mtDNA carrying the mutation in satellite cells suggests that regeneration of skeletal muscle fibres from satellite cells could restore a wild-type mtDNA genotype and normal muscle function.

INTRODUCTION

Mutations in mtDNA (deletions, duplications, point mutations), which produce deficiencies in one or more of the enzyme complexes in the mitochondrial respiratory chain, are the primary molecular defects in a heterogeneous group of disorders called the mitochondrial encephalomyopathies (reviews in refs 1,2). These are often multisystem disorders, with prominent involvement of highly aerobic, postmitotic tissues, such as skeletal and cardiac muscle and the central nervous system. Point mutations in the mtDNA-encoded tRNA genes are a common cause of these diseases and more than 25 such mutations have been reported in 10 of the 22 tRNA genes encoded in mtDNA (3). Some are associated with distinctive neurological syndromes such as mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonus epilepsy and ragged-red fibres (MERRF); others produce isolated myopathies, cardiomyopathies or multisystem disorders. Whereas particular mutations tend to be associated with specific clinical phenotypes, some, like the A3243G mutation in tRNA$^{leu(UUR)}$ can produce either MELAS, chronic progressive external ophthalmoplegia (CPEO) or type II diabetes and deafness in different pedigrees (1–3). The exact molecular basis for these different genotype–phenotype relationships remains obscure. The gene coding for tRNA$^{leu(UUR)}$, which also contains a transcription termination sequence, is a 'hotspot' for point mutations. At least 11 different mutations have been reported in this gene associated with several different phenotypes including MELAS, CPEO, diabetes and deafness, myopathy or cardiomyopathy.

Proliferation of mitochondria in skeletal muscle fibre segments, resulting in a 'ragged-red' appearance, is a characteristic feature of skeletal muscle pathology in patients with mtDNA tRNA point mutations or with large-scale deletions. Mitochon-

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drial protein translation defects have been demonstrated in cell lines carrying high proportions of these mutant mtDNAs (4–6) raising the possibility that a defect in mitochondrial translation, *per se*, may be causally related to the ragged-red fibre pathology. Recent demonstrations of this pathology in the context of a microdeletion (7) or point mutation in protein coding mtDNA genes (8) suggest that it may be a function of the severity of the respiratory chain defect, reflecting an attempt by the cell to compensate for an imbalance between oxidative ATP supply and demand.

Here we report on a sporadic patient with ragged-red muscle fibres, and a constellation of clinical features (CPEO, ptosis, pigmentary retinopathy, sensorineural hearing loss) that has previously been only found in association with large-scale deletions or the A3243G mutation in the $tRNA^{leu(UUR)}$ gene, in whom we detect a novel tRNA point mutation in the $tRNA^{leu(CUN)}$ gene. Surprisingly, the mutation was not detectable in other tissues available for investigation, including myoblasts cultured from muscle biopsy specimens. Our data suggest that mtDNAs carrying this mutation accumulate with time in postmitotic muscle cells, but are lost primarily by random genetic drift in mitotic cells. This pattern may be typical of patients with new germline mtDNA mutations in whom the overall load of mutations would be expected to be small at birth. These results raise the possibility of elimination of the mutant mtDNA genotype from skeletal muscle, and restoration of a normal muscle phenotype, by regeneration of mature muscle fibres from satellite cells.

RESULTS

A heteroplasmic G to A substitution at position 12315 in the $tRNA^{leu(CUN)}$ gene was identified by direct DNA sequencing of all 22 tRNA genes in the mitochondrial genome from skeletal muscle genomic DNA (Fig. 1A). This mutation disrupts base pairing in the TΨC stem of $tRNA^{leu(CUN)}$ (Fig. 1B) at a site that is conserved throughout evolution from sea urchins to humans (Fig. 1C). A PCR assay was designed to verify independently the presence of the mutation and to measure the relative proportions of mutant and wild-type genomes. The assay incorporates a mismatch reverse primer, creating a unique *Ban*I restriction site in the PCR product when the wild-type allele is present. Quantitative analysis showed that mtDNAs carrying the mutation constituted 94% of total mtDNAs in two independent muscle biopsies (Fig. 2). The mutation was undetectable in two blood samples (Fig. 2, confirmed by direct DNA sequencing) and cultured fibroblasts (data not shown). It was also not detected in 63 independent controls, or patients with a putative mitochondrial disorder without precise molecular diagnosis. The mutation appears to be a new germline mutation as no other family members are affected; however, we have not been able to verify this independently as other individuals in the maternal lineage were not available for investigation.

Primary myoblast (satellite cell) cultures were established from both biopsies. Myoblasts were cloned in the first biopsy by limiting dilution in 96 well plates. In the second biopsy, myoblasts were direct-cloned by limiting dilution from the trypsinized muscle specimen to avoid any possible growth advantage to clones containing only wild-type genomes, and the remaining myoblasts were cultured and purified by FACS. A total of 29 myoblast and 21 myotube clones were analyzed from the first biopsy. All were homoplasmic wild-type (data not shown). Neither the bulk or sorted culture, nor any of the 17 clones analyzed from the second biopsy contained any trace of the mutant DNA (data not shown).

Figure 1. Mutation analysis. (**A**) DNA cycle sequencing gel showing the heteroplasmic G to A transition mutation at position 12315 in the $tRNA^{leu(CUN)}$ gene. (**B**) Predicted two dimensional structure of $tRNA^{leu(CUN)}$ showing the position of the mutation in the TΨC stem. (**C**) Sequence of the TΨC stem and loop in the $tRNA^{leu(CUN)}$ gene showing evolutionary conservation of this structure. The mutated base in the patient is shown underlined in bold.

To demonstrate that the mutation was responsible for the COX–ve mosaic phenotype of the skeletal muscle (Fig. 4D), single-fibre PCR analysis was performed on cryostat sections of skeletal muscle (Fig. 3). Fibre segments that showed no detectable COX activity contained 95 ± 1% (range 93–98%) mutant mtDNAs while apparently normal fibres, which were COX+ve, contained from 11 to 90% (54 ± 33%) mutant genomes (Fig. 3B). These data are consistent with an overall proportion of ~92% mutants in muscle as COX+ve fibres make up ~10% of all fibres in the patient's muscle.

To test whether the mutation specifically affected translation of the mtDNA-encoded subunits of the respiratory chain, immunofluorescence studies were performed on skeletal muscle cryostat sections using antibodies directed against specific subunits of the COX complex: COX I and COX II (mtDNA-encoded) and COX IV (nuclear-encoded). The results show a specific reduction in immunoreactivity of the mitochondrially encoded cytochrome oxidase subunits (Fig. 4A–C).

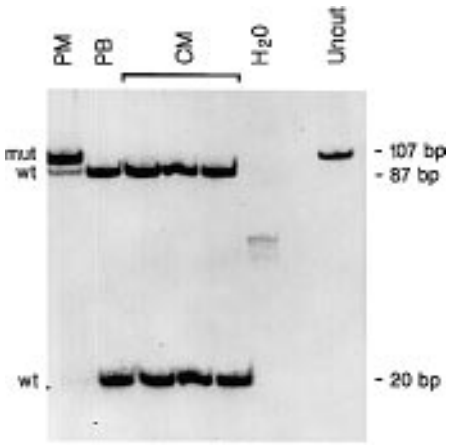

Figure 2. PCR analysis of the mutation. The majority of mtDNAs in the patient's muscle (PM) carry the mutation. The mutation is not detectable in the patient's blood (PB) or in control muscle samples (CM). H_2O is a water blank and the last track shows the PCR product undigested with *Ban*I.

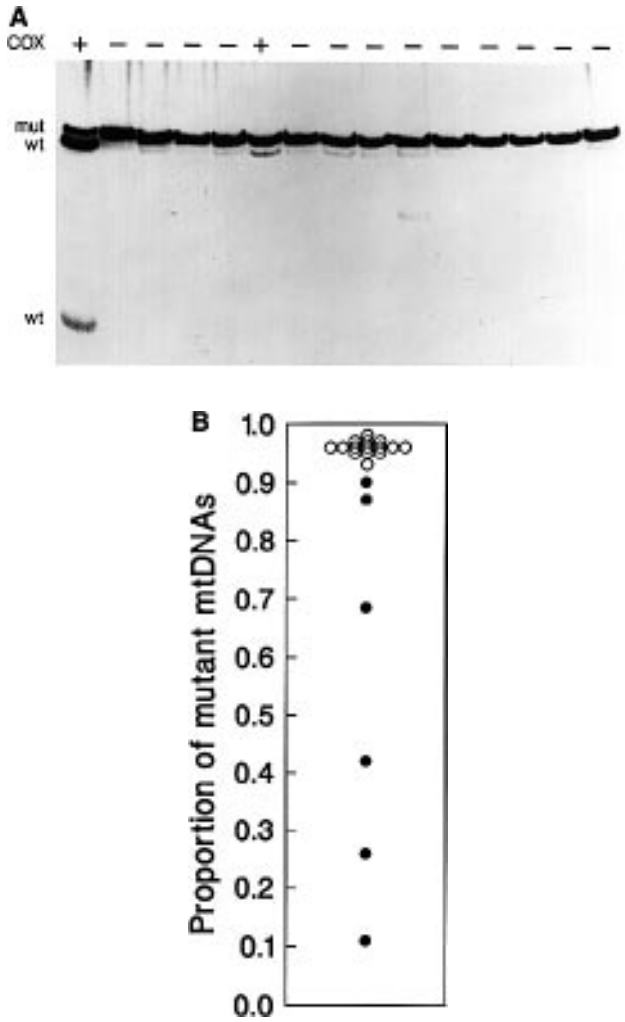

Figure 3. Single fibre PCR analysis. (**A**) PCR analysis for the presence of the G12315A mutation in single muscle fibres with positive (+) or negative (–) COX activity. (**B**) Proportion of mtDNAs carrying the G12315A mutation in COX+ve (●) or COX–ve (○) muscle fibres.

DISCUSSION

The $tRNA^{leu(CUN)}$ mutation which we report in this patient is very likely the cause of disease based on generally accepted criteria for pathogenicity of mtDNA mutations: (i) it disrupts a G-C base pair in the stem of the TΨC loop of the tRNA that is conserved in evolution from sea urchins to humans; (ii) it is heteroplasmic; (iii) there is an excellent correlation between the mtDNA genotype and the biochemical phenotype in single skeletal muscle fibres; and (iv) it has not been detected in a large number of controls from this laboratory, nor has it been reported in the literature.

This is the first pathogenic mutation reported in the $tRNA^{leu(CUN)}$ gene, although the other $tRNA^{leu}$ gene (UUR) is a 'hotspot' for mutations in mtDNA. The clinical phenotype of this patient is similar to that observed in patients carrying a point mutation in $tRNA^{leu(UUR)}$ at 3243 or large-scale deletions. Although it is tempting to speculate that pathogenic point mutations in either $tRNA^{leu}$ gene predispose to this clinical phenotype, this may be simply a fortuitous association.

An unexpected and surprising finding in this study is the nearly complete segregation of mtDNA in the satellite cells and mature muscle fibres: mutant mtDNAs were not detectable in satellite cells but accounted for 94% of total mtDNAs in skeletal muscle. Previous studies have demonstrated high and similar proportions of mutant mtDNAs in both satellite cells and mature myofibres in patients with MERRF (A8344G mutation in $tRNA^{lys}$) (4) or MELAS [A3243G mutation in $tRNA^{leu(UUR)}$] (5). The distribution of mutant mtDNAs in our patient is most similar to that reported in patients with large-scale mtDNA deletions and Kearns–Sayre syndrome (KSS), where deleted mtDNAs make up 20–80% of total mtDNAs in muscle, but <1–5% in the satellite cell population (9,10).

Satellite cells are mononuclear, committed myogenic cells that exit the cell cycle and remain dormant in a satellite position, between the basal lamina and plasma membrane of multinucleate myofibres (11). They are activated to re-enter the cell cycle in response to factors released on muscle damage (particularly necrosis) or to signals for muscle growth. Although we cannot rule out the possibility that satellite cells containing the mutant DNA species exist *in vivo* but are unable to grow in culture, a number of facts argue against this possibility. First, like other tRNA mutations, the G12315A mutation appears to be functionally recessive. Single fibres were observed with as many as 90% mutant mtDNAs and apparently normal COX activity. This threshold is similar to that observed in cells from MERRF patients with the A8344G mutation and from MELAS patients with the A3243G mutation (4–6). Cells carrying mutations below this threshold level have normal respiratory chain function and should be phenotypically indistinguishable from wild-type cells. Second, satellite cell clones homoplasmic for the pathogenic point mutations associated with MERRF or MELAS, both of which produce severe protein translation defects, have been grown under the same culture conditions (4; unpublished results). Third, the cell culture medium contains pyruvate and uridine and will support growth of rho° cells that have no mtDNA, and therefore no mitochondrial respiratory chain activity. It is therefore unlikely that selection for respiratory

chain function alone could account for the complete absence of mutants in the satellite cell population.

Large differences in the proportions of mutant mtDNAs in rapidly dividing tissues like peripheral blood cells as compared with post-mitotic tissues have been reported for other mtDNA mutations, and this has been ascribed to selection against cells carrying mutant mtDNAs in rapidly dividing tissues (12). The most dramatic examples of this are observed in sporadic cases. KSS is virtually always a sporadic disease (1,2). As mentioned above, large-scale mtDNA deletions account for a significant fraction of total muscle DNAs (20–80%) in these patients, but they are usually undetectable in blood by Southern blot analysis. There are also several examples of apparently sporadic cases of patients carrying tRNA point mutations in whom markedly different patterns of segregation of the mutant species have been reported in skeletal muscle and peripheral blood cells. Moraes and co-workers reported a G5703A mutation in $tRNA^{asn}$ with 69% mutants in muscle and 4% in blood, a C3256T mutation in $tRNA^{leu(UUR)}$ with 64% mutants in muscle 8% in blood (13) and a G15990A mutation in $tRNA^{pro}$ with 85% mutants in muscle and no detectable mutants in blood (14). Recently, a 15 bp microdeletion in the COIII subunit was reported in a patient with a mitochondrial myopathy; 92% mutant genomes were found in skeletal muscle, less than 1% in blood (7). Family history was negative in all of these cases and the mutation was not detected in tissues (usually blood) of maternal relatives. These examples contrast with MERRF and MELAS pedigrees where there is clear transmission of the pathogenic mtDNA mutation from a female carrier. In MERRF patients there is a linear correlation between the proportion of the A8344G mutation in muscle and blood (15). The proportion of mutants in blood is only slightly lower (~10%) than in muscle and is independent of patient age (16). In MELAS patients carrying the A3243G mutation there is no correlation between levels of the mutant in blood and muscle, but the difference in the proportion of mutant mtDNAs between blood and muscle increases linearly with age (17). These data demonstrate that mitotic segregation of mtDNA tRNA pathogenic point mutations is complex and that selection against cells carrying the mutation is not alone a sufficient explanation for the observed patterns. They further indicate that tissue to tissue variation in the level of heteroplasmy is greatest when the mutation arises sporadically in the ovum, but that segregation is less dramatic when the mutation is transmitted by a female carrier.

The most likely mechanism to explain the loss of mutant mtDNAs in lineages of dividing cells in sporadic cases is random genetic drift. Mitotic segregation of mtDNA results from relaxed control of replication of individual mtDNAs during the cell cycle, in which individual mtDNAs may replicate more than once or not at all, and random partitioning of daughter molecules at cytokinesis (18,19). In the patient described here the mutant mtDNA is presumably found in central nervous system tissues as evidenced by the pigmentary retinopathy and sensorineural hearing loss, both of which are common clinical features of mtDNA diseases. It must, therefore, have been present in early embryonic development before the three primary germ layers were differentiated and it likely represents a new germline mutation. Such a mutation would be expected to be at low frequency in all tissues of the embryo because of the high mtDNA copy number in the oocyte. The most probable outcome for a rare mtDNA mutation in a lineage of dividing cells is loss by random drift. This will occur with a probability of $1–1/n$, where $1/n$ is the frequency of the new mutation. The different patterns observed in patients with apparently sporadic mutations compared with those who have inherited a mtDNA mutation from a carrier mother may be due in large part to the relatively higher proportion of mtDNA mutants present in all cells when the mutation is transmitted by a carrier.

There are no recognized treatments for diseases due to mtDNA mutations, although vitamin therapy with compounds such as riboflavin, coenzyme Q, vitamins K and C have occasionally been helpful (2). The delayed onset and slow, progressive nature of most of these diseases suggests a continuous increase in the proportion of mutant mtDNAs in mature myofibres (and probably other post-mitotic cells), eventually exceeding the threshold for

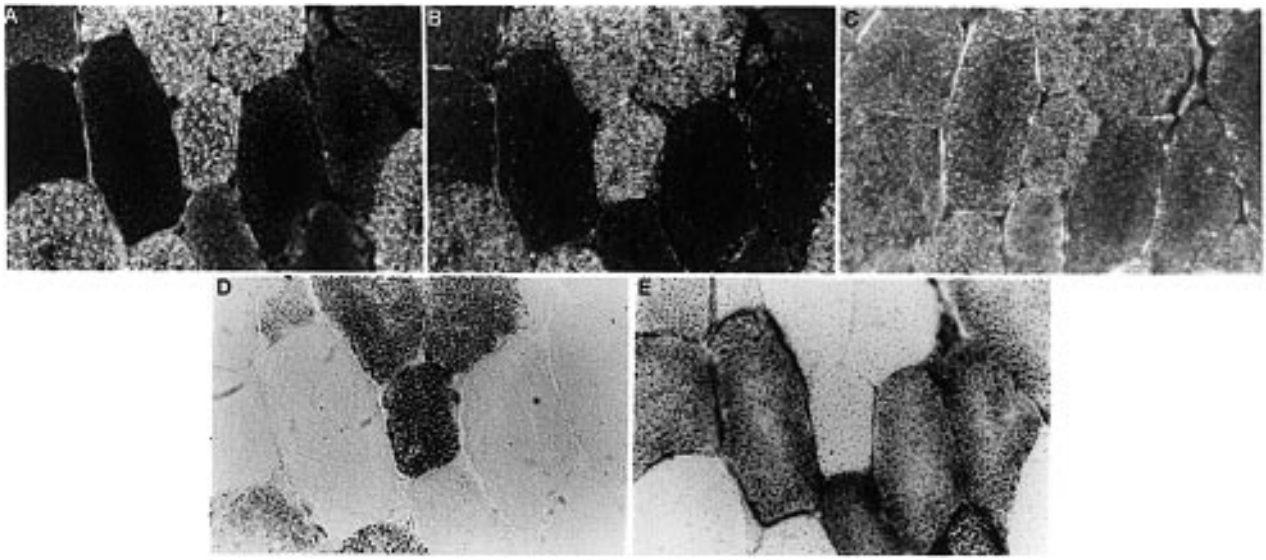

Figure 4. Immunofluorescence and cytochemical analysis in serial sections of skeletal muscle. Immunoreactivity for anti-COX I (**A**), anti-COX II (**B**) and anti-COX IV (**C**) antibodies. COX activity (**D**) and succinate dehydrogenase activity (**E**).

expression of a biochemical phenotype. If the proportion of mtDNAs carrying the G12315A mutation was initially rare in the myoblast population that contributed to the development of the limb muscles, then the inescapable conclusion is that there must be some form of selection for the mutant mtDNA in skeletal muscle. Direct evidence for such a process has been obtained for large-scale mtDNA deletions associated with the KSS (20).

The situation is less clear for other point mutations. The increase in the proportion of mtDNA mutants as a function of age in muscle vs. blood in MELAS patients (17) is suggestive of such a process in patients with this syndrome but there is no evidence for a similar increase in the proportion of mutant mtDNAs in the skeletal muscle of MERRF patients (16). Interestingly, one longitudinal study of a MELAS patient demonstrated a clear decrease in the proportion of mutants in muscle which was, however, accompanied by reversal of muscle pathology and clinical improvement (21). Although the mechanism that produced the favourable outcome in this patient is unclear, it indicates that it should be possible to change the course of disease by even a moderate reduction in the ratio of mutant/wild-type mtDNAs in existing myofibres using mtDNA from the satellite cell population. At least two possibilities could be envisioned. Simply promoting incorporation of satellite cells to mature muscle fibres by inducing muscle hypertrophy through strength training may help to change the proportion of wild-type to mutant mtDNAs, but the small number of mitochondria (and mtDNAs) in the satellite cell cytoplasm and limited fusion of satellite cells into muscle fibres during this process may not be sufficient to alter significantly the ratio in mature muscle fibres. A more drastic, but potentially more effective treatment, would involve a substantial replacement of existing muscle fibres by a single round of iatrogenic necrosis and regeneration from the satellite cell population. This strategy could be considered in any patient with a new mtDNA mutation in whom the proportion of mutant mtDNAs in satellite cells is low relative to the expression threshold for the pathogenic phenotype. It could be tested on a small muscle volume using a myotoxic agent, such as the local anaesthetic bipivacaine, which is capable of producing muscle necrosis, leaving the basal lamina, satellite cell population and motor nerve intact (22). Such a treatment would, in effect, set the molecular clock back closer to a wild-type mtDNA genotype and would be expected to restore normal muscle function.

MATERIALS AND METHODS

Patient

A 54 year old Italian-Canadian man presented with proximal limb muscle weakness and chronic progressive external ophthalmoplegia of 25–35 years duration. He had bilateral pigmentary retinopathy with severe bilateral ptosis. There was also significant dysmetria with broad-based gait and gait ataxia. Visual acuity was OD:20/100, OS:20/40. Serum CK activity (272 IU/l) and lactate levels (2.4 mM) were slightly elevated and CSF protein was normal. Electromyography showed prolonged F-wave latencies and myopathic motor unit potentials. Nerve conduction studies identified an axonal peripheral neuropathy. There was moderately severe sensorineural hearing loss which was greater on the left. MRI demonstrated a hyperintense signal on the left cerebellar hemisphere, suggestive of an ischaemic event. His parents were second cousins but no other family members were affected. He thus appears to be a sporadic case, but we have not been able to verify this directly because other members of the pedigree were unavailable for investigation. Muscle biopsies were obtained from the left and right biceps brachii muscles on two separate occasions approximately 1 year apart. About 90% of these fibres showed evidence of mitochondrial proliferation (equivalent to 'ragged-red fibres') as demonstrated by succinate dehydrogenase activity and virtually all of these had undetectable COX activity.

DNA sequencing and PCR analysis

Southern blot analysis and diagnostic testing for all described pathogenic tRNA point mutations on total genomic DNA from the skeletal muscle of the patient were negative. All 22 tRNA genes in the mitochondrial genome were sequenced by PCR cycle sequencing using the Circumvent kit from NEB. The primers used were those in reference (23). A PCR test incorporating a mismatch in the reverse primer was used to confirm the presence of the tRNA mutation. The sequences of the PCR primers used in this test were: forward 5′(12230)–ACTCATGCCCCCATGTCTAA–(12249) 3′ and reverse 5′(12336)–TACTTTTATTTGGAGTGGCA–(12317) 3′. The following PCR conditions were used to amplify the target sequence: 30 s denaturation at 94°C, 30 s annealing at 50°C and 30 s extension 72°C. To quantitate the proportions of mutant and wild-type mtDNAs, approximately 0.5 μCi of [α-^{32}P]-dCTP was added immediately before the last cycle. The T to G mismatch at position 12320 in the reverse primer creates a restriction site for the enzyme *Ban*I in the wild-type allele. The 107 bp PCR product from the wild-type allele is cut into fragments of 87 and 20 bp; the mutant allele remains uncut. Mixing experiments with different proportions of mutant and wild-type templates demonstrated that the relative proportions of mutant and wild-type mtDNAs measured in this assay was proportional to that in the sample.

Single fibre analysis

Single muscle fibre segments were dissected from 40 μM cryostat sections, which had been reacted for succinate dehydrogenase and fixed in 50% ethanol, using tungsten needles. Adjacent sections were reacted for COX to ensure that the biochemical phenotype (COX–ve or COX+ve) was uniform throughout the dissected fibre segment. DNA was extracted as described in reference (24) and tested for the presence of the mutation using the PCR test.

Immunofluorescence and cytochemistry

Unfixed cryostat sections (6 μM) were reacted with antibodies raised against the mtDNA-encoded COX I and COX II subunit and the nuclear-encoded COX IV subunit. The antihuman COX I mouse monoclonal antibody from Molecular Probes was used at 1:500. The antihuman COX II rabbit polyclonal antibody (a kind gift of Dr R. Doolittle,) was used at 1:500 and the antihuman mouse monoclonal COXIV antibody (a kind gift of Dr B. Kadenbach, Marburg, Germany) was used at 1:100. Biotinylated goat antirabbit IgG (Cedarlane Laboratories) or biotinylated horse antimouse IgG (Vector Laboratories) were used as secondary antibodies as appropriate at 1:100. Immunoreactivity was visualized using streptavidin-conjugated Cy3 (1:1000) (Jackson Immunoresearch Laboratories). COX and SDH activity was assessed as described in reference (25).

Muscle cell culture

Primary myoblast cultures were established from two independent biopsy specimens and ultimately purified by Fluorescent Activated Cell Sorting (FACS) (26). Clonal cultures were established by limiting dilution from the first biopsy after a brief bulk culture (10 days) and from the second biopsy by direct cloning from the biopsy specimen assuming 10^4 satellite cells per milligram of wet muscle weight.

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REFERENCES

1. Larsson, N.G. and Clayton, D.A. (1995) Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Genet.* **29**, 151–178.
2. Shoffner, J.M. and Wallace D.C. (1995) Oxidative phosphorylation diseases. In: Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds). *The Metabolic and Molecular Bases of Inherited Disease.* McGraw-Hill, New York, Vol. I. pp. 1535–1609.
3. Wallace, D.C., Lott, M.T., Brown, M.D., Huoponen, K. and Torrini, A. (1995) Report of the committee on human mitochondrial DNA. In: Cuticchia, A.J. (ed.). *Human Gene Mapping 1995: A Compendium.* Johns Hopkins University Press, Baltimore, pp. 910–954.
4. Boulet, L., Karpati, G. and Shoubridge, E.A. (1992) Distribution and threshold expression of the $tRNA^{Lys}$ mutation in skeletal muscle of patients with Myoclonic Epilepsy and Ragged-Red Fibers (MERRF). *Am. J. Hum. Genet.* **51**, 1187–1200.
5. Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S.T., Nonaka, I., Angelini, C. and Attardi, G. (1992) MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in the levels of upstream and downstream mature transcripts. *Proc. Natl Acad. Sci. USA* **89**, 4221–4225.
6. King, M.P., Koga, Y., Davidson, M. and Schon, E.A. (1992) Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the $tRNA^{Leu(UUR)}$ mutation associated with Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Strokelike Episodes. *Mol. Cell. Biol.* **12**, 480–490.
7. Keightley, J.A., Hoffbuh, K.C., Burton, M.D., Salas, V.M., Johnston, W.S.W., Penn, A.M.W., Buist, N.R.M. and Kennaway, N.G. (1996) A microdeletion in cytochrome c oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria. *Nature Genet.* **12**, 410–415.
8. Manfredi, G., Schon, E.A., Moraes, C.T., Bonilla, E., Berry, G.T., Sladky, J.T. and Dimauro, S. (1995) A new mutation associated with MELAS is located in a mitochondrial DNA polypeptide-coding gene. *Neuromusc. Disord.* **5**, 391–398.
9. Moraes, C.T., Schon, E.A., DiMauro, S. and Miranda, A.F. (1989) Heteroplasmy of mitochondrial genomes in clonal cultures from patients with Kearns-Sayre syndrome. *Biochem. Biophys. Res. Commun.* **160**, 765–771.
10. Shoubridge, E.A. (1993) Molecular histology of mitochondrial diseases. In: DiMauro, S. and Wallace, D.C. (eds). *Mitochondrial DNA in Human Pathology.* Raven Press, New York, pp. 109–123.
11. Manzanet, R. and Franzani-Armstrong C. (1986) The satellite cell. In: Engel, A.G., Bankier, B.Q. (eds). *Myology,* McGraw-Hill, New York, pp. 285–307.
12. Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1988) Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature* **331**, 717–719.
13. Moraes, C.T., Ciacci, F., Bonilla, E., Jansen, C., Hirano, M., Rao, N., Lovelace, R.E., Rowland, L.R., Schon, E.A. and Dimauro, S. (1993) Two novel pathogenic mitochondrial DNA mutations affecting organelle number and protein synthesis: is the $tRNA^{Leu(UUR)}$ gene an etiologic hotspot? *J. Clin. Invest.* **92**, 2906–2915.
14. Moraes, C.T., Ciacci, F., Bonilla, E., Ionasescu, V., Schon, E.A. and DiMauro S. (1993) A mitochondrial tRNA anticodon swap associated with a muscle disease. *Nature Genet.* **4**, 284–287.
15. Larsson, N.G., Tulinius, M.H., Holme, E. and Oldfors, A. (1995) Pathogenic aspects of the A8344G mutation of mitochondrial DNA associated with MERRF syndrome and multiple symmetric lipomas. *Muscle Nerve* Suppl. 3, S102–106.
16. Larsson, N.G., Tulinius, M.H., Holme, E.,Oldfors, A., Andersen, O., Wahlstrom, J. and Aasly, J. (1992) Segregation and manifestations of the mtDNA tRNA Lys A to G (8344) mutation of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome. *Am. J. Hum. Genet.* **51**, 1201–1212.
17. Poulton, J. and Morten, K. Noninvasive diagnosis of the MELAS syndrome from blood DNA. *Ann. Neurol.* **34**, 116.
18. Birky, C.W. Jr. (1994) Relaxed and stringent genomes: Why cytoplasmic genes don't obey Mendel's laws. *J. Hered.* **85**, 355–365.
19. Clayton, D.A. (1982) Replication of animal mitochondrial DNA. *Cell* **28**, 693–705.
20. Larsson, N.G, Holme, E., Kristiansson, B., Oldfours, A. and Tulinius, M. (1990) Progressive increase of the mutated mitochondrial DNA fraction in Kearns-Sayre Syndrome. *Pediatr. Res.* **28**, 131–136.
21. Kawakami, Y., Sakuta, R., Hashimoto, K., Fujino, O., Fujita, T., Hida, M., Horai, S., Goto, Y-I. and Nonaka, I.N. (1993) Mitochondrial myopathy with progressive decrease in mitochondrial $tRNA^{Leu(UUR)}$ mutant genomes. *Ann. Neurol.* **35**, 370–373.
22. Nonaka, I., Takagi, A., Ishiura, S., Nakase, H. and Sugita H. (1983) Pathophysiology of muscle fiber necrosis induced by bupivacaine hydrochloride (Marcaine). *Acta Neuropathol* **60**, 167–174.
23. Goto, Y-i., Nonaka, I. and Horai, S. (1990) A mutation in the $tRNA^{Leu(UUR)}$ gene associated with MELAS subgroup of mitochondrial encephalomyopathies. *Nature* **348**, 651–653.
24. Li, H., Cui, X. and Arnheim, N. (1991) Analysis of DNA sequence variation in single cells. *Methods:A Companion to Methods Enzymol.* **2**, 49–59.
25. Johnston, W., Karpati, K., Carpenter, S., Arnold, D. and Shoubridge, E.A. (1995) Late-onset mitochondrial myopathy. *Ann. Neurol.* **37**, 16–23.
26. Shoubridge, E.A., Johns, T. and Boulet, L. Use of myoblast cultures to study mitochondrial myopathies. *Methods Enzymol.* **264**, 465–475.