Am. J. Hum. Genet. 60:1430–1438, 1997

Intracellular Mitochondrial Triplasmy in a Patient with Two Heteroplasmic Base Changes

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Summary

We report the clinical, biochemical, and genetic investigation of a patient with a severe mitochondrial encephalomyopathy. Genetic studies identified a novel, heteroplasmic tRNA mutation at nt 10010. This T→C transition is located in the DHU loop of mitochondrial $tRNA^{Gly}$. In skeletal muscle, it was present at lower levels in cytochrome *c* oxidase (COX)–normal (87.2% ± 11%) compared with COX-deficient fibers (97.3% ± 2.6%); it was found in skin fibroblasts and blood cells, but at lower levels of heteroplasmy (15% ± 6% and 17% ± 10%, respectively). A second, heteroplasmic transition (A→G), at nt 5656, showed a different distribution than the $tRNA^{Gly}$ mutation, with very low levels in skeletal muscle (<3%) but higher levels in blood (22.7% ± 3%) and skin fibroblasts (21% ± 2%). These transitions were followed both in vivo, by repeat biopsy and blood sampling, and in vitro, by establishing primary cultures of myoblasts and skin fibroblasts. Repeat muscle biopsy showed a dramatic increase in COX-deficient fibers, but not of the $tRNA^{Gly}$ mutation. Indeed, no significant change in heteroplasmy was measured for either substitution in muscle or blood. In vitro analysis gave very different results. The T10010C was not found in cultured myoblasts, even at early passage. In uncloned fibroblasts, the T10010C was stable (~10%) for several passages but then gradually was lost. In contrast, the A5656G rose progressively from 27% to 91%. In cloned fibroblasts, different combinations of both base-pair changes and wild type could be identified, confirming the presence of clonal, intracellular triplasmy.

Introduction

mtDNA point mutations are well-recognized causes of disease, particularly within the nervous system (Wallace 1992, 1994). More than 50 mitochondrial point mutations have been reported, most commonly involving tRNA, particularly that for leucine (UUR) (Kadenbach et al. 1995). Different tRNA mutations can give rise to widely differing phenotypes, but it is evident that no one phenotype is specific for any one tRNA mutation. Pathological mitochondrial tRNA mutations are almost always heteroplasmic—that is, both mutant and wild type exist within the same individual and, usually, within the same tissue. Levels of heteroplasmy (i.e., the proportion of mutant mtDNA) are often highest in tissues that are postmitotic and are lowest in those that divide rapidly, presumably because dividing cells that contain high levels of a mtDNA mutation are energetically compromised and, therefore, at a selective disadvantage. The situation is different in cells dividing in culture where levels of heteroplasmy can remain stable, decline, or, indeed, increase, possibly influenced by nuclear background (Yoneda et al. 1992; Dunbar et al. 1995). The correlation between disease and level of mutant mtDNA is complex and often deviates from the linear. In cell-culture experiments and in single muscle fibers, however, the relationship between heteroplasmy and the biochemical phenotype is more consistent, with as little as 5%–10% wild-type mtDNA being sufficient to rescue respiratory function (Chomyn et al. 1992).

Although the finding of two different mtDNA molecules (mutant and wild type) is well recognized, the presence of two different, heteroplasmic changes in one individual is not. Recently, Howell and colleagues identified individuals with three base substitutions within the hypervariable region of the D-loop in a large pedigree having Leber hereditary optic neuropathy (Howell et al. 1996). Single base changes and length variations within the D-loop also have been found in controls and individuals carrying a polymorphism at nt 16189 (Bendall and Sykes 1995; Morten et al. 1995). In addition, D-loop changes also have been identified in individuals who carry mtDNA rearrangements (Brockington et al. 1993). Last, a patient with Kearns-Sayre syndrome and multiple endocrine abnormalities recently has been reported to be heteroplasmic for both a 2.5-kb deletion and the 3243 MELAS (myoclonic epilepsy, lactic acidosis, and strokelike episodes) mutation (Ohno et al. 1996). All

Received December 18, 1996; accepted for publication March 4, 1997.

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0002-9297/97/6006-0021$02.00

deleted molecules contained the mutant 3243 allele, which also was present in a subset of undeleted molecules. Whether more than one species of mtDNA was contained within the same cell was, unfortunately, not determined (Ohno et al. 1996).

We report here the investigation of a young woman with a clinical picture of severe mitochondrial encephalomyopathy in whom we identified two heteroplasmic base changes, one of which is a novel, pathological mutation involving mitochondrial $tRNA^{Gly}$. To our knowledge, there have been no previous reports identifying more than one heteroplasmic base change outside the D-loop. Variation in levels of both base substitutions was followed in vivo and in vitro. Our results also show that cells may be triplasmic, carrying at least three different mtDNA genotypes.

Case, Material, and Methods

Case Report

This 33-year-old female presented at age 20 years with severe generalized headache, nausea, and vomiting after the consumption of a moderate amount of alcohol. She had a brother with epilepsy and four healthy children. She was of short stature and of low IQ. Examination of the limbs showed normal tone and power but exaggerated reflexes with flexor plantars. Investigation showed mildly abnormal liver enzymes (raised γ glutamyl transferase and aspartate aminotransferase), acellular cerebrospinal fluid (CSF) with raised protein (0.5 g/liter), and an electroencephalogram (EEG) revealing a slow background with focal seizure discharges from the left temporal lobe. Her symptoms resolved, but she was readmitted 10 d later with choreoathetoid movements initially involving her right and then her left arm and then both legs. Computed-tomography (CT) brain scan was normal, as were serum copper, ceruloplasmin, and thyroid function. She was treated with tetrabenazine, and the movements gradually subsided.

Three years later she returned, suffering from unsteadiness, episodic loss of consciousness, and leg cramps, all of which had been present for several months. She had mild tandem ataxia, dystonic posturing of the right hand, a mild symmetrical tetraparesis, and both dysdidokokinesis and dysmetria of the upper limbs. Liver enzymes were again elevated, and an EEG showed generalized spike-and-wave discharges. Further CT brain scan, copper studies, and CSF analysis were all normal. She was treated with anticonvulsants but did not attend review.

Definitive investigation was performed in 1990. At this time she had developed bilateral optic atrophy and right-sided sensorineural hearing loss, in addition to a mild, left hemiparesis and an ataxic, spastic gait. Serum bicarbonate was low (15 mM; normal 22–29 mM), and serum lactate was elevated (5.3 mM; normal <1.7 mM). Creatine kinase activity varied and ranged between 1,800 U/liter and normal (<170 U/liter). Electromyelography showed changes compatible with a myopathy. Muscle biopsy was performed at this stage and confirmed a mitochondrial myopathy with ~30% ragged red fibers (RRF) and ~21% COX-deficient fibers.

She has continued to deteriorate, with progressive spastic ataxia and further seizures. Sequential CT brain scans have shown repeated cerebral infarction and progressive atrophy. She has also developed glucose intolerance. A second muscle biopsy was performed in 1995 and showed ~50% RRF and ~85% COX-deficient fibers.

Skin and Muscle Biopsy/Culture

Skin and quadriceps-muscle biopsies were performed under local anesthesia, with the informed consent of the patient. The first muscle biopsy was done in February 1990, the second in May 1995. Histological/histochemical analysis was performed on each skeletal muscle biopsy (Johnson et al. 1993), and the respiratory chain–complex activities were assessed on mitochondria fractions isolated both from the first muscle biopsy (Bindoff et al. 1991) and from skin fibroblasts (Lowerson et al. 1992).

Tissue Culture

Myoblast cultures were established from the first biopsy and were grown in standard media (Blau and Webster 1981) plus 50 μg uridine/ml and 110 μg pyruvate/ml. At ~80% confluency, the myoblasts were harvested, with half being replated and half being used for DNA extraction. Fibroblasts were grown in the presence of uridine and pyruvate until they were ~90% confluent. After being harvested, one-third of the cells were replated, whereas DNA was extracted from the remaining cells. The replating thereby constituted one passage. For cloning, fibroblasts (from passage 5) and myoblasts (from passage 5) either were subjected to serial dilutions in 96-well microtiter plates or were diluted into petri dishes, until the presence of single cells could be confirmed. Myoblast clones were confirmed by fusion to myotubes, on serum starvation.

DNA Analysis

Total genomic DNA from skeletal muscle, blood leukocytes, and cultured cells (myoblasts and fibroblasts) was extracted according to standard methods. Regions of the mitochondrial genome were amplified by use of PCR and were purified through a Centricon-100 filter. Biotinylated primers facilitated the production of single-strand template (DYNAL magnetic beads), which was subjected to dideoxy-chain terminating DNA sequence analysis following standard procedures (Jackson et al. 1994).

RFLP Analysis

10010 heteroplasmy.—A 1,252-bp mtDNA fragment spanning bp 8911–10163 (Anderson et al. 1981) was

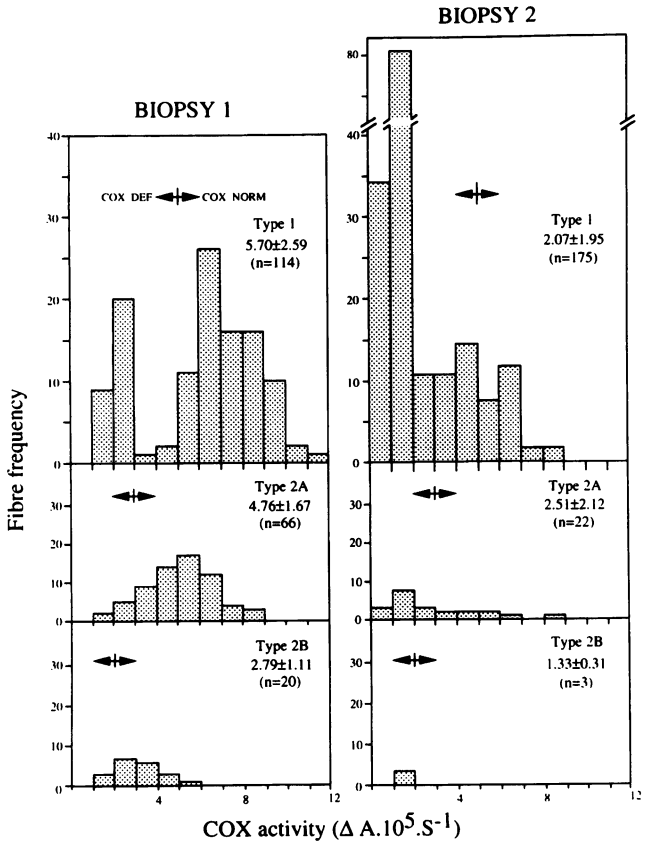

Figure 1 Cytochrome *c* oxidase activity of single muscle fibers. Ten-micrometer transverse sections were subjected to microspectrophotometric analysis in sequential muscle-biopsy samples taken from the patient. Two hundred fibers from each thin section are scored for COX activity, and fibers are represented as a function of activity and fiber type. Limits for COX-deficient activities and the mean COX activity (±SD) are shown for each fiber type (Johnson et al. 1993). The two biopsies were performed 63 mo apart. The results show a shift in fiber type, mainly to type I, and demonstrate marked loss of cytochrome oxidase activity, such that the majority of fibers fall under the lower limit of the normal range shown by the double arrow (←|→)

amplified from total DNA samples by use of the primers 5′-GGT GGA TCT TTC TAT GTA GGC-3′ (sense) and 5′-TTA CCA CAA GGC ACA CCT AC-3′ (antisense). The PCR profile consisted of an initial step of melting (4 min at 94°C), followed by 29 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C and a final cycle of amplification that contained 10 μCi of ^{32}P dCTP (3,000 Ci/mol). After precipitation, 25,000 cpm of DNA (Cerenkov) were digested with 10 units of *Rsa*I (Boehringer Mannheim) for 6 h at 37°C. The restriction endonuclease *Rsa*I normally would generate four fragments—748, 263, 153, and 88 bp— from this region of mtDNA, but, with the T10010C transition, a site is lost, leaving three fragments—748, 416, and 88 bp. Digested DNA was electrophoresed through a 3.5% nondenaturing polyacrylamide gel which was dried and exposed to a PhosphorImager cassette. For quantification of heteroplasmy, we used the ImageQuant software package (Molecular Dynamics) after normalizing the 416-, 263-, and 153-bp fragments for deoxycytosine content.

5656 heteroplasmy.—A 140-bp mtDNA fragment spanning bp 5538–5677 was amplified from total DNA samples by use of the primers 5′-CAC TAT AGC TAG CCC TCA GTA AGT TGC AAT AC-3′ (sense) and 5′-GTT TAA GTC CCA TTG GGC TAG-3′ (antisense). Both primers are mutated to create novel *Nhe*I sites (underlined). The restriction site within the sense-primer site acts as an internal control, whereas the antisense primer carries an A→G missense mutation five bases from the 3′ terminus, which creates an *Nhe*I site only when the A5656G transition is present. After an initial denaturation step (8 min at 94°C), PCR amplification was performed for 29 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C); a final hot cycle was done as detailed above, and the product was subjected to *Nhe*I digestion for several hours at 37°C. *Nhe*I produces two products—129 and 11 bp—from the wild-type mtDNA fragment, but, in the presence of the A5656G mutation, a novel restriction site is produced, and three fragments—109, 20, and 11 bp—are generated. Digested DNA was electrophoresed through a 16% nondenaturing gel, and heteroplasmy was quantified as for the T10010C transition after the 129- and 109-bp fragments were normalized for deoxycytosine content.

Single-Fiber and Single-Cell PCR

Transverse sections (30 μm) of frozen muscle were mounted on gelatin-coated slides and were double-stained for cytochrome c oxidase and succinate dehydrogenase activity, as described elsewhere (Weber et al. 1997). COX-deficient fibers appear blue, whereas COX-normal fibers are brown, enabling each fiber to be carefully identified and isolated with a borosilicate microcapillary under an inverted microscope. Fibers were placed in 20 μl of dH_20 and DNA isolated by either of the following two protocols: (1) fibers were boiled for 5 min before being added directly to the PCR reaction; or (2) the dH_20 was removed and the cells were lysed (200 mM KOH:50 mM DTT, for 1 h at 65°C) and neutralized (200 mM HCl:900 mM Tris-HCl pH 8.3) before being added to the PCR reaction. DNA also was extracted from single myoblasts and fibroblasts, for PCR amplification using these techniques. Amplification was as detailed in the RFLP Analysis section above.

For estimation of clonal triplasmy, DNA was isolated from a fraction of cells grown from a single clone. This DNA sample was then assessed for levels of both alleles, as detailed above.

Results

Histology/Biochemistry

Histochemical analysis readily identified the three main fiber types, and microspectrophotometric measure-

Table 1

Biochemical Analysis of Respiratory Chain–Complex Activities

	Skeletal Muscle		Fibroblasts	
	Patient	Control	Patient	Control
Complex I (nmol NADH oxidized/min/mg protein)	42	243 ± 59 (n = 20)	25.6	39.8 ± 16.6 (n = 9)
Complex II (nmol DCPIP reduced/min/mg protein)	250	304 ± 87 (n = 20)	42.7	41.1 ± 15.7 (n = 8)
Complex IV (k/s/mg protein)[a]	.9	2.19 ± .29 (n = 10)	.154	.214 ± .05 (n = 9)
Complex I/complex II ratio	.17	.8 ± .34 (n = 20)	.6	1.09 ± .6 (n = 8)

Note.—There is loss of activity of both complex I and complex IV in skeletal muscle, but not in fibroblast mitochondrial fractions.
[a] Expressed as an apparent first-order rate constant.

ment of enzyme activity showed a dramatic change, in both the quantity and distribution of COX-deficient fibers, between the two biopsies (fig. 1). In the first biopsy, the COX-deficient fibers were predominantly type I, and a clearly bimodal distribution can be seen in this fiber type. Five years later there were both an increase in the proportion of type I fibers relative to other fiber types and a major increase in COX-deficient fibers (21% vs. 85%; fig. 1). Biochemical measurement of respiratory-chain activity, by use of mitochondrial fractions from the first muscle biopsy, showed low complex I and IV activity (table 1). Respiratory-chain activity was normal in mitochondrial fractions from fibroblasts (table 1).

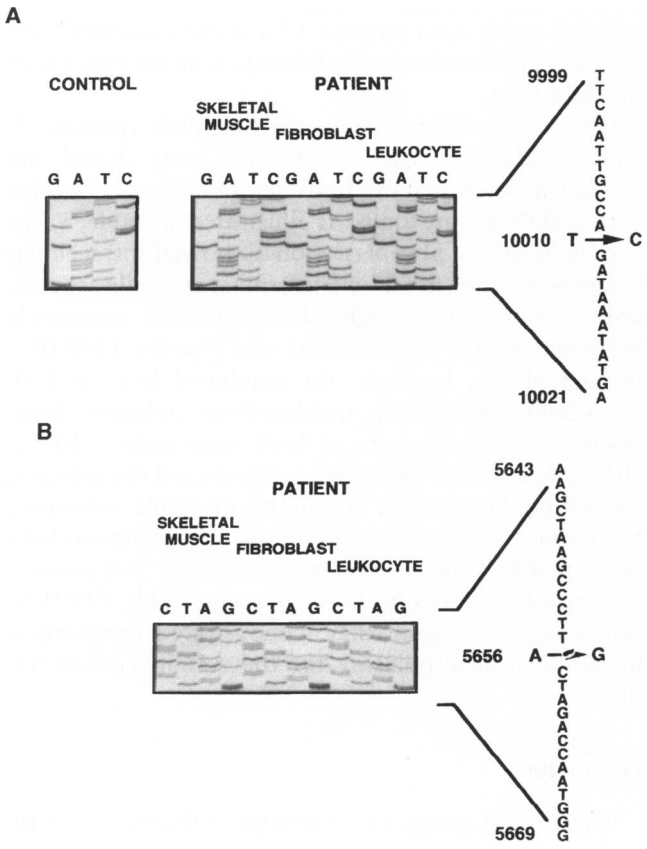

Figure 2 mtDNA sequence analysis of various tissues. *A*, Analysis of skeletal muscle DNA, clearly showing the T→C transition at nt 10010. Heteroplasmy for the T→C transition can be seen in mtDNA from fibroblasts and leukocytes. DNA isolated from a control contains no similar transition. *B*, Second heteroplasmic A→G transition at nt 5656, present in the patient's fibroblast and leukocyte DNA but absent in her skeletal muscle sample.

Genetic Studies

Southern blot analysis ruled out the presence of an mtDNA rearrangement. Each tRNA gene and various protein-coding regions of the mtDNA were sequenced. A novel T→C transition at nt 10010 was found. This is predicted to lie within a conserved region of the DHU loop of the $tRNA^{Gly}$ gene. No other mutation or polymorphism was apparent in skeletal muscle mtDNA, and initial DNA sequence analysis revealed little, if any, mtDNA wild type for the 10010 allele (fig. 2*A*). Although most pathological mitochondrial tRNA mutations are heteroplasmic, different mtDNA species may segregate during development or may not become fixed in rapidly dividing tissue. We looked, therefore, at blood and fibroblasts, for the presence of the T10010C transition. Figure 2*A* shows that peripheral blood leukocytes and fibroblasts are heteroplasmic for the transition but carry mainly the wild-type allele, explaining why the biochemical defect was not detectable in fibroblasts (table 1).

Sequence analysis of both fibroblast and leukocyte mtDNA revealed a second transition (A→G), at nt 5656, which was also heteroplasmic (fig. 2*B*). This residue is the single base pair that lies between two mitochondrial tRNA genes—$tRNA^{Ala}$ and $tRNA^{Asn}$—close to the origin of L-strand replication. To quantify the levels of both mutations in the various tissues, primers and restriction endonucleases were chosen to facilitate last-cycle-hot PCR and RFLP analysis. The approach is shown schematically in figure 3*A*, and the techniques are detailed in the Case, Material, and Methods section. The T10010C transition is close to 90% levels in homogenate from both skeletal muscle biopsies, whereas the A5656G tran-

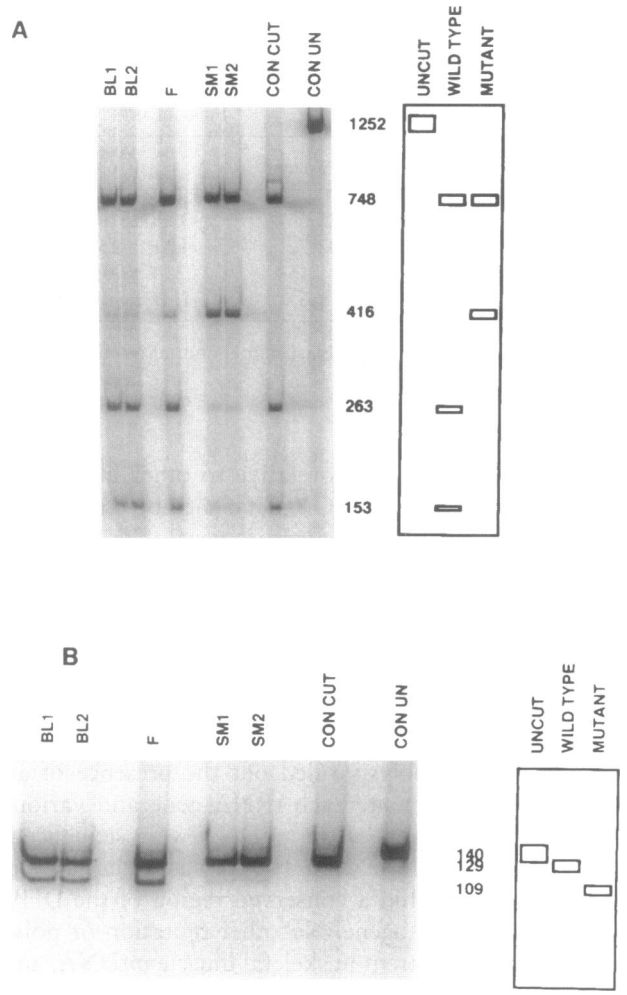

Figure 3 RFLP analysis of PCR-amplified mtDNA from various tissues. *A*, Analysis of DNA for the nt 10010 allele. A 1,252-bp fragment was amplified from two samples of blood leukocytes (BL1 and BL2), fibroblasts (F), and both skeletal muscle biopsies (SM1 and SM2). The two blood and muscle samples were taken 5 years apart (see text). Digestion with *RsaI* gives four fragments, shown schematically on the right-hand side the panel. The presence of the T→C transition at bp 10010 removes the site that permits digestion of the 416-bp fragment to 263- and 153-bp fragments. A high level of the T10010C transition is apparent in the skeletal muscle samples but not in blood cells or fibroblasts. DNA from control samples is shown both in the cut (CON CUT) and uncut (CON UNCUT) forms. *B*, Analysis of amplified DNA for the nt 5656 allele. A 140-bp fragment was isolated from the same types of tissues as are shown in panel *A*. Digestion with *NheI* generates two fragments (11 and 129 bp) in the presence of the wild-type allele and three fragments (11, 20, and 109 bp) in the presence of the A5656G transition. The two smaller fragments are not shown on the gel.

sition is <3% in both (table 2). Single-fiber analysis of the first biopsy shows that COX-normal fibers had a lower level of the T10010C transition than the COX-deficient fibers, which had a mean percentage of >95%. In contrast, the A5656G transition was present at extremely low levels, although it appears to be lower in COX-deficient fibers compared with COX-normal fibers (table 2). Despite the fact that the proportion of COX-deficient fibers increased dramatically between the two biopsies, the overall percentage of T10010C mtDNA did not differ markedly (92% vs. 88%; table 2). In addition, there was little change in heteroplasmy, for either allele, between successive blood samples (table 2).

The presence of two heteroplasmic mtDNA transitions in one individual raised the possibility that the patient has as many as four different mtDNA molecules: wild type, A5656G transition, T10010C transition, and a molecule containing both transitions. To investigate this and the question of whether mtDNA heteroplasmy was inter- or intracellular, primary cultures of fibroblasts and myoblasts were established. In the patient's myoblasts, the A5656G transition remained constant for a period, before declining (from 26% to 8%, between passage 4 and passage 21); the T10010C, however, was absent from the patient's myoblasts in culture, even at the earliest passage (passage 4) analyzed. Quantification of A5656G and T10010C heteroplasmy in cultured fibroblast is shown in figure 4. The data show a dramatic increase, in the level of the A5656G transition, between passage 5 and passage 10 (fig. 4). The level of T10010C remains stable until passage 17 and then declines, suggesting that the increase in A5656G is at the expense of the wild type.

Cells were removed from the fibroblast (passage 5) and myoblast (passage 5) lines and were cloned and expanded for several divisions before being assessed for each mtDNA transition. As illustrated in table 3, the vast majority of fibroblast clones showed intracellular heteroplasmy; of the 25 clones and single cells assayed, only 6 contained a single allele, all being apparently homoplasmic for A5656G and wild type for T10010C. In four clones, however, the combined level of both transitions was <100%, making them triplasmic. In no tissue type was the sum of both transitions >100%, although several clones appeared to exceed this number, suggesting the presence of a fourth molecule containing both transitions; however, preliminary experiments have been unable to detect this molecule (data not shown). No myoblast clones harbored any detectable T10010C transition, but, again, they demonstrated unequivocal intracellular heteroplasmy for the A5656G allele (table 3).

Discussion

The clinical symptoms, histological findings, and abnormal activities of complexes I and IV in the skeletal muscle mitochondrial respiratory chain are consistent with this patient having a pathological mtDNA mutation. The following criteria strongly suggest that the T10010C transition is indeed that mutation: (i) the mutation is heteroplasmic, has not been reported elsewhere, and is absent in all controls analyzed; (ii) the mutation

Table 2

Distribution and Change, with Time, of T10010C and A5656G Transitions

	Distribution (%)						
	In Tissue[a]					In Single Muscle Fibers	
	Skeletal Muscle 1	Skeletal Muscle 2	Fibroblasts	Peripheral Blood Leukocytes 1	Peripheral Blood Leukocytes 2	COX Normal	COX Deficient
T10010C	92 ± 3 (n = 4)	88 ± 5 (n = 4)	15 ± 6 (n = 4)	17 ± 10 (n = 4)	12 ± 6 (n = 4)	87.2 ± 11[b] (n = 17)	97.3 ± 2.6[c] (n = 22)
A5656G	2.3 ± 1 (n = 4)	1.2 ± 1 (n = 4)	21 ± 2 (n = 5)	28 ± 3 (n = 6)	27 ± 1 (n = 4)	2.3 ± 1.9 (n = 15)	<1 (n = 23)

[a] Designations "1" and "2" denote first and second biopsies, respectively, which were taken 5 years apart.
[b] Range 60–98.
[c] Range 95–100.

is present in very high levels (>90%) in skeletal muscle but is present at low levels in leukocytes and fibroblasts (<25%), a finding consistent with biochemical data showing low activity of complexes I and IV in skeletal muscle but not in fibroblast mitochondria; (iii) single-fiber analysis shows that the level of the T10010C transition is higher in COX-deficient fibers than in COX-normal fibers; and (iv) the T10010C mutation is predicted to fall within a highly conserved region of the DHU loop of an mtDNA-encoded tRNA gene, a position that is highly likely to disrupt its function and to interfere with mitochondrial protein synthesis.

The clinical features seen in this patient once again demonstrate the diversity of mitochondrial disease. She presented with an encephalopathy that may have been precipitated by alcohol and she went on to develop seizures, a movement disorder, and a progressive ataxic, spastic tetraparesis. It is likely that she also has liver involvement and that there is progressive cognitive impairment. One brother has been diagnosed as having epilepsy, and another brother is thought to be mentally slow; but neither are under neurological review. We did manage to obtain blood from an older, unaffected sister, and this showed neither of the transitions identified in the patient (data not shown).

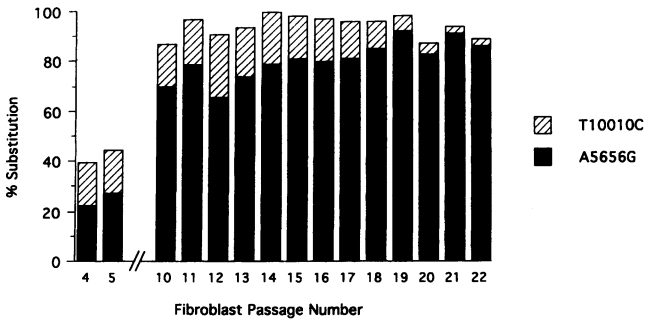

Figure 4 Histogram showing the levels of both transitions in uncloned cultured skin fibroblasts. T10010C and A5656G are present at similar levels in the earlier passages, but, between passages 5 and 10, the amount of A5656G increases dramatically. Since the level of T10010C remains stable, at least initially, this increase must be at the expense of the wild-type molecule.

The two muscle-biopsy samples, taken 5 years apart, showed a dramatic increase in COX-deficient fibers, but no increase in overall mutant load was detectable in homogenate. Most heteroplasmic defects of the mitochondrial genome are associated with progressive medical deterioration, as is the case with this patient. It has been shown elsewhere that deleted mtDNA can increase with time (Larsson et al. 1990), and, more recently, we have shown that this also is possible for an mtDNA point mutation (Weber et al. 1997). Work in vitro shows that mutated mtDNA may increase, decrease, or, indeed, stay the same; and it is postulated that this may reflect, in part, the nuclear background (Yoneda et al. 1992; Dunbar et al. 1995). One possible explanation of our observations is that most organelles in the earlier biopsy were heteroplasmic, with the wild-type mtDNA able to compensate for the mutant molecule. The recessive nature of mitochondrial tRNA mutations is well documented. After 5 years, mtDNA may have segregated to near homoplasmy between organelles in the same fiber (on the assumption that mitochondria remain discrete and do not fuse), allowing only a small subset of wild type–containing mitochondria to function correctly. These data are not necessarily consistent with a replicative advantage for the mutant genome, since segregation may have occurred stochastically. A second explanation, however, is that there has been a small but generalized increase in mutant load, sufficient to cross the threshold and cause COX deficiency. This increase may have been masked by the inherent error in quantification and, although small, may have been sufficient to exceed the protective capacity of intramitochondrial wild-type mtDNA molecules, which is calculated to be ~6%–10% in cell-culture experiments (Chomyn et al. 1991; Boulet et al. 1992).

Table 3

Analysis of Cultured Fibroblasts and Muscle Cells, for A5656G and T10010C Transitions

	Heteroplasmy[a] (%)		
	A5656G	T10010C	Total No. of Clones
Fibroblasts:			
Triplasmic:			
C6/C	58	30	4
D5/C	54	12	
E6/C	16	45	
F5/C	32	8	
Other heteroplasmic:[b]			
B2/C	90	10	15
F4/C	84	9	
C2/C	97	4	
C6/B	35	ND	
C8/B	48	ND	
G2/B	52	ND	
B8/C	43	56	
E3/C	78	31	
E7/C	ND	57	
E8/C	62	ND	
SF14	79	0	
SF17	74	ND	
SF18	71	ND	
SF19	4	95	
SF20	29	66	
Clones:			
Homoplasmic fibroblast:[b]			
	100	0	6
	0	100	0
	0	0	0
Heteroplasmic myoblast:			
C4/B	23	0	5
D6/A	38	0	
B5/B	9	0	
E11/B	9	0	
G10/B	21	0	
Homoplasmic myoblast:			
	100	0	0
	0	0	7

Note.—In four clones there was clear evidence of triplasmy, with varying amounts of both transitions and wild-type mtDNA. In a further five clones and two single cells isolated from clonal fibroblast lines, the analysis showed that the combined level of both transitions came to ~100%, suggesting that very little or no wild-type genome is present. Six fibroblast clones were homoplasmic for the A5656G transition, but we identified no lines that were homoplasmic for the T10010C transition. Analysis of cultured myoblasts showed five that were heteroplasmic for A5656G and wild type and seven that were homoplasmic wild type.

[a] ND = not done. 100% and 0% denote that no detectable levels of heteroplasmy could be determined within the limits of experimental error.

[b] SF = single fibroblast.

The absence of the T10010C transition in cultured myoblasts is noteworthy: we were unable to detect any, even when using the sensitive technique of last-cycle-hot PCR at the earliest stage of analysis (passage 4). Although it is feasible that satellite cells present in the biopsy did contain high levels of the T10010C mutation but were unable to thrive in culture, we feel that this is unlikely, since the high level of the mutation in muscle homogenate suggests that the vast majority of original muscle cells contained significant levels of mutation. If myoblasts and satellite cells come from a common progenitor, then the latter should also contain the mutation. An alternative explanation is that cells destined to be mature skeletal muscle and satellite cells come from different embryologi-

cal lineages (Schultz and McCormick 1993). Support for this hypothesis comes from (1) our recent observations in another patient with a selective mitochondrial myopathy, in whom the mtDNA mutation was present at high level in skeletal muscle and absent from satellite cells (Weber et al. 1997), and (2) the patient reported by Shoubridge and colleagues (Fu et al. 1996). Given the widespread nature of the clinical disease in our patient, we feel that it is surprising that our analysis of satellite cells failed to identify the T10010C mutation. Nevertheless, if this is indeed the case, then one intriguing therapy would be to encourage muscle degeneration, allowing subsequent regeneration from satellite cells containing substantial levels of wild-type mtDNA.

During our mtDNA analysis, we discovered a second, heteroplasmic, single base transition at nt 5656. To our knowledge, this is the first report of a patient harboring more than one heteroplasmic base substitution outside the D-loop. The A5656G was present at a very low level in skeletal muscle, an affected tissue; thus we feel that it is highly unlikely to be the pathological mutation in this patient. This transition recently has been reported in association with non–insulin-dependent diabetes (Thomas et al. 1996). Since it was homoplasmic in these individuals, it is likely to be a benign, silent polymorphism.

The two transitions identified in our study were present at different levels in the various tissues studied. Although analysis of whole tissues does not discriminate between inter- and intracellular heteroplasmy, our analysis of fibroblast clones confirms the presence of intracellular heteroplasmy. Moreover, several clones contained each of the transitions plus the wild-type molecule, confirming the presence of intracellular triplasmy. The distribution of both transitions in our patient, in conjunction with the absence of either in the only other family member studied, suggests that both must either have arisen early in embryogenesis or were present in the maternal germ line (Jenuth et al. 1996).

Our data show that dividing cells in vivo (leukocytes and fibroblasts) are amenable to the maintenance of intracellular heteroplasmy—and, indeed, triplasmy—for considerable periods. In contrast, our cell-culture experiments show a dramatic change in A5656G, which increases from 27% at passage 4 to 85% at passage 9, the increase occurring almost entirely at the expense of the wild-type molecule. In a recent review article, Birky (1994) concluded that, for most species undergoing cell division, the bulk of evidence is consistent with a "relaxed" mechanism for mtDNA replication and subsequent partitioning to progeny; that is, not every mtDNA is replicated, and not every daughter cell carries a copy of each mtDNA molecule. Even if a large number of segregating units are assumed, it is interesting to speculate why, given both that there is no selection for a given mtDNA allele and that a large number of cell divisions must have occurred, cells have not tended toward intracellular homoplasmy simply by random drift. These data suggest either that there may be a mechanism in vivo that selects for or maintains intracellular heteroplasmy or that there is indeed a more stringent mode of replication and partitioning than elsewhere has been reported in vitro.

The presence of three forms of mtDNA in at least some fibroblasts begs the following question: Is there any evidence for a recombinant molecule containing both transitions, or did the individual transitions occur independently and remain separate? Although recombination had been believed not to occur between mammalian mtDNA molecules, recent studies have suggested that a mechanism for homologous recombination may exist and that this may be important in mtDNA repair (Thyagarajan et al. 1996). We were unable to show unequivocally the presence of a molecule containing both transitions either in vivo or in cultured fibroblasts, although in two clones the quantification of both transitions was >100%. Since this increase may lie within the bounds of experimental error, we cannot formally prove or disprove the presence of a recombinant molecule.

Acknowledgments

Financial support for S.K.B. was provided by the Iranian government.

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