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Maintenance of Human Rearranged Mitochondrial DNAs in Long-Term Cultured Transmitochondrial Cell Lines

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Large-scale rearrangements of mitochondrial DNA (mtDNA; i.e., partial duplications [dup-mtDNAs] and deletions [Δ-mtDNAs]) coexist in tissues in a subset of patients with sporadic mitochondrial disorders. In order to study the dynamic relationship among rearranged and wild-type mtDNA (wt-mtDNA) species, we created transmitochondrial cell lines harboring various proportions of wt-, Δ-, and dup-mtDNAs from two patients. After prolonged culture in nonselective media, cells that contained initially 100% dup-mtDNAs became heteroplasmic, containing both wild-type and rearranged mtDNAs, likely generated via intramolecular recombination events. However, in cells that contained initially a mixture of both wt- and Δ-mtDNAs, we did not observe any dup-mtDNAs or other new forms of rearranged mtDNAs, perhaps because the two species were physically separated and were therefore unable to recombine. The ratio of wt-mtDNA to Δ-mtDNAs remained stable in all cells examined, suggesting that there was no replicative advantage for the smaller deleted molecules. Finally, in cells containing a mixture of monomeric and dimeric forms of a specific Δ-mtDNA, we found that the mtDNA population shifted towards homoplasmic dimers, suggesting that there may be circumstances under which the cells favor molecules with multiple replication origins, independent of the size of the molecule.

INTRODUCTION

The human mitochondrial genome is a 16.6 kilobase (kb) circle of double-stranded DNA (Anderson *et al.*, 1981). The gene organization is highly compact, except for a 1 kb control region (the "D-loop"), which is required for the initiation of DNA replication and of RNA transcription (reviewed in Shadel and Clayton, 1997). There are two modes of mtDNA replication, the orthodox "strand-asynchronous" model unique to mammalian mtDNAs (Clayton, 1982) and the standard "strand-synchronous" model ubiquitous in mammalian nuclear DNA replication, recently described by Holt and colleagues (Holt *et al.*, 2000).

According to the orthodox model (Clayton, 1982), a round of replication of a monomeric circle begins at the "origin of heavy-strand replication" (O_H), which is located in the control region at "12 o'clock" on the circle, and proceeds continuously and unidirectionally. As the DNA polymerase γ (and the displaced DNA) passes "8 o'clock," synthesis of the light strand begins, at the "origin of light-strand replication" (O_L). The two oppositely growing strands continue, eventually forming a catenated pair of rings. A topoisomerase II-like activity decatenates the circles, releasing the two daughter monomeric molecules. Recently, Holt *et al.*, (2000) found evidence supporting the existence of standard strand-synchronous replication in addition to orthodox replication. According to this model, synchronized leading- and lagging-strand replication starts at O_H and proceeds unidirectionally around the entire circular molecule. The replication intermediate derived from this mode of replication contains a standard replication fork, where replication occurs simultaneously on both strands, albeit discontinuously on one strand.

Both mechanisms coexist in mammalian cells (Holt *et al.*, 2000), but the mode of mitochondrial DNA (mtDNA) replication employed appears to depend on the conditions under which mtDNA repopulates the cell. The orthodox replication mode is predominant in cells maintaining their mtDNA

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copy number at steady state, whereas the standard replication mode operates almost exclusively in cells undergoing rapid mtDNA reamplification after partial depletion of their mtDNA copy numbers (Holt *et al.*, 2000). However, it is not clear whether these two modes of mtDNA replication are completely independent events, nor is it known how the switch between the two modes is regulated.

Besides monomeric circles, dimeric (and, to a lesser extent, multimeric) forms of mtDNA, which are composed of monomeric circles arranged head to tail (Bogenhagen *et al.*, 1981), are also normally present in mammalian cells; such dimers are particularly common in tumor cells (Clayton and Smith, 1975). As such, they contain four replication origins (i.e., 2 O_H's and 2 O_L's), but it appears that only one of the two pairs is used to initiate replication (Bogenhagen *et al.*, 1981).

Large-scale rearrangements of human mitochondrial DNA (i.e., kb-sized partial deletions and duplications) are found associated with a number of human disorders, including Kearns-Sayre syndrome (KSS), progressive external ophthalmoplegia, Pearson's syndrome, and some sporadic myopathies (reviewed in Schon *et al.*, 1997). Each patient usually harbors a heteroplasmic population of wild-type mitochondrial genomes (wt-mtDNA) together with a population of a specific partially deleted genome (Δ-mtDNA) in clinically affected tissues.

Often, however, these patients also harbor a third mtDNA species—a partial duplication (dup-mtDNA)—as well (Poulton *et al.*, 1989; Poulton *et al.*, 1993; Poulton *et al.*, 1994; Poulton *et al.*, 1995; Schon *et al.*, 1997). In all such "triplasmic" patients (i.e., containing wt-, Δ-, and dup-mtDNAs), the two rearranged species are always topologically related: the dup-mtDNA can be thought of as being composed of a wt-mtDNA and a Δ-mtDNA arranged head to tail (see example in Figure 1A), suggesting that the two rearranged species are generated through a common mechanism, or that one may be derived from the other (reviewed in Schon *et al.*, 1997). High levels of large-scale Δ-mtDNAs (which invariably remove at least one tRNA gene) are pathogenic (Mita *et al.*, 1989; Shoubridge *et al.*, 1990; Hayashi *et al.*, 1991; Tang *et al.*, 2000), but, to a first approximation, the corresponding dup-mtDNAs are not (Holt *et al.*, 1997; Manfredi *et al.*, 1997; Tang *et al.*, 2000). However, dup-mtDNAs may nevertheless be pathogenic in a secondary manner, especially if a dup-mtDNA could recombine to give rise to the corresponding Δ-mtDNA. Evidence in support of such recombination was reported by Holt *et al.* (1997), who found partial triplications of mtDNA arising in cells that had contained initially only dup-mtDNAs. They also found that the stability of dup-mtDNAs was affected by the nuclear background of the cells in which they resided.

In order to investigate the dynamic relationship among wild-type and rearranged mtDNAs, and whether mtDNAs with different structural features (e.g., different lengths and/or numbers of replication origins) can coexist, we created transmitochondrial cell lines harboring homoplasmic rearranged and wt-mtDNAs, as well as heteroplasmic cells containing wt- and Δ-mtDNAs (Tang *et al.*, 2000), and asked if we could detect qualitative or quantitative changes among the relevant mtDNA species over long periods of time in culture.

MATERIALS AND METHODS

Patients

We studied rearranged mtDNAs from two patients. Patient 1, with KSS, who was described previously (patient 4 in Wilichowski *et al.*, 1997; patient 1 in Tang *et al.*, 2000), harbored both Δ- and dup-mtDNAs. The Δ-mtDNA was 8756 base-pairs (bp) long, lacking 7813 bp from nt-7883 in the cytochrome *c* oxidase II (COX II) gene to nt-15696 in the cytochrome *b* (Cyt *b*) gene (Figure 1A). The deletion was flanked by imperfect direct repeats located near the rearrangement breakpoint (i.e., a class II rearrangement [Mita *et al.*, 1990]). The corresponding dup-mtDNA was 25325 bp long (Figure 1A). We call this organization of the two rearranged molecules a "2–4" rearrangement, based on the number of replication origins present on the rearranged circles: like wt-mtDNA, which contains 2 origins of replication (O_{H1} and O_{L1}), the Δ-mtDNA in this patient also contained 2 origins (O_{H2} and O_{L2}); the corresponding dup-mtDNA thus contained 4 origins of replication, O_{H1}, O_{H2}, O_{L1}, and O_{L2} (Figure 1A).

Patient 2, with late-onset myopathy, was described previously (Manfredi *et al.*, 1997), and also contained both Δ- and dup-mtDNAs in mature muscle. The Δ-mtDNA was 4589 bp long, lacking 11980 bp from nt-3567 in the NADH dehydrogenase-CoQ oxidoreductase subunit 1 (ND1) gene to nt-15547 in the cyt *b* gene (Figure 2A). The deletion was flanked by perfect 10 bp direct repeats at the breakpoint (i.e., a class I deletion [Mita *et al.*, 1990]). The corresponding dup-mtDNA was 21158 bp long. Importantly, the deletion removed O_L, which is located around nt-5750. We therefore call the mtDNA organization in this patient a "1–3" rearrangement, as the Δ-mtDNA contains only 1 origin of replication (O_{H2}) and the corresponding dup-mtDNA contains 3 origins of replication, 2 for the heavy strand (O_{H1} and O_{H2}), but only one for the light strand (O_{L1}).

Cell Culture

The 143B (ρ^+) and 143B206 (ρ^0) cell lines have been described previously (King and Attardi, 1989). Cloned transmitochondrial cell lines from patient 1 containing 100% wt-mtDNA, 100% dup-mtDNA, and 100% Δ-mtDNA were characterized previously (Tang *et al.*, 2000; see Table 1). In addition, three new clonal heteroplasmic lines were generated, containing wt-mtDNA plus Δ-mtDNA monomers: two lines contained 30% Δ-mtDNA and one line contained 47% Δ-mtDNA (Table 1). One transmitochondrial cell line from patient 2, containing 90% dup-mtDNA and 10% wt-mtDNA (CH125.25), was treated with ethidium bromide for 11 days (King, 1996), followed by repopulation of the mtDNAs. Cells were cloned and a line containing 100% dup-mtDNA (CH125.25EB11.5-U) was isolated. This line was then subjected to a second 11- to 13-d regimen of ethidium bromide treatment, and three clones, each containing 100% dup-mtDNA, were expanded for further study (Table 1).

All cell lines were grown in DMEM high-glucose medium supplemented with 50 μg/ml uridine and 5% FBS (nonselective medium). Cell pellets were collected at selected intervals for mtDNA analyses, as described below.

DNA Analyses

Total DNA was extracted from exponentially growing cells, as described (King and Attardi, 1989). Southern blot analyses were performed to characterize and quantify the mtDNA in the cybrid clones, as described (Zeviani *et al.*, 1988).

Two μg of total DNA were digested with the restriction enzymes *Pvu*II, or *Bam*HI, or *Sna*BI (Boehringer Mannheim). *Pvu*II has only one recognition site in wt-mtDNA (at nt-2650) and in Δ-mtDNA monomers, but has two recognition sites in dup-mtDNA and in Δ-mtDNA dimers. Thus, it is not possible to distinguish between dup-mtDNA and Δ-mtDNA, or between Δ-mtDNA monomers and dimers, using this enzyme. However, *Bam*HI (at nt-14258) and *Sna*BI (at nt-10734) have a single restriction recognition site in both wt-

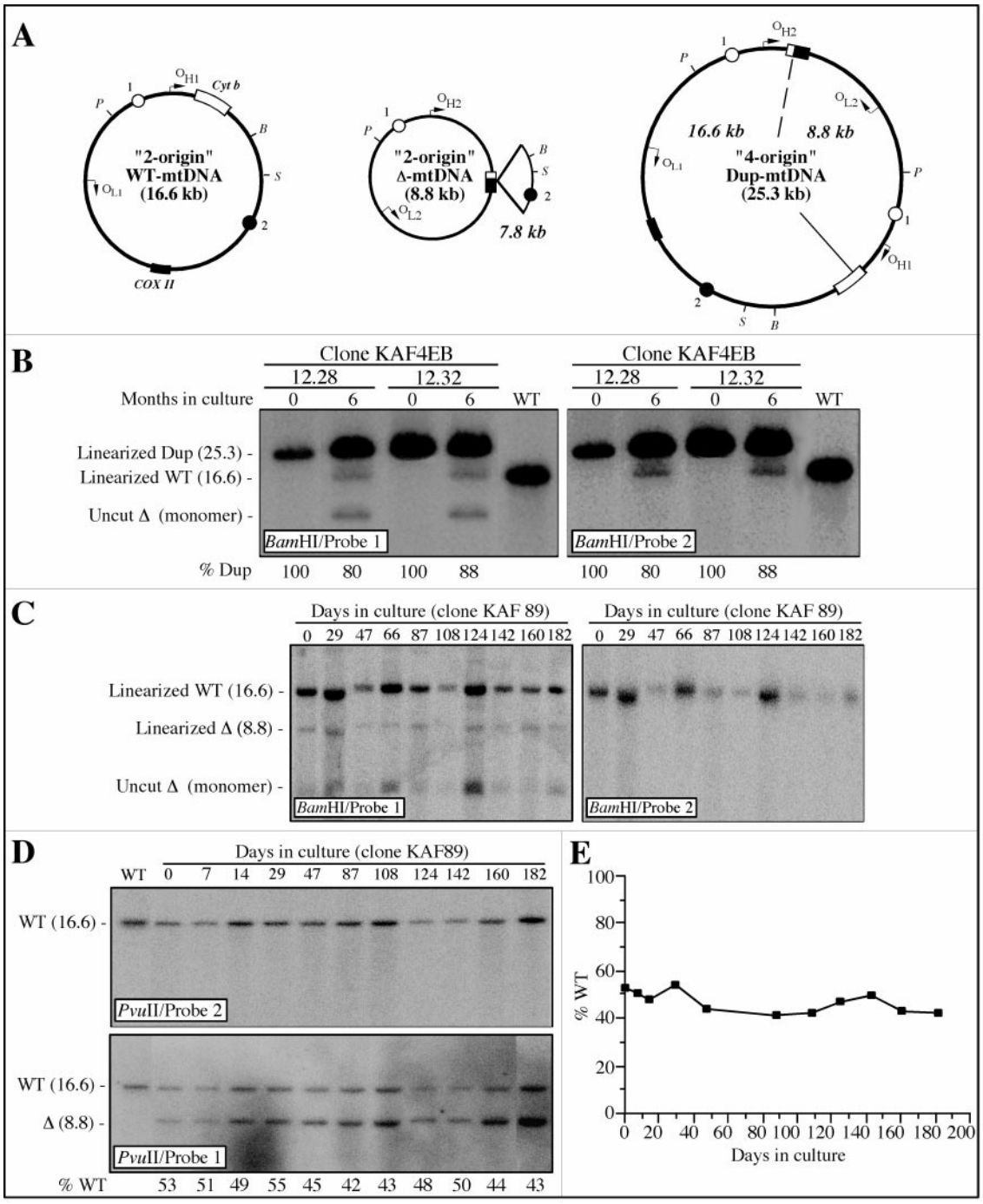

Figure 1. Long-term culture of transmitochondrial cell lines from patient 1. (A) Maps of the mtDNA species, i.e., deleted mtDNA (Δ-mtDNA), duplicated mtDNA (Dup-mtDNA), and wild-type mtDNA (WT-mtDNA). The protruding "pie section" on the Δ-mtDNA denotes the deleted region. Only the genes involved in the rearrangement are shown, i.e., COX II (solid box) and Cyt *b* (open box); a dashed line indicates the breakpoint. Note that the dup-mtDNA is composed of a full-length wild-type mtDNA into which a Δ-mtDNA has been inserted. Also shown are the origins of replication (O_H, O_L), the locations of the *Bam*HI (*B*), *Pvu*II (*P*), and *Sna*BI (*S*) restriction sites, and probes 1 (open circle) and 2 (solid circle) used in the Southern blot analyses. (B) Southern blot analysis of the 100% duplication lines (KAF4EB12.28 and KAF4EB12.32) cultured for 6 mo. DNA was digested with *Bam*HI, and hybridized with probes 1 and 2. The identity of each hybridizing fragment and its size (in kb) is indicated at left. The percentage of dup-mtDNA is indicated below each lane. (C) Southern blot analysis of a heteroplasmic cell line (KAF89, initially containing 53% wt-mtDNA and 47% Δ-mtDNA) cultured for 182 days, and analyzed as in (B). (D) Southern blot analysis of long-term passage of heteroplasmic cell line KAF89, using *Pvu*II instead of *Bam*HI for the Southern blot analysis. The percentage of wt-mtDNA (based upon number of molecules) is indicated below each lane. (E) Curve drawn from the % wt-mtDNA data shown in (D).

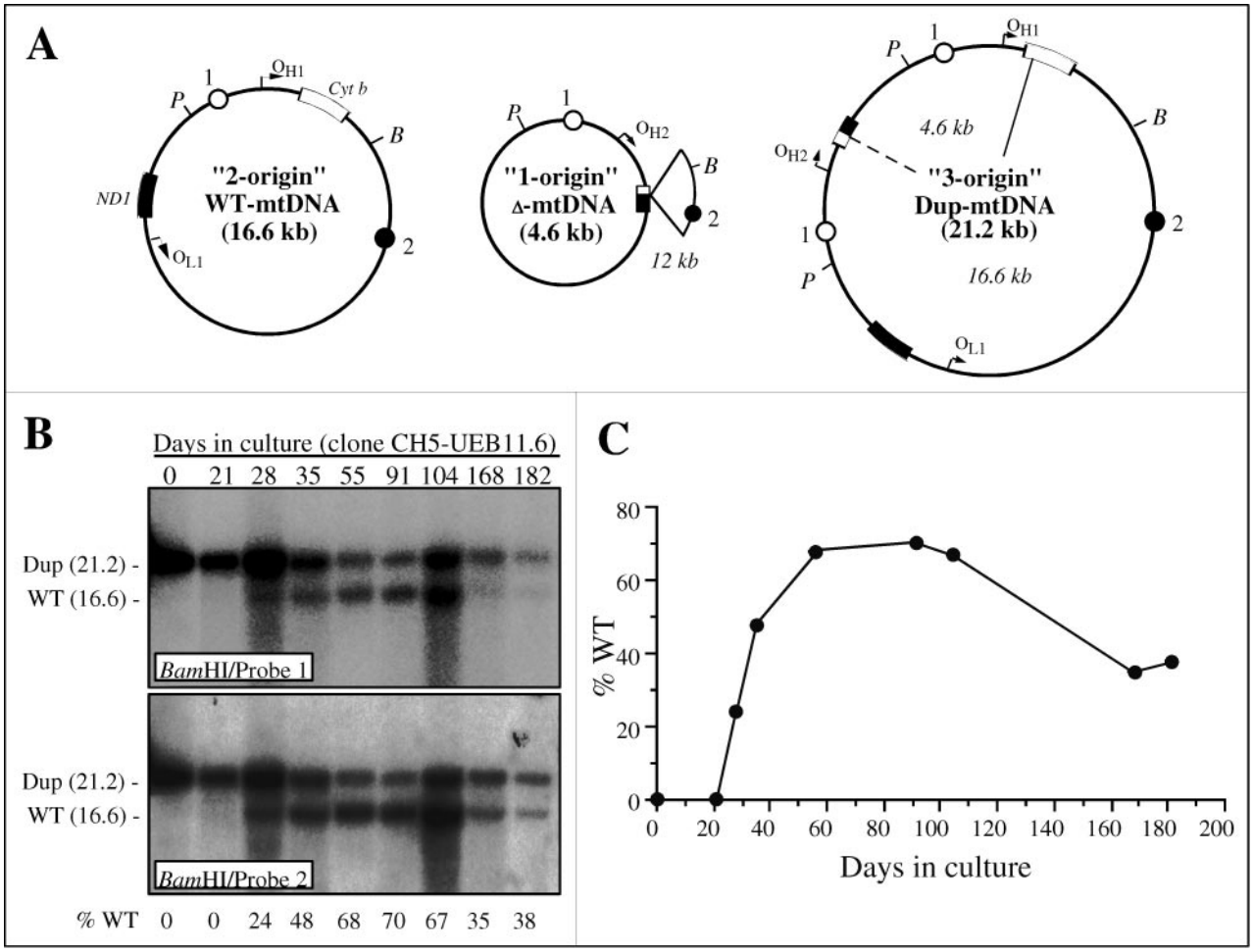

Figure 2. Long-term culture of a homoplasmic duplication cell line from patient 2. (A) Maps of the relevant mtDNA species. (B) Southern blot analysis of long-term culture of 100% duplication line CH5-UEB11.6. (C) Curve drawn from the % wt-mtDNA (based upon number of molecules) data shown in (B). All other notation as in Figure 1.

mtDNA and dup-mtDNA. Thus, digestion of either molecule with *Bam*HI or *Sna*BI linearizes the respective circles, which can be distinguished by their different lengths. Furthermore, there are no *Bam*HI or *Sna*BI sites in the Δ-mtDNAs, and these molecules remain uncut after treatment with these enzymes (however, a small proportion of these molecules are linearized randomly during the DNA extraction procedure, and appear as linearized Δ-mtDNAs on Southern blots). Thus, *Bam*HI or *Sna*BI can be used to distinguish among all three species. Because *Bam*HI and *Sna*BI do not cut Δ-mtDNA, both Δ-mtDNA monomers and dimers remain uncut, and these, too, can be distinguished, owing to their distinct gel migration patterns.

Gel electrophoresis and capillary transfer were performed as described (Zeviani *et al.*, 1988). The blots were probed sequentially with three randomly primed [α-^{32}P]-labeled human mtDNA fragments generated by PCR: probe 1 (988 bp, nt-1460–2447 [Anderson *et al.*, 1981]), which detects all mtDNA species; probe 2 (1,060 bp, nt-8239–9298), which hybridizes to wt- and dup-mtDNA, but not to Δ-mtDNA (Figures 1A, 2A, and 3A); and a nuclear 18S rDNA probe (Moraes *et al.*, 1991). Hybridizing bands were quantified using a GS-363 Molecular Imager System (Bio-Rad).

Direct sequencing of PCR products of mtDNA from the cybrids of patient 1, using primers corresponding to nt-7407–7429 (forward), and nt-15885–15860 (reverse), was performed with an automatic sequencer (ABI Prism 310, Perkin Elmer-Cetus) using the manufacturer's dye terminator cycle sequencing kit.

RESULTS

Long-Term Culture of Homoplasmic dup-mtDNAs from Patient 1

If mtDNA can undergo intramolecular recombination, one would predict that a dup-mtDNA should give rise to a wt-mtDNA plus the corresponding Δ-mtDNA (Figure 1A). In order to test this hypothesis, we cultured three transmitochondrial cell lines containing 100% dup-mtDNA from patient 1 for six months, and then analyzed the mtDNA in those cells by Southern blot analysis (Figure 1B).

Southern blot analysis of DNA extracted from two lines (KAF4EB12.28 and KAF4EB12.32) at the first passage (arbitrarily defined as time 0) and digested with *Bam*HI (which cuts once in wt- and dup-mtDNA, but does not cut Δ-mtDNA) revealed a single band of 25.3 kb with both

Table 1. Cell lines used in this study

Cell line	%mtDNA		
	WT	Δ	Dup
143B (ρ^+)	100	0	0
143B206 (ρ^0)	0	0	0
KAF4EB12.22	100	0	0
KAF4EB12.26	100	0	0
KAF4EB12.39	100	0	0
KAF4EB12.17	0	100	0
KAF4EB12.49	0	100	0
KAF4EB12.28	0	0	100
KAF4EB12.32	0	0	100
KAF4EB12.42	0	0	100
KAF89	53	47	0
KAF30	70	30	0
KAF94	70	30	0
CH125.25	10	0	90
CH125.25EB11.5-U	0	0	100
CH5-UEB11.6	0	0	100
CH5-UEB11.22	0	0	100
CH5-UEB13.8	0	0	100

probes 1 and 2, indicating that initially, both cell lines contained exclusively dup-mtDNA (Figure 1B). However, after 6 mo of culture (~ 210–240 cell divisions), we detected two additional bands when the blots were hybridized with probe 1 (located outside the deleted region): one band, 16.6 kb in size, corresponded to wt-mtDNA; the other band, migrating at a lower position in the gel, corresponded to [uncut] Δ-mtDNA. Rehybridization of the blot with probe 2 (located inside the deleted region; Figure 1B) confirmed the identities of these two species. We did not detect any other bands in the gel, implying that no other mtDNA rearrangements (e.g., multimers of the deleted mtDNA or multimers of the duplicated region) had arisen in these cells.

After 6 mo in culture, clone KAF4EB12.28 contained, on a molar basis, ~ 80% dup-mtDNA, 10% wt-mtDNA, and 10% Δ-mtDNA, and clone KAF4EB12.32 contained ~ 88% dup-mtDNA, 6% wt-mtDNA, and 6% Δ-mtDNA. The breakpoint of the newly generated Δ-mtDNA was identical to that in the parental dup-mtDNA, based on two criteria. First, the sequence of the PCR-amplified region flanking the breakpoint was identical in both the homoplasmic and hetroplasmic (i.e., 6-mo) DNA samples, and second, the Southern blot pattern following digestion of the DNA isolated from both the homoplasmic and heteroplasmic samples with *Sna*BI (which, like *Bam*HI, is located inside the deleted region) was identical to the pattern obtained with *Bam*HI digestion (data not shown).

The fact that the breakpoint of the Δ-mtDNA was the same as that in the dup-mtDNA, and that the number of wt-mtDNA molecules was essentially equal to the number of Δ-mtDNA molecules, are consistent with the prediction that intramolecular homologous recombination of one dup-mtDNA should give rise to one wt-mtDNA plus one Δ-mtDNA.

During this long-term culture, these two cell lines had growth characteristics comparable to those of wild-type lines (including doubling times [Tang *et al.*, 2000]). However, the third cell line (KAF4EB12.42) grew well only for ~ 2–1/2 mo (80–90 cell divisions), but within a 2-wk period thereafter the cells went into crisis and died. The reason for the demise of this line is unknown, as Southern blot analysis revealed that the cells had contained 100% dup-mtDNA as late as the last cell pellet collected (data not shown).

Long-Term Culture of Heteroplasmic mtDNAs from Patient 1

It is unclear at present how duplicated molecules arise in the first place. A reasonable mechanism is that a wt-mtDNA recombines with a preexisting Δ-mtDNA to give rise to a dup-mtDNA, via an intermolecular recombination event (in essence, this is the reverse of the above reaction). In order to investigate this question, transmitochondrial cell lines from patient 1 containing a mixture of wild-type and deleted mtDNA monomers were cultured long term, and their mtDNA composition was assayed at various times.

Two transmitochondrial cell lines from patient 1 (KAF30 and KAF94), both of which contained 70% wt-mtDNA and 30% Δ-mtDNAs (as monomers), and one cell line (KAF89), containing 53% wt-mtDNA and 47% Δ-mtDNA (also as monomers; see Table 1), were grown for 6 mo. Aliquots of the cells from each line were analyzed at various time points by Southern blot. We digested total cellular DNA with *Bam*HI and hybridized the blots with both probes 1 and 2, in order to detect unambiguously all three potential mtDNA species (i.e., wt-mtDNA, Δ-mtDNA, and, if present, dup-mtDNA). Analysis of all three long-term cultures (a representative analysis, on KAF89, is shown in Figure 1C) indicated that no dup-mtDNAs, or any other new mtDNA species, arose during this time period.

A second mechanism by which dup-mtDNAs could arise is through dimerization of two monomeric wt-mtDNAs, followed by a spontaneous intramolecular deletion event. We therefore grew three transmitochondrial cell lines from patient 1 containing 100% wt-mtDNAs (see Table 1) over a two-year period, and analyzed cell aliquots as above. We did not detect any new species of mtDNA arising in any of the lines (data not shown); the cells remained homoplasmic for wt-mtDNA.

Since there was no qualitative change in the types of mtDNA species present in the three heteroplasmic cell lines described above (containing only wt-mtDNA and Δ-mtDNA monomers), we deemed these cell lines to be useful for addressing a separate issue, namely, whether Δ-mtDNAs accumulate over time at the expense of the larger wild-type molecules. We therefore analyzed the same three heteroplasmic lines over time, but with a slight variation in the Southern blot analysis that allowed us to quantitate the two mtDNA species (i.e., wt- and Δ-mtDNA) more easily. Using *Pvu*II to linearize both wt and Δ-mtDNA, and regional probes 1 and 2 to distinguish the two species, we quantitated the amount of each species at each time point. For cell line KAF89, originally containing 53% wt-mtDNA (and 47% Δ-mtDNA), the percentage of wt-mtDNA remained essentially stable, ranging between 42 and 53% over the 6-mo period (Figure 1D, 1E). The percentage of wt-mtDNA also remained essentially stable in the other two cell lines (KAF94 and KAF30, both originally containing 70% wt-mtDNA), ranging between 60 and 73% over the 6-mo period (data not shown).

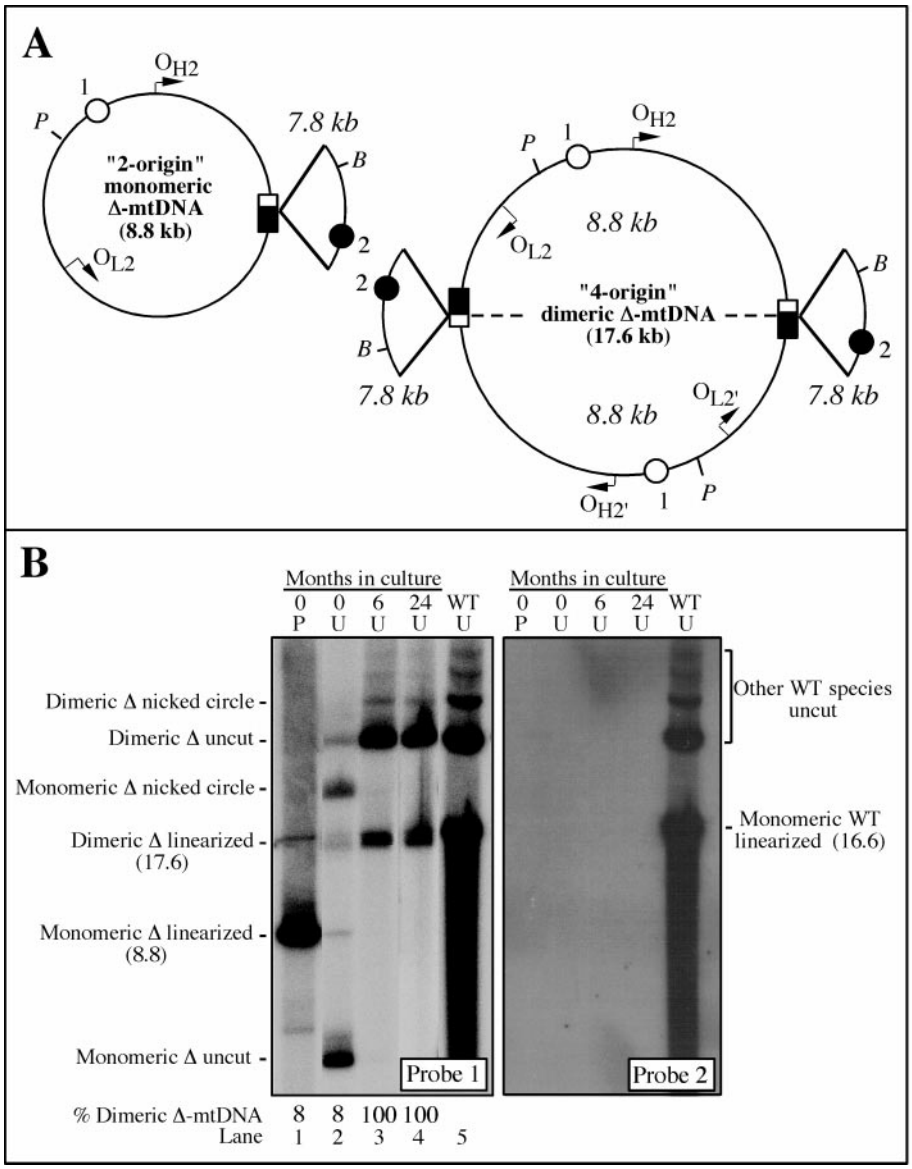

Figure 3. Long-term culture of a homoplasmic deletion cell line from patient 1. (A) Maps of the monomeric and dimeric forms of the Δ-mtDNA species present in 100% deletion lines. One of the two identical pairs of replication origins in the dimer is indicated with prime notation ($O_{H2'}$ and $O_{L2'}$). (B) Southern blot analysis of long-term culture of a 100% deletion line (KAF4EB12.49), using either DNA digested partially with *Pvu*II (P; lane 1), or undigested (U; lanes 2–4). The blot was then hybridized first with probe 2 and then with probe 1. The identity of the major hybridizing fragment is indicated at left. Uncut wt-mtDNA was also loaded as a comparative control; the identification of each band is indicated at right. The % dimeric Δ-mtDNA is indicated below. All other notation as in Figure 1.

Long-Term Culture of Homoplasmic dup-mtDNAs from Patient 2

In order to assess the generality of the finding that a dup-mtDNA can give rise to a wt-mtDNA plus the corresponding Δ-mtDNA (Figure 1B), we performed the same long-term culturing experiment on cells containing homoplasmic levels of a different dup-mtDNA, from patient 2 (see Table 1). Note that the rearrangement in this patient is unusual, in that it is a "1–3" rearrangement (see Figure 2A) rather than the more typical "2–4" rearrangement found in most patients (see definition in patient section of Materials and Methods). The dup-mtDNA in patient 2 contains only one O_L instead of the two found in the dup-mtDNA of patient 1. Furthermore, the absence of O_L in the corresponding Δ-mtDNA in patient 2 could potentially render this molecule unreplicatable, unless a "cryptic O_L " were present on the molecule, but only if mtDNA replicated via the orthodox mode; if the mtDNA replicated via the standard mode, the presence of O_L should not be crucial, and daughter light-strand synthesis should proceed even in its absence.

Southern blot analysis of *Bam*HI-digested DNA extracted from clone CH5-UEB11.6 at the first passage (arbitrarily defined as time 0) revealed a single band of 21.2 kb when hybridized with both probes 1 and 2 (Figure 2B), indicating that this cell line was initially homoplasmic for dup-mtDNA. Beginning with the analysis at day 28, we detected varying amounts of a 16.6 kb band, corresponding to wt-mtDNA. However, we never detected the corresponding Δ-mtDNA at any time point. The amount of wt-mtDNA, on a molar basis, increased to a maximum of 70% of the total mtDNA after 3 mo (i.e., ~ 110–120 cell divisions). During the subsequent 3-mo period, the amount of wt-mtDNA ap-

peared to reach a steady state, leveling out at ~ 40% wt-mtDNA (Figure 2B, 2C; the faint mtDNA band at day 182 was due to underloading, not to depletion of mtDNA in the cells). Similar results (including the 3- to 4-wk time lag before the wt-mtDNA appeared, the peak percentage of wild-type molecules, and the steady-state levels of wt-mtDNAs) were obtained with the other two 100% duplication cell lines (data not shown).

Long-Term Culture of Homoplasmic Δ-mtDNA Cell Lines from Patient 1

We had available to us two cell lines from patient 1 (KAF4EB12.17 and KAF4EB12.49 [Tang *et al.*, 2000]) that allowed us to test the hypothesis that the replication of mtDNA circles with multiple pairs of replication origins might, under some growth conditions, be favored over circles with one pair of origins. Both lines contained 100% Δ-mtDNA, and both had growth characteristics similar to those of 100% wild-type lines in nonselective medium (Tang *et al.*, 2000). Importantly, both lines contained initially a mixture of monomeric Δ-mtDNAs (containing 1 O_H and 1 O_L) and dimeric Δ-mtDNAs (i.e., two monomeric molecules arranged head to tail, and containing 2 O_H's and 2 O_L's; Figure 3A).

An example of a Southern blot analysis to distinguish among these topoisomers is shown in Figure 3B. A blot of *Pvu*II-digested DNA from cells collected at time 0 showed one major hybridizing band with probe 1, migrating at 8.8 kb (representing unit-length linearized species derived from complete *Pvu*II digestion of both monomers and dimers), and a minor band migrating at 17.6 kb (representing partial *Pvu*II digestion of the deletion dimer; Figure 3B, lane 1). Note that because *Pvu*II can cleave both monomers and dimers, this analysis cannot be used to quantitate the relative amounts of the two species present in the cells, and can only be used as a marker on the blot to identify the migration patterns of linearized monomer and dimer in the gel. The hybridization pattern of *uncut* DNA from the initial aliquot at time 0 showed that the Δ-mtDNA monomers and dimers migrated as multiple distinct (and quantitatable) bands in the gel, representing different topological isoforms: uncut monomeric and dimeric circles (the majority) plus a smaller amount of nicked and linearized monomeric and dimeric circles (compare lanes 1 and 2 in Figure 3B). Clone KAF4EB12.49 contained ~ 92% monomeric Δ-mtDNAs and 8% dimeric Δ-mtDNAs; similar values were obtained with line KAF4EB12.17 (data not shown).

The two clones were then cultured for more than 2 years in nonselective medium, and cells were collected at different time points. Aliquots analyzed after 6, 8, 12, and 24 mo (months 6 and 24 are shown in Figure 3B) showed that both clones still contained 100% Δ-mtDNAs (i.e., no hybridizing bands were detected with probe 2; Figure 3B), but that only the dimeric form was now present (see also Tang *et al.*, 2000). Furthermore, no new mtDNA species were detected.

All cell lines from patients 1 and 2, which were subjected to long-term culture for this study, showed no change in their total mtDNA content over time, as demonstrated by Southern blot quantification of mtDNA hybridizing bands relative to a nuclear DNA standard (data not shown).

DISCUSSION

DNA Recombination Activity in Human Mitochondria

Large-scale rearrangements of mtDNA have been associated with a number of neuromuscular disorders. Many patients harbor, besides wild-type mtDNAs, both mtDNA deletions and duplications. Because the two types of rearranged molecules are related (i.e., they have the identical rearrangement breakpoint), it had long been speculated that the two molecules are generated via a common mechanism, or that one is derived from the other (Poulton *et al.*, 1993; Schon *et al.*, 1997).

While slipped mispairing has been invoked as a mechanism to explain the generation of mtDNA deletions (Shoffner *et al.*, 1989; Madsen *et al.*, 1993), it alone cannot explain the coexistence of topologically related deleted and duplicated mtDNAs, especially when the rearrangements span many kb. A more likely mechanism is recombination. Although the existence of recombination in mammalian mtDNAs has been controversial (Horak *et al.*, 1974; Zuckerman *et al.*, 1984; Howell *et al.*, 1996; Ohno *et al.*, 1996; Bidooki *et al.*, 1997; Holt *et al.*, 1997; Lunt and Hyman, 1997), evidence has been accumulating steadily in support of the presence of recombinational activity in mammalian mitochondria (Holt *et al.*, 1997; Awadalla *et al.*, 1999; our unpublished observations). In particular, recent biochemical data show that both homologous (Thyagarajan *et al.*, 1996) and nonhomologous (i.e., end joining) recombination (Lakshmipathy and Campbell *et al.*, 1999a) activities have been found in mammalian mitochondrial protein extracts, and human DNA ligase III, which plays a central role in replication, recombination, and DNA repair, has been found to localize not only to the nucleus but to mitochondria as well (Lakshmipathy and Campbell *et al.*, 1999b).

Our ability to isolate pure populations of wild-type, duplicated, and deleted mtDNAs, and of selected heteroplasmic mixtures of specific mtDNA species, all derived from the same patient, allowed us to determine whether a dynamic relationship actually exists among the three types of molecules. The experiments reported here strongly support the concept that, as is the case with the mtDNAs of plants (Kanazawa *et al.*, 1998) and lower eukaryotes (MacAlpine *et al.*, 2000), human mtDNAs can undergo recombination.

We found that homoplasmic populations of duplicated mtDNAs can give rise to equimolar amounts of both wild-type mtDNA and the corresponding deleted mtDNA species. Specifically, the conversion of a 25.3 kb dup-mtDNA to a 16.6 kb wt-mtDNA plus an 8.8 kb Δ-mtDNA in patient 1 cybrids, and of a 21.3 kb dup-mtDNA to a 16.6 kb wt-mtDNA in patient 2 cybrids, was most likely due to an *intramolecular* recombination event, mediated by the kb-sized regions of tandemly repeated mtDNA sequences flanking the duplication breakpoint. In the case of the "1–3" rearrangement found in patient 2, we believe that Δ-mtDNAs failed to accumulate in cybrids because the loss of O_L from this molecule compromised its ability to replicate as an independent circle. Under our tissue culture conditions, in which mtDNA is maintained at a steady-state level, the "orthodox" model of mtDNA replication predominates, and could well be the only mode of mtDNA replication operating. It has been noted that different modes of mtDNA syn-

thesis operate in human cells under different conditions, depending on how mtDNA copy number is being modulated (Holt *et al.*, 2000). In situations where mtDNA undergoes rapid reamplification (e.g., induced by prior partial depletion of mtDNA copy number), the "standard" model of mtDNA replication is used almost exclusively, whereas when mtDNA copy number is merely maintained from one cell generation to the next, the "orthodox" model prevails (Holt *et al.*, 2000). The failure to accumulate a Δ-mtDNA molecule that lacks O_L was also obtained during long-term culture of a different "1–3" rearrangement (Holt *et al.*, 1997).

Surprisingly, however, we found no evidence that the reverse reaction—*intermolecular* recombination of a wt-mtDNA and a Δ-mtDNA to give rise to a dup-mtDNA—occurred in our system, as we observed no new species of mtDNA generated during long-term culture of heteroplasmic cells containing a mixture of wild-type and deleted [monomeric] mtDNAs from patient 1. As opposed to intramolecular recombination (which is a zero-order unimolecular reaction), intermolecular recombination requires not only recombinases (e.g., enzymes functionally similar to yeast mitochondrial Cce1p/Mgt1p [Zweifel and Fangman, 1991; Lockshon *et al.*, 1995]), Mhr1p (Ling *et al.*, 1995), Abf2p [MacAlpine *et al.*, 1998], and Ilv5p [MacAlpine *et al.*, 2000]), but also physical proximity of the two interacting mtDNAs for the recombination event to occur (i.e., it is a first-order bimolecular reaction).

It is worth noting that since the heteroplasmic cell lines carrying wt- and Δ-mtDNAs were obtained directly by fusion of patient fibroblasts with ρ^0 cells, it is possible that the two mtDNA species never coexisted within the same organelle initially, or were present within the same organelle but were attached to the mitochondrial inner membrane at physically separated positions (Albring *et al.*, 1977). The idea that mtDNAs are physically segregated within cells and individual mitochondria is supported by the observation that complementation of function did not occur when mitochondria with different mtDNA point mutations were introduced into the same cells, whereas functional complementation did occur if the mutant mtDNA molecules arose within the same population of organelles (Yoneda *et al.*, 1994; Shoubridge, 1994), although intramitochondrial complementation does not necessarily demonstrate that the genomes are close enough to recombine. In fact, mtDNAs cannot migrate freely in yeast mitochondria; rather, they tend to cluster into nucleoids that are attached to the inner mitochondrial membrane (Newman *et al.*, 1996). The same immobility of mtDNAs may also be true for mammalian mitochondria, as human mtDNA also tends to cluster into nucleoids containing 2 to 10 genomes (Nass, 1969; Satoh and Kuroiwa, 1991).

In spite of the fact that we did not find evidence for intermolecular recombination in our experiments, intermolecular recombination is consistent with the very existence of duplicated mtDNAs in the first place, as they were likely formed by one of two mechanisms, both of which almost certainly involve intermolecular recombination: (1) intermolecular recombination of two monomeric wt-mtDNAs to give a dimeric wt-mtDNA, followed by an intramolecular deletion event to give a dup-mtDNA plus a Δ-mtDNA, or (2) intramolecular deletion of a wt-mtDNA to give a Δ-mtDNA, followed by an intermolecular recombination event between a wt-mtDNA and Δ-mtDNA, to give a dup-mtDNA. In support of this concept, Holt *et al.* (1997) found that homoplasmic dup-mtDNAs present in osteosarcoma-based cybrids gave rise to a subpopulation of triplicated mtDNAs (i.e., containing two extra segments of rearranged mtDNA). It would be difficult to envision an intramolecular mechanism for the generation of the triplication from the duplication. More likely, the triplication arose via an intermolecular recombination event: a monomeric Δ-mtDNA that had arisen (together with a wt-mtDNA) from the dup-mtDNA recombined with a second dup-mtDNA, thereby forming the triplicated species.

Factors Conferring Replicative Advantage to mtDNAs

After 6 mo of culture of the initially homoplasmic 3-origin dup-mtDNA from patient 2, we observed a steady-state level of approximate 40% wt-mtDNA, a level that was much higher than the 6–10% level of wt-mtDNA found after 6-mo culture of the initially homoplasmic 4-origin dup-mtDNA from patient 1. While many processes may account for this difference, we note that in the case of a "2–4" rearrangement, all 3 mtDNA species are present, and there is an equilibrium between the "forward" and "reverse" reactions (i.e., dup $\leftrightarrow$ wt + Δ). However, in the case of a "1–3" rearrangement, the forward reaction is highly favored, owing to the removal of unreplicatable deletions from the system (i.e., dup $\rightarrow$ wt + Δ [$\downarrow$]). This process alone, however, would predict that in the case of the "1–3" rearrangement, the wt-mtDNA should accumulate inexorably, and eventually reach homoplasmy. The fact that the amount of wt-mtDNA remained steady at 40% in patient 2, and at only 6–10% in patient 1, implies that other factors were also operating to counteract the increases in wt-mtDNA.

One such possible factor may be the number of replication origins present on the molecules. For example, we found that in an initially heteroplasmic population of monomeric and dimeric forms of Δ-mtDNA, grown in the absence of selection for respiratory function, the monomeric form eventually disappeared completely (Figure 3B), for unknown reasons. However, since both the monomeric and dimeric forms were genotypically identical (Tang *et al.*, 2000), one possible explanation is that molecules with more origins of replication (i.e., the dimeric Δ-mtDNAs) were favored over those with fewer origins (i.e., the monomeric Δ-mtDNAs), in spite of the fact that the multi-origin mtDNAs were larger. The simplest explanation of those results is that 4-origin molecules have a replicative advantage over 2-origin molecules if both species are competing for a limiting level of one or more replication factors that bind to the origins (for example, mitochondrial DNA polymerase γ, RNA polymerase, mitochondrial transcription factor A, RNA processing enzymes, factors that bind to termination-associated sequences, topoisomerases, and single-stranded DNA-binding protein [reviewed in Shadel and Clayton, 1997]). This could explain how the monomeric Δ-mtDNAs (with one pair of origins) disappeared in favor of the dimeric forms (with two pairs of origins).

This "competition" or "titration" hypothesis implies that a dup-mtDNA containing two O_H/O_L pairs can still replicate successfully even if the necessary replication factors bind only to one pair of origins. In the case of the replication of

dimeric versus monomeric wt-mtDNAs (Bogenhagen *et al.*, 1981), and of the replication of yeast wt-mtDNA versus hypersuppressive petite mtDNAs containing multiple *rep/ori* sequences (Baldacci *et al.*, 1984), this indeed seems to be the case. A titration model is also consistent with the fact that alterations in cellular growth conditions (e.g., concentration of metabolites, inhibitors of protein synthesis, cell density) can shift drastically the composition of wild-type mtDNAs from monomeric to dimeric forms (reviewed in Clayton and Smith, 1975). Finally, competition for rate-limiting amounts of *trans*-acting replication factors was invoked by Moraes *et al.* (1999) to reconcile the paradoxical findings that ape mtDNAs were able to replicate in human "xenomitochondrial" cybrids when present as a homoplasmic population (Kenyon and Moraes, 1997), but were unable to be maintained when introduced into cybrids containing a preexisting population of human mtDNAs (Moraes *et al.*, 1999). Other factors besides the number of origin of replications, however, must also play a role in controlling the balance of mtDNA topoisomers, because these shifts occurred in some of our experiments but not in others.

We found that the steady-state amounts of Δ-mtDNA and wt-mtDNA remained stable over time (Figure 1D, 1E), suggesting that in cultured cells there was no replicative advantage for the shorter Δ-mtDNAs. In contrast, Moraes *et al.* (1999) reported that in cells that had been depleted of mtDNA by ethidium bromide treatment, deleted mtDNAs repopulated the cells faster than did full-length mtDNAs. A possible explanation for this discrepancy could be that Moraes *et al.* (1999) monitored the *rate* of mtDNA repopulation, whereas we measured the *steady-state level* of mtDNA.

In summary, we have obtained strong evidence that human mtDNAs can undergo homologous recombination. This finding has potentially important implications for the use of the mitochondrial genome in evolutionary, genealogical, and even forensic analyses, as mtDNA has been the molecule of choice in these fields, owing, in part, to its putative "nonplasticity" as compared with highly recombinogenic nuclear DNA. Recombination in mammalian mtDNAs also has relevance to the etiology and pathogenesis of disorders associated with mtDNA rearrangements. Finally, it may be possible to exploit mtDNA recombination as a way to replace mutated mtDNA sequences, or to manipulate the mtDNA genetically by introducing exogenous sequences.

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