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Distribution and Threshold Expression of the tRNA^{Lys} Mutation in Skeletal Muscle of Patients with Myoclonic Epilepsy and Ragged-Red Fibers (MERRF)

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Summary

We investigated the distribution and expression of mutant mtDNAs carrying the A-to-G mutation at position 8344 in the tRNA^{Lys} gene in the skeletal muscle of four patients with myoclonus epilepsy and ragged-red fibers (MERRF). The proportion of mutant genomes was greater than 80% of total mtDNAs in muscle samples of all patients and was associated with a decrease in the activity of cytochrome c oxidase (COX). The vast majority of myoblasts, cloned from the satellite-cell population in the same muscles, were homoplasmic for the mutation. The overall proportion of mutant mtDNAs in this population was similar to that in differentiated muscle, suggesting that the ratio of mutant to wild-type mtDNAs in skeletal muscle is determined either in the ovum or during early development and changes little with age. Translation of all mtDNA-encoded genes was severely depressed in homoplasmic mutant myoblast clones but not in heteroplasmic or wild-type clones. The threshold for biochemical expression of the mutation was determined in heteroplasmic myotubes formed by fusion of different proportions of mutant and wild-type myoblasts. The magnitude of the decrease in translation in myotubes containing mutant mtDNAs was protein specific. Complex I and IV subunits were more affected than complex V subunits, and there was a rough correlation with both protein size and number of lysine residues. Approximately 15% wild-type mtDNAs restored translation and COX activity to near normal levels. These results show that the A-to-G substitution in tRNA^{Lys} is a functionally recessive mutation that can be rescued by intraorganellar complementation with a small proportion of wild-type mtDNAs and explain the steep threshold for expression of the MERRF clinical phenotype.

Introduction

Myoclonus epilepsy with ragged-red fibers (MERRF) is a maternally inherited mitochondrial encephalomyopathy characterized by myoclonus epilepsy, generalized seizures, ataxia, and myopathy (Fukuhara et al. 1980; Rosing et al. 1985). The syndrome was first shown to be due to an A-to-G transition mutation at position 8344 in mtDNA in the gene coding for tRNA^{Lys} (Shoffner et al. 1990), and the same mutation has now been reported in numerous other MERRF pedigrees (Berkovic et al. 1991; Noer et al. 1991; Seibel et al. 1991; Shih et al. 1991; Tanno et al. 1991; Zeviani et al. 1991). Biochemically the mutation produces multiple deficiencies in the enzyme complexes of the respiratory chain, most prominently involving NADH-CoQ reductase (complex I) and cytochrome c oxidase (COX) (complex IV) (Wallace et al. 1988; Bindoff et al. 1991), consistent with a defect in translation of all mtDNA-encoded genes. Transfer of mtDNAs carrying the mutation to human cell lines lacking their own mtDNA (rho^{o} cells) has been shown to produce a severe defect in mitochondrial translation in the recipient cells, independent of nuclear background, implying that the tRNA mutation itself is sufficient to cause the disease (Chomyn et al. 1991).

Although the genetic defect is transmitted along a maternal lineage, the clinical phenotype varies greatly within a pedigree, consistent with a heteroplasmic

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population of mtDNAs some of which are wild type and others of which are mutant. In skeletal muscle the biochemical defect is often segmental (Matsuoka et al. 1991), suggesting a nonrandom distribution of mutant and wild-type mtDNAs within a muscle cell, a situation similar to that observed with large-scale mtDNA deletions (Shoubridge et al. 1990). However, the proportion of mutant mtDNAs sufficient to produce the biochemical phenotype (threshold for expression) is unknown. Although the skeletal muscles of MERRF patients contain predominantly mutant mtDNAs, differences in the proportions of mutant and wild-type mtDNAs between patients and their unaffected maternal relatives are often very small. Shoffner et al. (1990) observed that virtually the entire range of clinical phenotypes occurs when the wild-type mtDNAs are 2%–27% of total mtDNAs, suggesting that the threshold for phenotypic expression of the $tRNA^{Lys}$ mutation is steep and that the clinical phenotype can be rescued by complementation with a small proportion of wild-type mtDNAs. On the other hand, the almost complete segregation of genotype and phenotype observed on fusion of rho^o cells with myoblasts cultured from the muscle of MERRF patients suggests that heteroplasmy, at least in the myoblast population, is largely intercellular (Chomyn et al. 1991).

In the present study we have investigated the distribution of mtDNAs carrying the $tRNA^{Lys}$ mutation in skeletal muscle and in cloned myoblasts from MERRF patients, and we have determined the threshold for expression of the biochemical phenotype in heteroplasmic myotubes. We found that the majority of satellite cells are homoplasmic for the mutant, despite the fact that these cells have a severe translational defect and minimal oxidative phosphorylation potential. Further, we show that translation of the mtDNA-encoded proteins and COX activity in heteroplasmic myotubes is similar to that in controls when approximately 15% wild-type mtDNAs are present. These data demonstrate intraorganellar complementation of the $tRNA^{Lys}$ mutation and explain the steep threshold for the clinical expression of the disease.

Patients, Material, and Methods

Patients

Muscles from four MERRF patients in three independent pedigrees were studied. Muscle (biceps) biopsies were obtained for microscopic cytochemistry, DNA and biochemical analysis, and myoblast culture.

Patient 1 was a 42-year-old man who presented with increasingly severe exercise-induced pain and fatigability of limb muscles, of many years duration. At age 39 years, large lipomata of the neck region were noted. At the same time, mild bilateral sensorineural hearing loss became evident. Ataxia, fixed-muscle weakness, and myoclonus or extraocular muscle paresis were absent. Serum creatine kinase activity was consistently elevated 4–10 times above normal. Resting serum lactate was normal. Two biopsies of each biceps muscle showed an abundance of ragged-red fibers (RRFs) with the modified trichrome stain. These fibers exhibited abnormally high succinate dehydrogenase (SDH) activity, and many had reduced or absent COX activity.

Patient 2 was a 22-year-old man who presented with infrequent generalized myoclonic jerks and subtle global cognitive deficits. An electroencephalogram showed generalized multifocal paroxysmal abnormalities. Resting serum lactate was normal. A biopsy of the biceps muscles showed no RRFs or other abnormalities.

Patient 3 was a 21-year-old woman (first cousin of patient 2) who presented at the age of 15 years with seizures and mild myoclonic episodes, which were provoked by exercise, and exercise intolerance. Muscle biopsy showed numerous RRFs, and the majority of fibers had reduced or absent COX activity.

Patient 4 was a 44-year-old woman who developed progressive ataxia of gait, diffuse myoclonic jerks, and infrequent generalized convulsive seizures late in the third decade of life. Conspicuous bilateral sensorineural hearing loss supervened, and prominent cervical cutaneous lipomata were later noted. A biopsy of the biceps at age 44 years showed numerous RRFs and strongly SDH-positive fibers, most of which were negative for COX activity.

Cell Culture

Myoblast cultures were established from dissociated muscle biopsy specimens (Blau and Webster 1981) by using supplemented growth medium (HAM F10, 15% FCS, 0.5 mg BSA/ml, 0.39 μg dexamethasone/ml, 10 ng epidermal growth factor/ml, 0.5 mg fetuin/ml, 0.18 mg insulin/ml, and 2.5 μg gentamicin/ml) according to a method described by Ham et al. (1988). Once a countable number of cells were obtained (i.e., after four or five divisions), cells were cloned in 96-well plates and were expanded. To control for a possible bias in the distribution of mutant

mtDNAs, related to differential growth prior to cloning, myoblasts from patient 1 were directly cloned from the dissociated biopsy, under the assumption of a satellite-cell density of 50–100 cells/mg of muscle. Clones were identified as myoblasts by their ability to fuse in Dulbecco's modified Eagle medium (DMEM) containing 2% horse serum. In patient 2, a skin fibroblast culture was established from an explant and was grown in modified Eagle medium supplemented with 10% FCS. Fibroblasts were cloned from early-passage cultures in supplemented growth medium according to the method described above and were expanded in DMEM plus 10% FCS.

mtDNA Analysis

A PCR-based assay was used to determine the proportion of mutant and wild-type mtDNAs. The primers used were as follows: forward primer 5′-8155–8176-3′ in the Cambridge sequence (Andersen et al. 1981) and reverse primer 3′-8345–8366-5′. The reverse primer contains a mismatch (G for A) at position 8347, creating a restriction site for the enzyme *Ban*II when the $tRNA^{Lys}$ mutant is present. PCR amplification was carried out on a Perkin Elmer Cetus thermocycler by using the following protocol: 30 s denaturation at 94°C, 30 s annealing at 50°C, and 30 s (plus 1 s per cycle) extension at 72°C, for 35 cycles. To quantitate the relative proportions of mutant and wild-type mtDNAs, approximately 0.5 μCi of [α^{32}P]dCTP was added immediately before the last cycle. A sample of the PCR product was digested with *Ban*II for at least 4 h and was run on a 12% acrylamide gel. The gel was dried, exposed, and analyzed on a Molecular Dynamics phosphoimager. *Ban*II digestion produces bands of 95, 76, and 41 bp in wild-type mtDNA. When the A-to-G mutation is present, the 76-bp band is cut into two bands, of 52 and 24 bp. A standard curve constructed by mixing different proportions of mutant and wild-type mtDNAs was linear over the entire range of mutant and wild-type proportions. If the radiotracer was added in the first cycle, the relative proportion of wild-type mtDNAs was consistently underestimated.

Enzyme Assays

COX was measured in muscle biopsies and in either isolated mitochondria or whole-cell extracts from myoblasts or myotubes. The activity was measured at 30°C in the presence of 1% Lubrol WX (Sigma), by the method of Wharton and Tzagoloff (1967), by using dithionite to reduce cytochrome c and was expressed per milligram of mitochondrial or cellular protein. Citrate synthase (CS), a marker of mitochondrial matrix space, was measured by the method of Srere (1969). Protein was measured with a micro-BCA protein assay kit (Pierce).

Analysis of Mitochondrial Translation Products

Translation of mitochondrially encoded genes was studied using methods similar to those developed by Attardi and Ching (1979). In the initial experiments, exponentially growing myoblasts were trypsinized and resuspended in DMEM lacking methionine (2×10^6 cells/ml), in 24-well plates, for labeling. In later experiments, labeling was performed in 2 ml of the same medium in 60-mm culture plates without prior trypsinization and resuspension of myoblasts; all myotubes were labeled this way. Similar results were obtained using both methods. Cells were incubated at 37°C for 30 min, and emetine (100 μg/ml), an inhibitor of cytoplasmic translation, was added 5 min prior to labeling. A control, parallel culture was labeled in the presence of emetine and chloramphenicol (100 μg/ml), to inhibit mitochondrial protein synthesis. Cells were labeled for 60 min at 37°C in the presence of 200 μCi of ^{35}S-methionine/ml, and the label was chased with 30 μg "cold" methionine/ml for 10 min. Labeling of suspended cells was stopped by centrifugation at 2,000 *g* for 2 min, and cell pellets were washed three times in NKM buffer (130 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$) at 0–4°C. Cells labeled directly on plates were washed three times in PBS, trypsinized, and resuspended in PBS. Myotubes were selectively harvested by a brief trypsinization that did not release unfused myoblasts. This allowed comparison of essentially pure populations of myoblasts and multinucleate myotubes. Isolated mitochondria (Smith et al. 1971) or cell pellets were suspended in 10 μl of water plus 10 μl of loading solution (186 mM Tris-HCl pH 6.7, 15% glycerol, 7% SDS, 6% mercaptoethanol, and 0.5 mg bromophenol blue/ml) and were sonicated for 3 s, and 30–50 μg of protein was loaded and run on 12%–20% gradient gels (Laemmli 1970). Gels were fixed, treated with EN^{3}HANCE (Dupont), dried, and exposed at −80°C for 2–5 d on preflashed Kodak X-OMAT AR film (Laskey and Mills 1975). In some experiments, direct autoradiography was used, and gels were scanned on a Molecular Dynamics phosphoimager. Mitochondrial translation products were assigned as described by Chomyn et al. (1991).

Formation of Heteroplasmic Myotubes

To determine the threshold for expression of the tRNA mutation, heteroplasmic myotubes were formed by mixing together different proportions of homoplasmic wild-type or mutant myoblasts. In one experiment, myoblasts from a control patient that were purified to approximately 99% purity by fluorescence-activated cell sorting (FACS) as described by Webster et al. (1988) were mixed with mutant clones. To set up the experiments, we needed to take account of (*a*) doubling-time differences between mutant and wild-type clones and (*b*) possible differences in mtDNA copy number. We observed that wild-type clones grew more vigorously than mutant clones in culture; however, because of the rarity of wild-type clones, it was not possible to precisely determine differences in doubling time. We estimated that wild-type clones were doubling at twice the rate of mutant clones. A similar difference was reported, by Moraes et al. (1989), in myoblasts that were heteroplasmic for mtDNA deletions. mtDNA copy number, determined relative to the copy number of nuclear-encoded 28s rDNA genes, was not significantly different in wild-type, heteroplasmic, or mutant clones (data not shown). This was further corroborated by analyzing samples of mixed myoblasts at the start of the experiments. Mutant/wild-type ratios were not significantly different than those calculated on the basis of identical copy numbers. The relative proportions of different clones mixed together at the start of the experiment were such that homoplasmic wild-type cells would undergo four divisions to reach confluence, and homoplasmic mutant cells two divisions. When myoblasts reached confluence, the medium was changed to fusion medium (DMEM plus 2% horse serum). mtDNA studies, labeling experiments, and enzyme assays as described above were performed 72 h later.

Results

Distribution of Mutant and Wild-Type mtDNAs

The relative proportion of mutant and wild-type mtDNAs was determined in the biceps muscle of four MERRF patients and in approximately 40–50 myoblast clones cultured from the satellite-cell population of the same muscle in three patients (fig. 1). The proportion of mutant mtDNAs in three patients whose muscle was clearly heteroplasmic for the mutation was 80%–91% (table 1). The muscle of patient 3 was virtually homoplasmic for the $tRNA^{Lys}$ mutant. The majority (74%–89%) of myoblast clones in all patients were homoplasmic for the mutation; homoplasmic wild-type and heteroplasmic clones were rare (table 1). The proportion of mutant mtDNAs in the satellite-

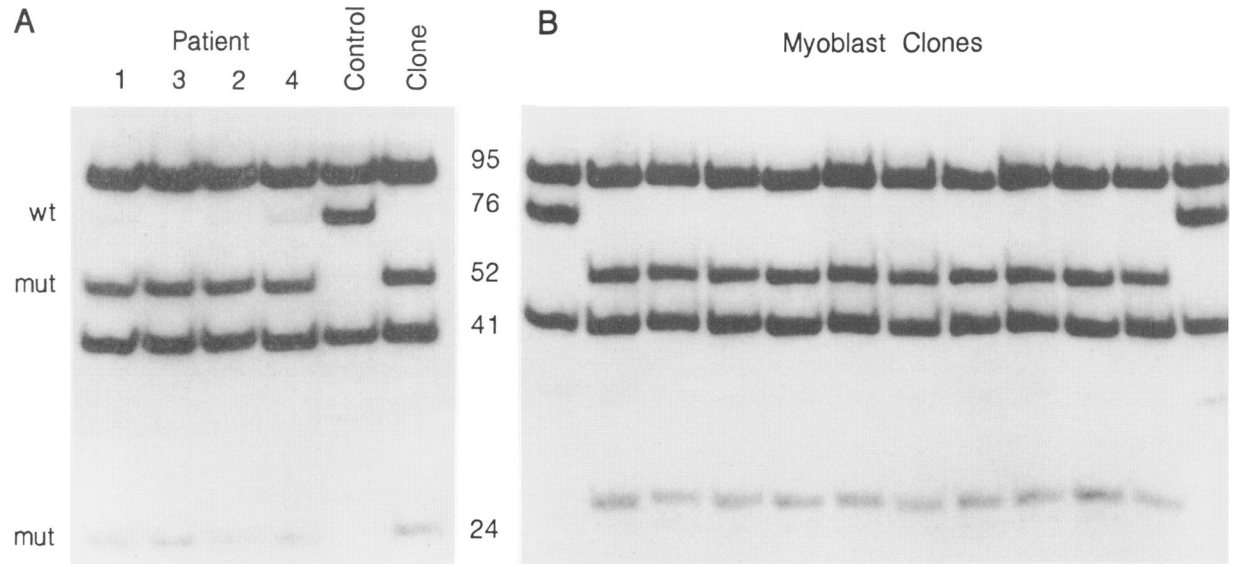

Figure 1 Analysis of the distribution of mutant and wild-type mtDNAs in (*A*) muscle biopsies and (*B*) myoblast clones. Autoradiograms of *Ban*II-digested PCR fragments amplified from the $tRNA^{Lys}$ gene are shown. Myoblast clones in *B* are a sample of those analyzed from patient 1. Control = patient without the mutation; Clone = cloned DNA fragment containing the $tRNA^{Lys}$ mutation; wt = wild type; and mut = mutant.

Table 1

Distribution of $tRNA^{Lys}$ Mutants and Wild-Type mtDNAs in Skeletal Muscle and Myoblast Clones

Patient	% of Mutant mtDNAs In Muscle	No. (%) of Myoblast Clones[a]			
		Total	Homoplasmic Wild Type	Heteroplasmic	Homoplasmic Mutant
1	83.1	47	2 (4.3)	3 (6.4)	42 (89.4)
2	90.9	38	9 (23.7)	1 (2.6)	28 (73.7)
3	>99	48	2 (4.2)	6 (12.5)	40 (83.3)
4	80.0		ND	ND	ND

[a] ND = not determined.

cell population was similar to that in differentiated muscle, in all patients. This was most evident in patient 1, whose myoblasts were directly cloned from the dissociated muscle-biopsy specimen. To test whether the clonal distribution of mutant mtDNAs was cell-type specific, fibroblast clones (N = 18) were examined in patient 2. All clones were found to be either homoplasmic wild-type (56%) or heteroplasmic (44%); no homoplasmic mutant clones were found.

Muscle Biochemical Phenotype

COX and CS were measured in the muscles of three patients (table 2). COX activities were about 40% of control activities, in all patients. When normalized to total mitochondrial volume by using CS, a marker enzyme of the mitochondrial matrix space, they were 44%–88% of control values, but they did not correlate well with the proportion of wild-type mtDNAs found in the muscle.

Analysis of Mitochondrial Translation Products

The translation products of mitochondrially encoded genes were examined by pulse labeling exponentially growing myoblasts with ^{35}S-methionine in the presence of emetine, an inhibitor of cytoplasmic translation (fig. 2). Mitochondrial translation in homoplasmic mutant myoblasts was severely impaired, whereas translation in wild-type clones from patients was indistinguishable from that in controls. Similar results were obtained from myoblast clones in patients 1–3. The magnitude of the translation defect was not related in any obvious way to either the size or lysine content of the individual translation products. The labeling intensity of the translation products was primarily a measure of the rate of translation of individual proteins, rather than a measure of steady-state protein concentration, as shown by the time course of incorporation of ^{35}S-methionine in wild-type and mutant myoblast clones (fig. 3).

To determine the threshold for expression of the translation defect, homoplasmic wild-type and mutant clones from patient 1 were mixed in different proportions and were allowed to fuse to form multinucleate, heteroplasmic myotubes with different mutant/wild-type mtDNA ratios (fig. 4*A*). In a similar experiment, FACS-sorted myoblasts from a control patient were mixed, in various proportions, with homoplasmic mutant clones from patient 3 (fig. 4*B*). Previous experiments, in which postfusion myotubes were exposed to the vital stain rhodamine 123 to visualize mitochondria, suggested that mitochondria from individual myoblasts would randomly mix in multinucle-

Table 2

Enzyme Activities in MERRF Muscle

Subject(s)	CS (μmol/min/g)	COX (μmol/min/g)	% Control	COX/CS	% Control
Patient 1	7.2	3.6	41	.50	60
Patient 2	5.2	3.8	44	.73	88
Patient 4	9.5	3.5	40	.37	44
Controls	10.5 ± 2.1	8.7 ± 3.6		.83	

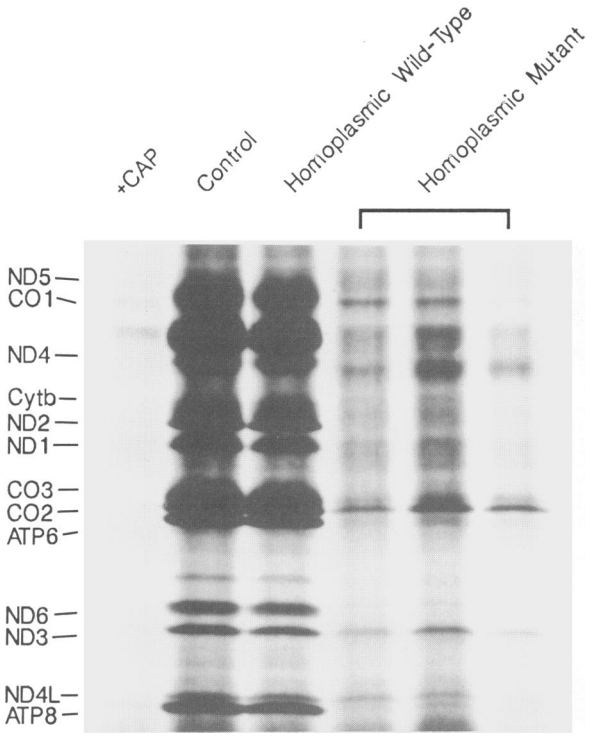

Figure 2 Fluorogram of mitochondrial translation products from exponentially growing myoblast clones labeled for 60 min with ^{35}S-methionine and run on a 12%–20% linear gradient acrylamide gel. Myoblast clones were from patient 1 and a control patient. Equal amounts of protein (50 μg) from whole-cell extracts were loaded in each lane. Translation products were assigned as described by Chomyn et al. (1991). CAP = chloramphenicol; ND1–ND6 = subunits of NADH CoQ reductase (complex I); Cytb = cytochrome b; CO1–CO3 = subunits of COX (complex IV); and ATP6 and ATP8 = subunits of ATP synthase (complex V).

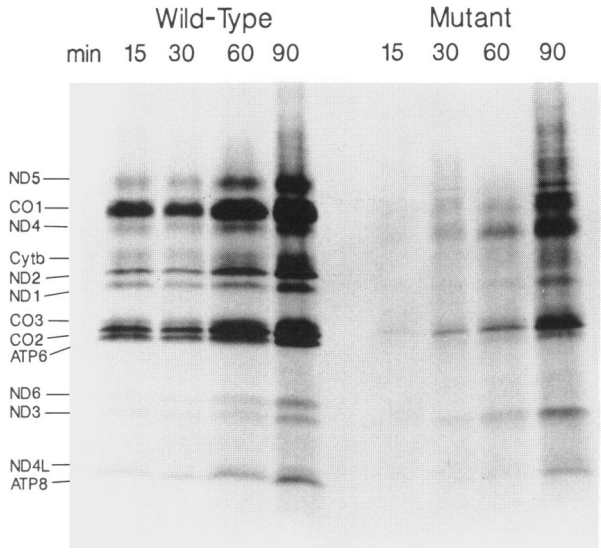

Figure 3 Fluorogram of mitochondrial translation products from exponentially growing wild-type or mutant myoblast clones after 15–90 min of labeling with ^{35}S-methionine, run on a 12%–20% linear gradient acrylamide gel. Equal amounts of protein (30 μg) from whole-cell extracts were loaded in each lane.

ate myotubes, forming an interconnected network (authors' unpublished observations). Postmitotic myotubes were pulse labeled with ^{35}S-methionine, and COX and CS activities were determined in isolated mitochondria. COX activity, normalized for mitochondrial matrix space, was restored to near control values, in the presence of 15%–16% wild-type mtDNAs in both experiments (figs. 4 and 5). Below this threshold, COX/CS activity decreased linearly as the proportion of mutant genomes increased to 100% (fig. 5). Translation of CO1 correlated with the COX/CS ratio, in both experiments (fig. 5); however, in the experiment in which myoblasts from different patients were used (fig. 4*B*), translation of CO1 in heteroplasmic myotubes was proportionately greater than COX/CS activity. The overall labeling pattern in myotubes containing mutant mtDNAs was similar to that in controls, except for the presence of a strong abnormal band running below, but not well resolved from, the normal ND4 translation product (arrow in fig. 4*A*). Results of time-course experiments on homoplasmic wild-type and mutant myotubes showed that, as in myoblasts, we were measuring rates of translation rather than steady-state levels (data not shown). Labeling intensities of individual translation products in myotubes varied with the proportion of mutant mtDNAs, in a protein-specific fashion. Labeling intensity of some subunits (ND1, ND3, ND4L, ATP6, and ATP8) was similar in homoplasmic wild-type and homoplasmic mutant myotubes, sharply contrasting with results obtained in corresponding myoblast populations (compare figs. 3 and 4), whereas other proteins were undetectable in mutant myotubes (ND5 and ND6). Except for the presence of the abnormal translation product, translation was nearly wild type at 36% or 16% wild-type mtDNAs (fig. 4*A* and *B*, respectively). In general, larger polypeptides with the greatest lysine content were more affected by the presence of the mutation, and complex I and IV subunits were more affected than complex V subunits (fig. 4). For instance, ND5, the largest mtDNA-encoded protein with the most lysine residues (21 lysines and 603 amino acids), is completely absent in homoplasmic mutant myotubes. ATP8, the smallest translation

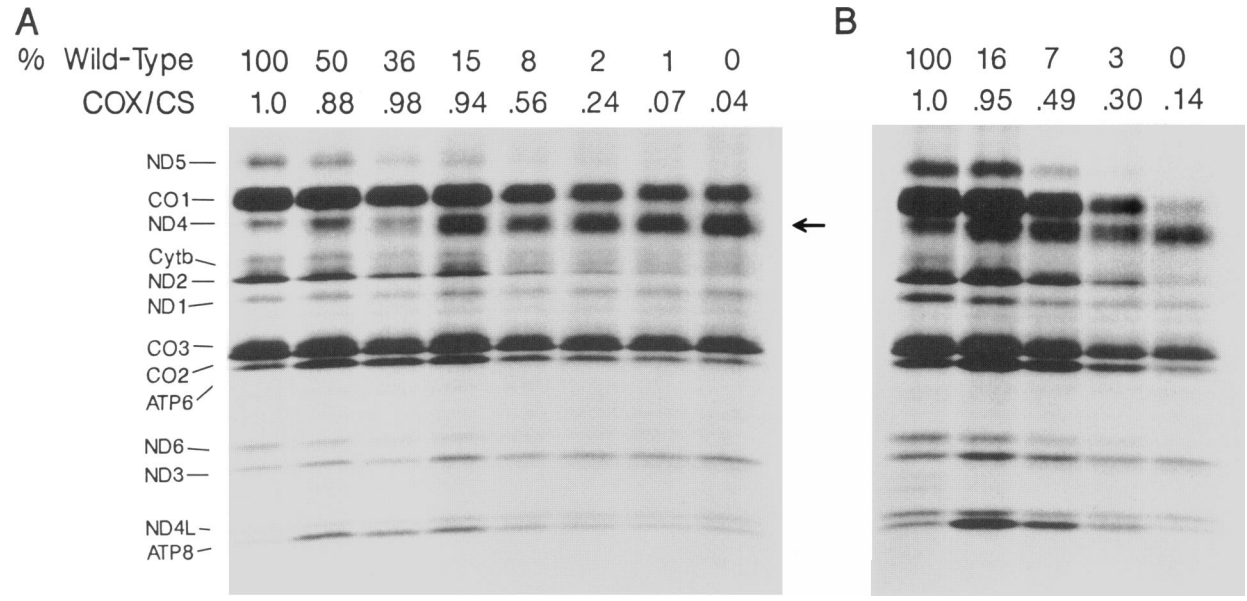

Figure 4 Fluorograms of mitochondrial translation products in heteroplasmic myotubes. *A*, Myotubes formed by mixing homoplasmic wild-type or mutant myoblast clones from patient 1. *B*, Myotubes formed by mixing FACS-sorted control myoblasts with homoplasmic mutant myoblast clones from patient 3. Equal amounts of protein (30 μg) were loaded in each lane. The proportion of wild-type genomes, as well as COX/CS ratios measured in isolated mitochondria, are indicated above individual lanes. The arrow indicates the presence of an abnormal translation product.

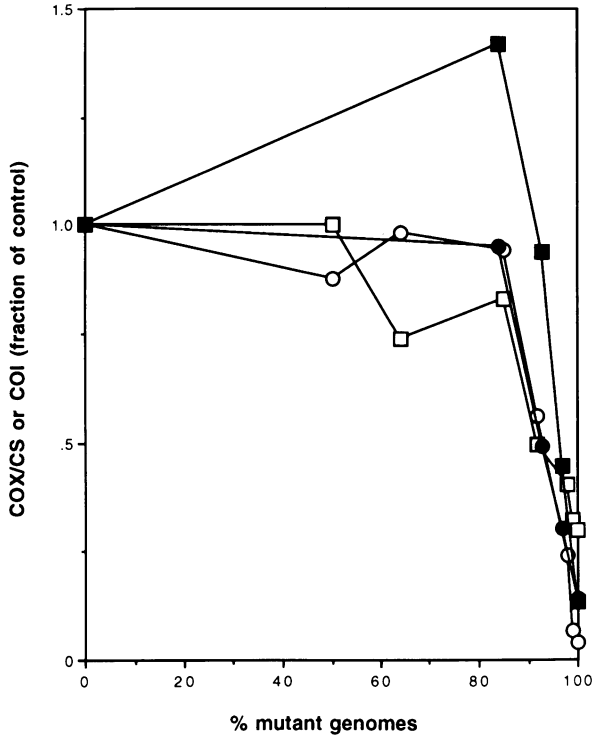

Figure 5 Plot of COX/CS activity and labeling intensity of CO1 as a function of relative proportion of mutant mtDNAs in heteroplasmic myotubes. The ratio of enzyme activity or COI labeling intensity is expressed as a fraction of the control value obtained in homoplasmic wild-type myotubes. Results are from the two experiments in fig. 4: ○ = COX/CS from experiment whose results are shown in fig. 4*A*; ● = COX/CS from experiment whose results are shown in fig. 4*B*; □ = COI labeling intensity from experiment whose results are shown in fig. 4*A*; ■ = COI labeling intensity from experiment whose results are shown in fig 4*B*.

product (68 amino acids), has proportionately the largest number of lysine residues (10%) but is virtually unaffected by the mutation. ND4L has no lysine residues and is also unaffected by the mutation. The only real exception to this pattern is ND6, a protein of 174 amino acids with only two lysines, which is labeled in a fashion almost identical to that for ND5.

The above results suggested that the tRNA^{Lys} mutation could be rescued by intraorganellar complementation of the mutant mtDNAs by a minority of wild-type mtDNAs. To test whether similar complementation was present in naturally occurring heteroplasmic cells, we examined the translation of the mitochondrially encoded genes in heteroplasmic myoblasts and myotubes in clones isolated from patient 3 (fig. 6). COX activity was measured in heteroplasmic myotubes. The pattern and intensity of labeling of translation

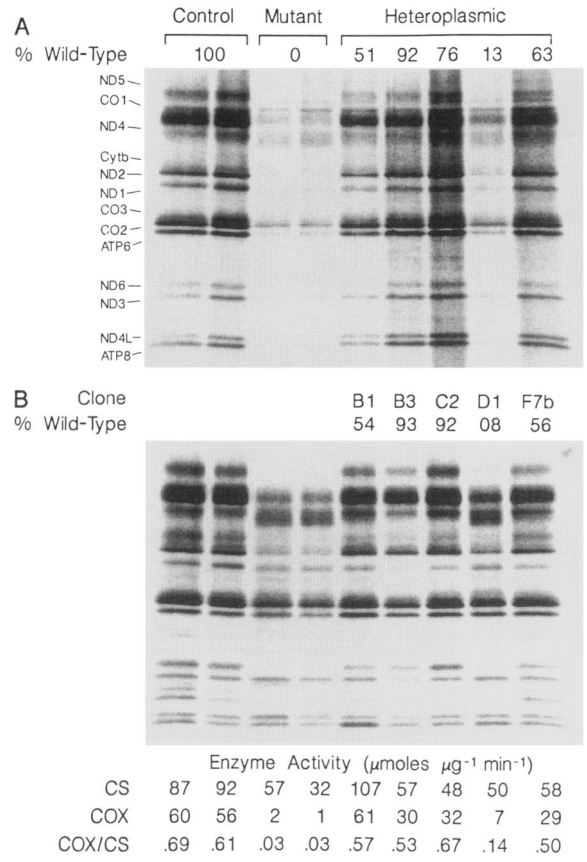

Figure 6 Autoradiograms of mitochondrial translation products from (*A*) exponentially growing myoblast clones or (*B*) myotubes, after 60 min of labeling with ^{35}S-methionine, run on a 12%–20% linear-gradient acrylamide gel. Myoblasts and myotubes came from the same clones in each experiment. Heteroplasmic clones are identified, between *A* and *B*, as in fig. 7. Equal amounts of protein (30 μg) from whole-cell extracts were loaded in each lane. COX and CS activities were measured in whole-cell extracts and are expressed per milligram of total cellular protein.

products in heteroplasmic myoblasts and myotubes with greater than 50% wild-type mtDNAs were similar to those in controls. The COX/CS ratio was also near control levels in myotubes. Clone D1, with 8% wild-type mtDNAs, had a COX/CS ratio significantly lower than would have been predicted from the complementation seen in figure 4. The marked difference in labeling intensities, which was consistently observed between myoblasts and myotubes containing a high proportion of mutant mtDNAs, is particularly striking in these experiments in which the same clones have been compared. In homoplasmic mutant clones and in clone D1, the labeling intensity of many translation products (e.g., ND1, ND3, ND4L, ATP6, and

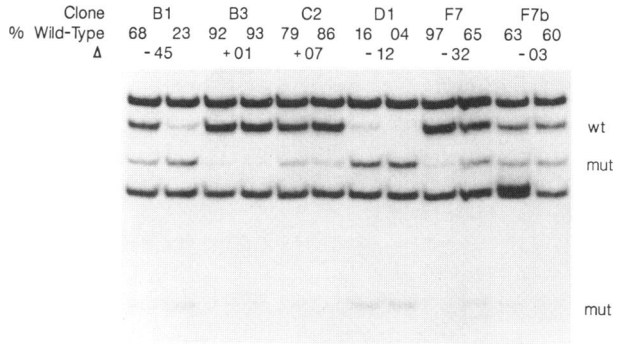

Figure 7 Analysis of mtDNA genotype in early-passage heteroplasmic clones pre- and postfusion. Paired tracks for each clone show the results of *Ban*II restriction analysis of PCR-amplified targets in myoblasts and myotubes, respectively.

ATP8) was very similar to that in control myotubes, whereas marked reductions in the labeling of these translation products were observed in myoblasts (compare fig. 6*A* and *B*).

Segregation of Mutant mtDNAs

The overwhelming preponderance, in the satellite-cell population, of myoblasts homoplasmic for the tRNA^{Lys} mutant was surprising in light of both the defect in mitochondrial translation and diminished oxidative phosphorylation potential. These results suggested a mechanism favoring selection—and ultimate fixation—of the mutant mtDNAs in heteroplasmic cells. To test this, we examined the stability of mutant mtDNAs in heteroplasmic clones. We first compared the frequency of mutant mtDNAs in expanded clones (after approximately 20 doublings) with that observed in the fusion test (2–3 further divisions 4 d after transfer to fusion medium). Several different outcomes were observed: stability of the heteroplasmic state (B3 and F7B), large increases in the frequency of the mutant mtDNA (B1 and F7), and smaller shifts toward wild-type (C2) or mutant-mtDNA (D1) homoplasmy (fig. 7). To test whether changes in the genotype were occurring either in the population of dividing myoblasts or postfusion, some of the same clones were expanded (approximately 25 doublings in total) for the labeling experiments in figure 6. Myotubes were analyzed after 3 d in fusion medium. The genotype of clones B3 and F7b remained stable, and the shifts toward homoplasmy in C2 and D1 occurred postfusion. In clone B1 the proportion of mutant mtDNAs increased in the dividing myoblast population and was essentially unchanged postfusion.

Discussion

Threshold for Expression of the $tRNA^{Lys}$ Mutation

Our results demonstrate that the A-to-G substitution in $tRNA^{Lys}$, which is associated with the MERRF phenotype, is a functionally recessive mutation that can be rescued by approximately 15% wild-type mtDNAs. This likely occurs by intraorganellar complementation, since there is no evidence that tRNAs can be exchanged between mitochondria. It is important to emphasize that this represents the threshold for biochemical—and not necessarily clinical—expression of the mutation. In general, one would expect the threshold for clinical expression to be more variable than the biochemical threshold, depending on tissue-specific requirements for oxidative phosphorylation. Even a small percentage of wild-type mtDNAs, if uniformly distributed, could restore partial oxidative phosphorylation function. When this falls below tissue-specific oxidative-phosphorylation requirements, one would expect to observe a clinical phenotype. These results provide a molecular explanation for the strong protective effect of a small proportion of wild-type mtDNAs in MERRF and explain why a wide range of clinical phenotypes is observed over a narrow range of proportions of wild-type mtDNA (Shoffner et al. 1990).

The relationship between the biochemical phenotype (COX/CS activity) and the proportion of mutant genomes was approximately linear between 0 and approximately 15% wild-type mtDNAs (fig. 5). Because only integer numbers of wild-type mtDNAs can complement the mutant mtDNAs, these data indicate that the number of interacting copies of mtDNA (functional expression unit) is approximately six in human myotubes. This is within the range of estimates of the number of mtDNAs/organelle (2–10) in a variety of mammalian cell types (Bogenhagen and Clayton 1974; Oliver and Wallace 1982; Shmookler-Reiss and Goldstein 1983). It is a minimum estimate, since we cannot be sure that postfusion myoblast mtDNAs form a truly panmictic population, although the reproducibility of the biochemical threshold when cells from different patients are used suggests that this may be nearly so. Similarly steep thresholds for phenotypic expression have been reported in other mtDNA mutants that act at the level of mitochondrial translation. For instance, the chloramphenicol-resistance phenotype, which results from a point mutation in 16s rRNA, has been shown to change abruptly when the proportion of chloramphenicol-resistant mtDNAs falls below 10% of total mtDNAs (Wallace 1986). A more complex relationship between mutant/wild-type mtDNA ratio and phenotype has been reported for large-scale mtDNA deletions (Hayashi et al. 1991). When the proportion of deletion mutants is relatively low (below 55%), wild-type mtDNAs can complement the deletion mutants, as evidenced by the appearance of a fusion protein encoded by genes spanning the deletion breakpoint. However, when deletion mutants predominate (i.e., are greater than 65%), they act as dominant mutations and suppress translation of wild-type genomes (Shoubridge et al. 1990; Hayashi et al. 1991). For a given mutation, the size of the mtDNA expression unit would be expected to set limits on the steepness of the threshold for phenotypic expression in heteroplasmic mtDNA populations. For instance, if the unit of expression were only two, then at least 50% wild-type mtDNAs would be necessary to restore COX activity in MERRF myotubes; obviously, the larger the complementation unit, the fewer wild-type mtDNAs are necessary for rescue and the steeper is the possible threshold. This could assume some importance in the pathogenesis of mtDNA diseases if the size of the functional expression unit for mtDNA either varies during embryological development or is specific to different cell types.

Nature of the Translational Defect

The defect in mitochondrial translation that we observed in skeletal myoblasts and myotubes is very similar to that demonstrated by Chomyn et al. (1991) in rho^o cells reconstituted with cytoplasts derived from MERRF patients. This reinforces the conclusion that the mtDNA mutation is itself sufficient to cause the disease. The mutation occurs at a conserved site in the TψC arm of $tRNA^{Lys}$, a part of the molecule that is thought to interact with the ribosome surface (Rich and RajBhandary 1976); however, the exact mechanism by which it causes the translation defect remains unknown. When homoplasmic, the mutation produces both a large decrease in the overall rate of mitochondrial translation and the appearance of a prominent abnormal translation product. We did not observe two other, minor abnormal bands reported by Chomyn et al. (1991), but this may be a reflection of the shorter labeling times used in the present study. The decrease in the rate of translation in myotubes was protein specific, and there was a rough correlation between protein size, lysine content, and the magnitude of the decrease. Translation of large proteins with many lysine residues (e.g., ND5) was severely cur-

tailed, whereas small proteins with (e.g., ATP8) or without (e.g., ND4L) lysine residues were largely unaffected. The labeling of ND6, which does not fit this pattern is unexplained; however, the fact that changes in labeling intensity of this protein exactly paralleled those in ND5 suggests that translation of the former is somehow linked to the latter. There was a close correlation between translation of CO subunits and COX activity, especially in heteroplasmic myotubes formed from myoblasts cloned from the same patient (fig. 5). The consistently higher rate of CO1 translation compared with COX/CS activity, which was observed when myoblasts from different patients were used, may reflect differences in nuclear or mtDNA background. These data indicate that the defect in myotubes is directly related to abnormal function of $tRNA^{Lys}$ itself. The correlation with protein size and lysine content, as well as the appearance of abnormal translation products, suggest that translational stalling and premature termination of nascent proteins may be important.

Evidence for a protein-specific effect was less obvious in myoblasts where all translation products were drastically reduced. The difference between myoblasts and myotubes is most clearly apparent in the experiments where the same clones were compared (fig. 6). In these experiments the labeling intensity of some translation products in myoblasts containing a high proportion of mutants increased substantially, relative to control values, after fusion to form multinucleate myotubes. Myoblast fusion is associated with an approximately threefold increase in both mtDNA copy number and steady-state mRNA levels (authors' unpublished observations); however, an increase in either mtDNA copy number or mRNA level cannot by itself explain the relative change in rate of translation, since the increases occur in both mutant and wild-type myotubes. The differences in labeling between myoblasts and myotubes may reflect differences in translation control in exponentially growing versus terminally differentiated cells. Translational control is thought to be important in the regulation of mitochondrial gene expression in brain and muscle (Attardi et al. 1990).

The translational defect would be expected to produce deficiencies in all of the complexes of the electron-transport chain, except in complex II, which has no mtDNA-encoded subunits. A variety of such deficiencies have been reported in several MERRF pedigrees (summarized in Bindoff et al. 1991). In the present study, we used the activity of COX, normalized for mitochondrial matrix space, as a marker of the biochemical phenotype. Although our sample size was small, we did not observe a convincing correlation between genotype and biochemical phenotype in the skeletal muscle of the patients studied. For instance, normalized COX activity in patient 2 was only slightly less than control values, but it was reduced by 60% in patient 4, despite the fact that the proportion of mutant mtDNAs was approximately 90% in the former and 80% in the latter. These data suggest that factors other than the wild-type/mutant mtDNA ratio are important in determining the biochemical phenotype. Such factors could include other mtDNA mutations, nuclear mutations, or age-related decreases in oxidative-phosphorylation capacity. It is unlikely that the $tRNA^{Lys}$ mutation is cosegregating with another mtDNA mutation, since a number of mitochondrial genomes in independent MERRF pedigrees have now been sequenced and no other consistent candidate mutations have been uncovered (Shoffner et al. 1990; Noer et al. 1991). Also, the mosaic nature of the biochemical defect in muscle argues against a mutation in a nuclear-encoded gene. Shoffner et al. (1990) have suggested that age-related decreases in oxidative-phosphorylation potential can account for at least part of the apparent discrepancy between genotype and phenotype in MERRF. In normal individuals, only a small proportion of the variation in oxidative-phosphorylation potential can be accounted for by age; and, in addition, convincing age-related changes are not seen in individuals under 50 years of age (Trounce et al. 1989). The age range of our patients was 21–44 years at the time of biopsy. It is possible, however, that age-related changes in oxidative phosphorylation are accelerated in patients carrying a pathogenic mtDNA mutation (Corral-Debrinski et al. 1991).

The apparent lack of correlation between genotype and biochemical phenotype may reflect differences in the overall level of intra- versus interorganellar heteroplasmy. An abnormal biochemical or clinical phenotype could occur if heteroplasmy were mainly interorganellar, even if the proportion of wild-type mtDNAs is above the estimated 15% rescue level. When the proportion of wild-type mtDNAs is near or below the threshold for rescue of the biochemical phenotype, any nonrandom distribution of wild-type genomes would result in COX activities below control values. One would expect the extent of nonrandom mixing of mtDNAs to be particularly acute when the proportion of wild-type mtDNAs is low. This could explain occasional observations of relatively low COX activity in

heteroplasmic myotubes with a low proportion of wild-type mtDNAs (fig. 6). The hypothesis predicts that mtDNA genotype in individual fiber segments will be independent of the mosaic biochemical phenotype, and we are currently testing this by using single-fiber PCR techniques.

Segregation of $tRNA^{Lys}$ Mutants in Satellite Cells

The most surprising result of this study was the finding that the vast majority of satellite-cell clones in all patients were homoplasmic for the $tRNA^{Lys}$ mutation. This is opposite to results reported for mtDNA deletions (Moraes et al. 1989). Deleted mtDNAs are rare (1%–5% of total mtDNAs) in myoblast cultures from patients with Kearns-Sayre syndrome (KSS), and myoblast clones homoplasmic for large mtDNA deletions have never been observed (Moraes et al. 1989; authors' unpublished observations). Satellite cells are dormant myoblasts that arise from embryonic myogenic cells, most of which fused to form multinuclear myotubes during muscle development (Manzenet and Franzini-Armstrong 1986). We have previously argued that the proportion of mtDNA mutants in this population may be representative of the proportion in the initial myoblast population (Shoubridge et al. 1990). Because of their widespread tissue distribution and sporadic pattern of inheritance, mtDNA deletions are thought to arise as clonal events either in the egg or very early in development. There is now clear evidence, from longitudinal studies of KSS patients, for a slow and progressive increase in the proportion of deleted mtDNAs in skeletal muscle with age (Larsson et al. 1990). No such studies have been reported for MERRF patients; however, the similarity in the proportion of mutant mtDNAs in the satellite-cell population and in differentiated muscle suggests that it is not necessary to postulate a large increase in the proportion of mutant mtDNAs with age. Thus, although both deletion mutants and the $tRNA^{Lys}$ mutation are present early in embryological development, and although both impair mitochondrial translation, the selection rules that determine the distribution and proportion of deletion versus $tRNA^{Lys}$ mutants in muscle cells appear to be different. This could be related either to the relative proportion of mutant genomes initially present in the very early embryo or to the exact nature of the molecular defect. It is unlikely that the patients we studied are unusual with respect to the distribution of mutant mtDNAs in myoblast clones. The proportion of mutant mtDNAs is reported to be high in the muscles of all individuals affected with MERRF (Shoffner et al. 1990; Seibel et al. 1991; Tanno et al. 1991), and indirect studies of patient myoblasts in an Italian pedigree predicted that heteroplasmy would be largely intercellular (Chomyn et al. 1991). The fact that heteroplasmy was observed to be entirely intracellular in the fibroblast clones in one patient suggests that this may be a cell type–specific effect.

How can one explain the high proportion of homoplasmic mutant clones in the satellite-cell population? Myoblasts in the satellite-cell population must have arisen from a stem-cell population that was heteroplasmic for the mutation, so segregation of $tRNA^{Lys}$ mutants could be explained by (*a*) some form of growth advantage for mutant mtDNAs, (*b*) a high proportion of mutant mtDNAs initially present in the ovum, or (*c*) a nonrandom sampling of the mtDNA population in early embryological development. A replicative advantage for mutant mtDNAs has been shown both for mtDNAs harboring large-scale deletions (Hayashi et al. 1991) and for petite mutants in yeast (de Zamaroczy et al. 1981). In both cases the replication origins in the mutant genomes are intact, and the suggestion is that the smaller size of the mutant species allows it to replicate faster than a wild-type genome. In this context it is difficult to envision a selective advantage for mtDNAs carrying the $tRNA^{Lys}$ mutation. If, however, selection occurred at the level of the organelle, and if dysfunctional mitochondria were selectively propagated, then the degree of intra- versus interorganellar heteroplasmy could determine the direction of shifts in genotype, depending on whether individual mitochondria contained a sufficient proportion of wild-type mtDNAs to rescue the phenotype. We looked for, but could not find, evidence for differences (reflecting different degrees of intraorganellar heteroplasmy) in mitochondrial translation in heteroplasmic myoblast clones. Such differences might have predicted the direction of the change in genotype we observed in these cells (figs. 6 and 7). We cannot rule out subtle differences in the distribution of wild-type mtDNAs, as these might have been hard to detect as a change in labeling pattern or intensity of the mitochondrial translation products. However, the independence of mtDNA copy number and genotype suggests that the cellular mechanisms that regulate mtDNA copy number are not sensitive to oxidative-phosphorylation phenotype, at least in aneural cultures. mtDNA copy number has also been observed to be independent of genotype in HeLa cells heteroplasmic for mtDNA deletions (Hayashi et al. 1991). This is clearly not the case in muscle in vivo where

large accumulations of deleted mtDNAs, producing the characteristic cytological abnormalities (i.e., RRFs) associated with these diseases, are seen in muscle fiber segments (Shoubridge et al. 1990).

The experiments designed to examine segregation of mutant mtDNAs in heteroplasmic clones (fig. 7) provided no clear evidence for positive selection favoring selection of the $tRNA^{Lys}$ mutant, although the largest changes in genotype resulted in an increased frequency of the mutant mtDNA. It is likely that random replicative segregation of mtDNAs, such as has been observed in studies of chloramphenicol-resistant hybrid human cell lines (Wallace 1986), can explain these results. It is unlikely that such a mechanism alone could explain the distribution of mutant mtDNAs in the satellite-cell population MERRF patients. Random segregation of a heteroplasmic population of mtDNAs in daughter myoblasts could lead to eventual fixation of either wild-type or mutant genomes, given enough generations, but would not itself produce such a skewed distribution of homoplasmic clones. Also, the clear growth advantage of wild-type or heteroplasmic clones in culture would suggest that they would overtake homoplasmic mutant clones in an expanding myoblast population. Such a phenomenon has been reported for mtDNA deletions in cultured myoblasts (Moraes et al. 1989).

We suggest that both the proportion of mtDNAs carrying the $tRNA^{Lys}$ mutation and their distribution in the satellite cells of MERRF patients are determined largely by the proportion of mutant mtDNAs initially present in the ovum and by mitotic segregation in early development. Although mammalian eggs are estimated to contain approximately 10^5 copies of mtDNA (Michaels et al. 1982), studies of segregation of neutral polymorphisms in cows have demonstrated rapid shifts in mtDNA genotype in a single generation and have indicated that the segregating unit of mtDNAs may be as few as 20–100 copies (Ashley et al. 1989). Such a genetic bottleneck could produce highly skewed distributions of mutant mtDNAs in different stem-cell populations; however, such a sampling error should equally likely favor wild-type mtDNAs. Because we have only examined satellite cells from MERRF patients, and not those from their unaffected maternal relatives, our data almost certainly contain an ascertainment bias. In this view, individuals expressing a clinical phenotype inherit a large proportion of mutant mtDNAs from their mothers, either because the ovum contained a high proportion of mutant mtDNAs or from a nonrandom sampling of mtDNAs in early development, and the genotype remains relatively static with age. The proportion of wild-type mtDNAs in skeletal muscle (and in other tissues) would then be determined by the relative growth advantage of presumptive myoblasts containing wild-type mtDNAs and by the number of cell divisions in the blast population before terminal differentiation. Such a model predicts that the proportion of mutant mtDNAs in satellite cells in clinically unaffected maternal relatives in MERRF families should be (*a*) below the threshold for expression of the biochemical phenotype and (*b*) similar to that in differentiated muscle. This hypothesis, which is easily testable, has important implications for predicting the course of disease and for prenatal diagnosis in MERRF.

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