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Mechanisms of Human Mitochondrial DNA Maintenance: The Determining Role of Primary Sequence and Length over Function

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Although the regulation of mitochondrial DNA (mtDNA) copy number is performed by nuclear-coded factors, very little is known about the mechanisms controlling this process. We attempted to introduce nonhuman ape mtDNA into human cells harboring either no mtDNA or mutated mtDNAs (partial deletion and tRNA gene point mutation). Unexpectedly, only cells containing no mtDNA could be repopulated with nonhuman ape mtDNA. Cells containing a defective human mtDNA did not incorporate or maintain ape mtDNA and therefore died under selection for oxidative phosphorylation function. On the other hand, foreign human mtDNA was readily incorporated and maintained in these cells. The suicidal preference for self-mtDNA showed that functional parameters associated with oxidative phosphorylation are less relevant to mtDNA maintenance and copy number control than recognition of mtDNA self-determinants. Non–self-mtDNA could not be maintained into cells with mtDNA even if no selection for oxidative phosphorylation was applied. The repopulation kinetics of several mtDNA forms after severe depletion by ethidium bromide treatment showed that replication and maintenance of mtDNA in human cells are highly dependent on molecular features, because partially deleted mtDNA molecules repopulated cells significantly faster than full-length mtDNA. Taken together, our results suggest that mtDNA copy number may be controlled by competition for limiting levels of *trans*-acting factors that recognize primarily mtDNA molecular features. In agreement with this hypothesis, marked variations in mtDNA levels did not affect the transcription of nuclear-coded factors involved in mtDNA replication.

INTRODUCTION

The human mitochondrial genome is a 16,569-bp circular molecule, containing genes that are necessary for the synthesis of the catalytic components of the oxidative phosphorylation (OXPHOS) system. Although the mtDNA-coded subunits of the OXPHOS system are essential for mitochondrial respiration and ATP production, they are intrinsically dependent on factors encoded by the nuclear DNA, synthesized in the cytosolic ribosomes, and imported into the mitochondria. These include factors that regulate mitochondrial DNA (mtDNA) gene expression, including mitochondrial DNA and RNA polymerases, mitochondrial transcription factors, RNA-processing and -modifying enzymes, transcription termination factors, mitochondrial ribosomal proteins, aminoacyl-tRNA synthetases, and translation factors (Attardi *et al.*, 1990; Shadel and Clayton, 1997). All these factors have to recognize specific mtDNA (or mtRNA) sequences to perform their functions. In addition, an equally large number of nuclear DNA (nDNA)-coded factors have to interact with mtDNA-coded polypeptides for the correct assembly and function of the OXPHOS system.

The mechanisms underlying mtDNA copy number control are not fully understood. Regulation of mtDNA replication seems to be controlled mainly by the frequency of transcription initiation at the mtDNA light-strand promoters (Shadel and Clayton, 1997), and the only known mammalian mitochondrial transcription factor was recently shown to be required for mtDNA maintenance (Larsson *et al.*, 1998). Mitochondrial transcription, in turn, is controlled by a number of factors, including metabolic cues, such as thyroid hormone levels (Pillar and Seitz, 1997). The main regulator of mitochondrial gene expression, however, is mtDNA copy number, which is controlled by the nucleus (Williams, 1986). Although defects in OXPHOS have been associated with mitochondrial proliferation (Wallace, 1997), it is unclear whether deficiencies in ATP production induce changes in mtDNA levels. The present study helped clarify these issues

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by analyzing mtDNA maintenance and copy number control in cell lines harboring different mtDNA genotypes.

MATERIALS AND METHODS

Human Cells Containing Homoplasmic Levels of Pathogenic mtDNA Mutations or Primate mtDNA

The 143B (TK^-) and its mtDNA-less derivative (143B/206 ρ°) were a kind gift of Michael King (Thomas Jefferson University, Philadelphis, PA). We have used several characterized cell lines containing the osteosarcoma 143B nuclear background and various mtDNAs. Cell lines harboring nonhuman ape mtDNAs have been previously described (Kenyon and Moraes, 1997). Human transmitochondrial cybrids harboring gorilla (HG-13), common chimpanzee (HC-14), and pigmy chimpanzee (HP-4) mtDNAs were used in this study. Primate primary fibroblasts were obtained from the Coriell Repository. Human fibroblasts were from a patient with an infantile metabolic disorder without OXPHOS deficiency. A cell line harboring a pathogenic mtDNA point mutation in the $tRNA^{Asn}$ gene (W72) and a cell line containing the same haplotype with the wild-type version of the $tRNA^{Asn}$ gene (W20) were also previously characterized (Hao and Moraes, 1997). Cell lines harboring homoplasmic mtDNA deletions were obtained by fusing 143B/206 with enucleated fibroblasts from a patient harboring a 7.5-kb deletion (spanning positions 7982–15,504; Anderson *et al.*, 1981) and a HeLa/fibroblast hybrid line harboring ~ 0% of the 4.9-kb "common deletion" (Sancho *et al.*, 1992). Clones containing heteroplasmic levels of the 7.5-kb deleted mtDNA were treated with ethidium bromide for 21 d and allowed to recover in nonselective medium as described (King, 1996). Several clones (termed Δ16.10.n) containing exclusively partially deleted molecules were isolated. 143B and Δ16.10.40 were transfected with a plasmid containing a zeocin resistance gene (pVgRXR; Invitrogen, San Diego, CA). HG13 was transfected with a plasmid containing a neomycin resistance gene (pSV2neo). Resulting clones obtained under selection for the respective drugs were used in fusion experiments in which no selection for respiratory function was applied.

Characterization of Fusion Products between Human Cells and Nonhuman Ape Cytoplasts or Intact Cells

The TK^- osteosarcoma-derived human cell lines harboring no or exclusively mutated mtDNAs (1.5×10^6 cells) were fused with cytoplasts from different apes (gorilla, common chimpanzee, pigmy chimpanzee, and human). Cytoplasts were produced by enucleating $\sim 3 \times 10^5$ fibroblasts in a 35-mm^2 dish as described (King and Attardi, 1989). Fusion products were allowed to recover overnight and plated into 20×100 mm^2 dishes in selective media (King and Attardi, 1989). Parental cell lines were killed either by bromodeoxyuridine (fibroblasts) or by the lack of uridine in the medium (143B/206 ρ° or homoplasmic deleted). After 20 d, either surviving clones were picked with the help of cloning rings, or the dishes were stained with toluidine blue for colony counting. Selected clones were expanded and analyzed by Southern blot as indicated in the legend to Figure 1. Fusion between W72 (human transmitochondrial cybrid harboring a pathogenic point mutation in the $tRNA^{Asn}$ [G5703A]) gave rise to nine clones in a medium in which glucose was replaced by galactose (5 mM; Robinson *et al.*, 1992). These clones were analyzed by Southern blot after digestion with *Xba*I and probing with a long PCR fragment corresponding to the whole human mtDNA (positions 10–16,496). In all experiments, enucleated human fibroblasts were used in parallel fusions as controls.

Hybrid clones obtained from the fusion between 143B or Δ16.10.40 and HG13 cells after selection in the presence of G418 and zeocin were analyzed by Southern blot after digestion with *Pvu*II and probing with a cloned DNA fragment corresponding to gorilla mtDNA promoter region (positions 293–797).

mtDNA Depletion and Repopulation Analysis

The various cell lines were grown in complete medium (Dulbecco's modified Eagle's medium supplemented with 4.5 mg/ml glucose, 50 μg/ml uridine, 10% FCS, 1 mM pyruvate, 10 μg/ml gentamicin) in the presence of 50 ng/ml ethidium bromide (EtBr). EtBr was kept in the media for 15 d, after which cells were fed with complete media without EtBr. Cells were kept between 30 and 80% confluence with excess fresh medium to assure exponential growth. At selected days (0, 6, 15, 22, 30, and 45) cells were harvested for DNA extraction. Doubling time was similar for all cell lines in complete medium (18–22 h).

For each cell line (obtained from all selected treatment days) DNA samples (~2 μg) were digested with *Hin*dIII and slot blotted at three different dilutions (~0.5, 0.25, and 0.12 μg) in two nylon filters (Zeta probe; Bio-Rad, Hercules, CA). Every filter set also included two concentrations of the 143B-digested DNA and 1 μg of yeast tRNA. One filter was initially hybridized with a probe corresponding to a nondeleted region (mtDNA position 3305–4261). The second filter was hybridized to a probe corresponding to the 18S rDNA gene (Moraes *et al.*, 1991). The first filter was stripped and hybridized to the nuclear probe, and the second filter was stripped and hybridized to the mtDNA probe. All probes were prepared by the random primer method. Hybridizing bands were quantitated in a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. Signals were used to determine the ratios between mtDNA:nDNA corresponding to the three concentrations. The intensity of the signal was normalized to day 0 in the repopulation analyses.

Transcriptional Studies

Oligonucleotide primers (~22–24 mer) were designed to amplify the following regions of nuclear-coded genes involved in mtDNA replication (numbers correspond to coding region as described in GenBank): large subunit of DNA polymerase γ (U60325; nucleotides [nt] 3211–3746); mitochondrial single-strand binding protein (M94556; nt 79–525); mitochondrial transcription factor A (M62810; nt 133–873); and mitochondrial RNA polymerase (U75370; nt 2564–3721). PCR products were amplified from a HeLa cDNA library and their identity confirmed by the expected size and restriction fragment length polymorphism (RFLP). The fragments were gel purified and used as templates to synthesize ^{32}P-labeled probes. The complete human apocytochrome *b* gene (positions 14,747–15,886) was also amplified by PCR from total DNA from human white blood cells. A 1.9-kb γ-actin mRNA probe was obtained by *Eco*RI digestion of a cloned insert (Erba *et al.*, 1988). The purified fragment was ^{32}P-labeled by the random primer method (Boehringer Mannheim, Indianapolis, IN). These probes were used in Northern blot studies in independent membranes, with the follow exceptions: the steady-state levels of γ-actin were determined in every blot. Also, single-stranded DNA-binding protein, γ-actin and apocytochrome *b* RNAs steady-state levels were determined in the same blot, respectively (after stripping the previous probe).

RESULTS

Competition between Functional Non–Self-mtDNA and Partially Deleted, Nonfunctional Self-mtDNA

We have previously shown that mtDNA from gorilla and chimpanzees can replace human mtDNA and restore respiration in cultured human osteosarcoma cells to almost normal levels (Kenyon and Moraes, 1997; Barrientos *et al.*, 1998). We now attempted to introduce these ape mtDNAs into a human cell line homoplasmic for a 7.5-kb mtDNA deletion

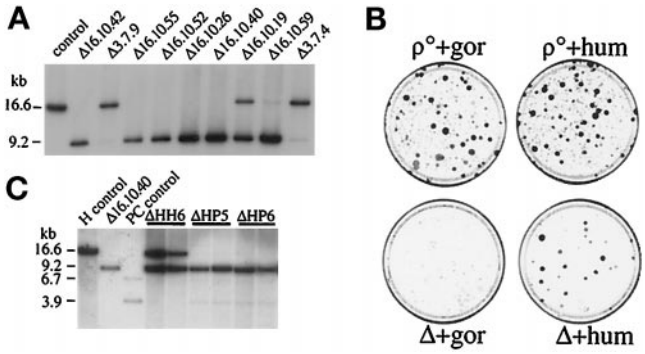

Figure 1. Competition between human partially deleted mtDNA and primate wild-type mtDNA. (A) Southern analysis of mtDNA in homoplasmic deleted cells. Cell lines homoplasmic for a 7.5-kb deletion were obtained by treating a heteroplasmic transmitochondrial cybrid with ethidium bromide. Shown is a Southern analysis of mtDNA from selected clones after digestion of total DNA with *Pvu*II and probing with an mtDNA rRNA region probe (positions 1462–2463). (B) The cell lines Δ16.10.40 (bottom) and 206ρ° (top) were used as mitochondrial recipients in cybrid fusions with gorilla (left) and human (right) cytoplasts. Fifteen days after fusion, plates were stained with toluidine blue to identify colonies of growing cells. (C) Southern blot analyses of respiration-competent cybrids between Δ16.10.40 and pigmy chimpanzee cytoplasts. DNA from the only two identified clones (ΔHP5 and ΔHP6) and from a control cybrid produced at the same time but with human cytoplasts (ΔHH6) was prepared after 5 wk (left lane) and 10 wk (right lane) after fusion and digested with *Pvu*II before the Southern analyses with a human mtDNA probe (positions 1462–2463). DNA from a normal human cell line (H control), pigmy chimpanzee fibroblasts (PC control), and Δ16.10.40 was analyzed in the same manner.

(termed Δ16.10.40). This cell line was obtained by treating a transmitochondrial line heteroplasmic for the deletion with ethidium bromide and allowing the cell to repopulate with the residual mtDNAs. This procedure led to the isolation of several clones containing exclusively partially deleted mtDNA (Figure 1A). These cells were unable to survive in medium lacking uridine because of a complete lack of OXPHOS function (our unpublished results). To investigate for interactions between gorilla mtDNA and a partially deleted human mtDNA, we fused gorilla cytoplasts with Δ16.10.40 cells. Cybrid fusion products, obtained as described in MATERIALS AND METHODS, were subjected to a selection medium for OXPHOS function (i.e., lack of uridine). No growing clones were observed using gorilla cytoplasts as mtDNA donor, whereas >100 uridine-independent clones were obtained from a control fusion between Δ16.10.40 and enucleated human fibroblasts (Figure 1B and Table 1).

The fusion between gorilla cytoplasts and Δ16.10.40 was repeated six independent times. In all experiments >100 clones were obtained with the Δ16.10.40–human cytoplast control fusion, and none was obtained with the Δ16.10.40–gorilla cytoplast fusion. Fusions between the human ρ°143B/206 and gorilla cytoplasts were also repeated and consistently yielded >300 uridine-independent clones (Table 1). Two additional human clones containing the 7.5-kb mtDNA deletion (Δ16.10.26 and Δ16.10.52; Figure 1A) were tested in similar fusions, and the results were identical to those observed with clone Δ16.10.40. Fusions using common chimpanzee and pigmy chimpanzee as mtDNA donors yielded similar results (i.e., essentially no uridine-independent colonies were obtained; Table 1). In the fusion experiment between Δ16.10.40 and pigmy chimpanzee cytoplasts, two uridine-independent clones were identified and analyzed (ΔHP5 and ΔHP6). These clones showed a small percentage of the wild-type pigmy chimp mtDNA coexisting with the deleted human mtDNA (Figure 1C). These cell lines were cultivated continuously in selective media for 35 d, and no change in the phenotype or genotype was observed (Figure 1C). By the sixth week in culture both cell lines went into "crisis" and died. A control Δ16.10.40–human cytoplast fusion clone (ΔHH6) obtained and cultured in parallel continued to grow normally. Because only two uridine-independent clones were obtained, it was possible that in these cells the pigmy chimpanzee mtDNA became better adapted to a human nuclear environment by recombining with the partially deleted human mtDNA. To investigate for the presence of mtDNA recombination in the heteroplasmic clones, DNA from ΔHP5 and ΔHP6 was analyzed by Southern blot after digestion with *Xba*I (DNA obtained before crisis). The restriction fragments obtained did not show any bands with abnormal size, suggesting the absence of recombinant molecules (our unpublished results). We estimated our detection limit to be ~1–2% of abnormal mtDNA. In total, 10 controlled experiments failed to generate stable cell lines containing, in the same cell, human partially deleted mtDNA and ape mtDNAs.

Table 1. Generation of OXPHOS-competent clones after fusion of cytoplasts from different apes with human cell lines harboring no or homoplasmic mutated mtDNA

	mtDNA donor			
Human recipient	Human	Common chimp	Pigmy chimp	Gorilla
143B/206 (ρ°)	449	385	392	408
Δ16.10.40	141	0	2[a]	0
W72	226	ND	ND	3[b]

Data are from representative fusion experiments using similar amounts of mtDNA donors (~3×10^5 enucleated fibroblasts) and recipient donors (1.5×10^6 osteosarcoma-derived cell). ND, not done.

[a] Two clones containing low levels of pigmy chimp mtDNA were isolated and grown for 30 d, at which point they became unable to sustain growth in uridine-lacking selective medium and died.

[b] A fusion between W72 and gorilla cytoplasts yielded nine clones after galactose selection, but only three contained gorilla mtDNA. One of them contained <10% gorilla mtDNA, whereas the other two were homoplasmic.

Competition between Non–Self-mtDNA and a Point Mutated Function-impaired Self-mtDNA

We also attempted to introduce gorilla mtDNA in a cell line containing homoplasmic levels of an mtDNA containing a G5703A transition in the $tRNA^{Asn}$ gene (clone W72). This cell line has been previously characterized at the molecular

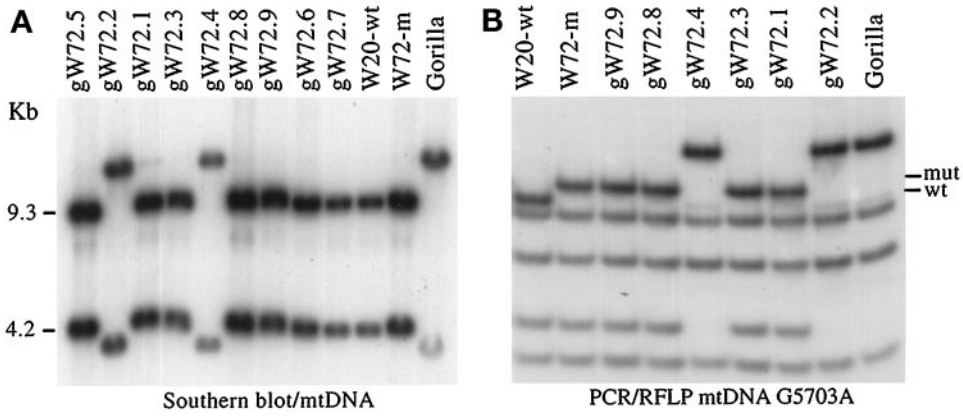

Figure 2. Competition between human point-mutated mtDNA and primate wild-type mtDNA. (A) Southern analysis of fusion products between a human cell line harboring a pathogenic G5703A mutation and gorilla's cytoplasts. DNA from nine clones able to grow in galactose medium were digested with *Xba*I and probed with a whole human mtDNA (positions 10–16,496) probe. (B) Analysis of the G5703A mutation by PCR and RFLP. A DNA fragment encompassing the $tRNA^{Asn}$ gene was amplified by PCR and digested with *Dde*I as described (Hao and Moraes, 1997). There are five *Dde*I sites in the human DNA fragment, but the one at position 5699 allows the differentiation of human mutated and wild-type molecules, whereas two additional absent sites allow the identification of gorilla mtDNA. The small levels of gorilla mtDNA observed by Southern analysis of clone gW72.1 cannot be observed in the PCR and RFLP analysis, probably because the human-based PCR primers may be slightly biased toward human templates due of to a few mismatches in the 5′ region of the oligonucleotides. Note the absence of heteroplasmic or recombinant clones.

level in our laboratory (Hao and Moraes, 1997), and although it can grow in the absence of uridine, it is unable to grow in a medium containing galactose as a carbon source (Hao *et al.*, 1999). Fusions between W72 and enucleated fibroblasts from human or gorilla were placed under galactose selection. Two hundred twenty-six clones were obtained when human mitochondria were introduced into W72. On the other hand, only nine galactose-resistant clones were obtained from the introduction of gorilla mitochondria into W72. Of the nine growing clones, only two had gorilla mtDNA, and both were homoplasmic (Figure 2). Only one heteroplasmic clone containing a small percentage of gorilla mtDNA was identified (gW72.1). The identification of several galactose-resistant clones containing only G5703A mutated mtDNA is puzzling, and it appears to be related to changes in the nuclear background (Hao *et al.*, 1999).

Competition between Non–Self- and Self-mtDNAs under Nonselective Pressure for OXPHOS Function

We investigated whether the competition observed between self- and non–self-mtDNA was not due to a functional interference between species-specific mtDNA-coded factors during the assembly of the multisubunit OXPHOS complexes. To do so, we devised an experiment to introduce gorilla mtDNA into a human nuclear background without the requirement for OXPHOS function after fusion. We fused a human xenomitochondrial cybrid harboring gorilla mtDNA (HG13) containing a neomycin resistance nuclear marker with a human cell (143B or Δ16.10.40) containing a zeocin resistance nuclear marker. Fusion products were selected in the presence of complete medium supplemented with uridine, G418, and zeocin. Clones resistant to both nuclear markers were isolated and expanded, and their mtDNA was analyzed by Southern blot. Figure 3 shows that all 12 selected hybrids resistant to zeocin and G418 (six from the fusion with 143B and six from the fusion with Δ16.10.40) contained only human mtDNA. For maximum sensitivity, we used a gorilla D-loop region as a probe. We found traces of what could be gorilla mtDNA in only one clone (clone 6 of 143B/HG13 fusion). These results indicate that the gorilla mtDNA was outcompeted by the nuclear cognate human mtDNA also without selective pressure for respiratory function.

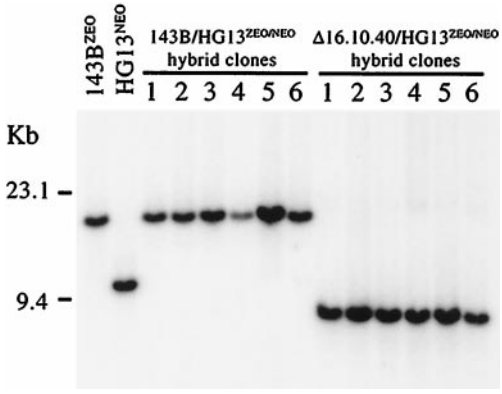

Figure 3. Competition between self- and non–self-mtDNA under nonselective conditions for respiratory function. 143B or Δ16.10.40 cells containing a zeocin resistance marker were fused to HG13 cells (harboring human nDNA and gorilla mtDNA) containing a neomycin resistance marker. Six of the resulting hybrids from each fusion resistant to both zeocin and the neomycin analogue G418 were isolated in complete medium supplemented with uridine. DNA was extracted from these hybrid clones and parental lines, digested with *Pvu*II, and analyzed by Southern blot using a ^{32}P-labeled gorilla D-loop mtDNA region as a probe. The human samples showed the expected band corresponding to the 16.6-kb linearized mtDNA. The gorilla mtDNA was digested into the expected 10.5-kb fragment and three smaller fragments that were not detected by the probe. Note that none of the 12 zeo-neo hybrids retained the gorilla mtDNA.

Table 2. Characterization of several mitochondrial and mtDNA features in transmitochondrial cybrids

Cell line	mtDNA	mtDNA levels (mean ± SD)[a]	Mitochondrial content (CS/LDH) (mean ± SD)	mtDNA/Mitochondrial content	mtDNA repopulation rate (% rec/d)[b]	Repopulation rate/wild-type rate[c]
143B	Wild-type	2.35 ± 0.12	0.22 ± 0.03	10.7	1.7	1.4
143B/206	ρ°	0	0.36 ± 0.06	NA	NA	NA
W20	Wild-type	2.49 ± 0.26	0.33 ± 0.07	7.6	0.8	0.6
W72	$tRNA^{Asn}$ G5703A	3.27 ± 0.69	0.35 ± 0.09	9.3	2.3	1.8
Δ16.10.40	100% 7.5-kb deletion	4.17 ± 0.15	0.31 ± 0.04	13.5	6.0	4.8
ΔBH10.5.9	88% 4.9-kb deletion	3.43 ± 0.31	0.37 ± 0.11	9.3	9.4	7.5
ΔHH6.1	89% 7.5-kb deletion	6.27 ± 1.20	0.28 ± 0.03	22.4	9.2	7.4
HC14	Common chimp	NA	0.33 ± 0.04	NA	2.0	1.6
HP4	Pigmy chimp	NA	0.40 ± 0.02	NA	0.8	0.6
HG13	Gorilla	NA	0.31 ± 0.02	NA	2.0	1.6

All cell lines have the same nuclear background (143B/TK^-). NA, not applicable.

[a] Ratio of mtDNA hybridization (ND1 region) and 18S rDNA (arbitrary units). The levels observed in nonhuman apes cannot be directly compared with the human cell lines, because the hybridized region (ND1) has ~84% identity with the ape mtDNAs.

[b] mtDNA repopulation rates were calculated for the initial 7 d after EtBr removal (see Figure 5).

[c] The wild-type rate was assumed to be the average between 143B and W20 values.

Relative mtDNA Repopulation Rates in Cells Containing Different Mitochondrial Genotypes

To test whether the preference for self-mtDNA was associated with relative mtDNA replication rates, we measured mtDNA repopulation rates in different cell lines after severe mtDNA depletion by EtBr treatment. Different cell lines were treated with EtBr in nonselective media for 15 d, after which the drug was removed, and the cells were allowed to continue to grow exponentially for an additional 30 d. Cell samples were collected at different time intervals, and the relative mtDNA levels were determined as described in MATERIALS AND METHODS. Before starting the experiment, we characterized several functional parameters in our cell lines (Table 2). The population doubling of all cell lines was similar in complete medium (~18–22 h; our unpublished results). The mitochondrial content was estimated by the ratio of citrate synthase (a mitochondrial matrix enzyme) to lactate dehydrogenase (a cytosolic enzyme). The mitochondrial content was also similar among all cell lines, with the parental 143B having slightly lower values (Table 2). MtDNA relative values, measured as the ratio between mtDNA and the nuclear rDNA (see MATERIALS AND METHODS) and normalized to mitochondrial content, were slightly higher in one cell line containing close to homoplasmic levels of a partially deleted mtDNA but were essentially doubled in another one. Another cell line containing almost homoplasmic levels of the common deletion (ΔBH10.5.9) had mtDNA relative levels comparable to controls. The mtDNA:rDNA ratios in xenomitochondrial cybrids could not be compared with the "all-human" cell lines because of a lower degree of homology between the probe (human mtDNA) and the other ape mtDNAs. However, previous experiments showed that when normalized for total DNA, mtDNA values in xenomitochondrial cybrids were similar to human–human transmitochondrial cybrids (Kenyon and Moraes, 1997).

The kinetics of mtDNA depletion and repopulation of these different cell lines in nonselective medium showed a dramatic difference only for the partially deleted mtDNAs. The relative levels of partially deleted mtDNAs recovered to pretreatment levels in ~5 d after EtBr removal, whereas full-length mtDNA needed a much longer period to approach original levels (Figure 4). A cell line containing a point mutated mtDNA and a severe functional defect (W72) showed a kinetic of depletion and repopulation that was not markedly different from that of cell lines harboring wild-type mtDNA. Likewise, human xenomitochondrial cybrids harboring common chimpanzee and gorilla mtDNA showed kinetics of mtDNA depletion and repopulation similar to those of human controls (Figure 4).

The initial repopulation rate of mtDNA relative to nuclear DNA was estimated by the slope of mtDNA:nDNA ratios after EtBr removal (Figure 5). Considering the mtDNA repopulation linear (which clearly was not the case), the mean repopulation rates of the combined cells harboring mtDNA deletions were sevenfold higher than the control cell lines during the first 7 d ($p = 0.006$) and fourfold higher during the first 15 d ($p = 0.002$). Ape mtDNA (chimpanzee and gorilla) repopulation rates were not different from human wild-type controls at either 7 d ($p = 0.11$) or 15 d ($p = 0.12$). Likewise, the mtDNA repopulation rates in cell line W72 were not markedly different from those in cell lines containing wild-type or ape mtDNAs. It is likely that the mtDNA repopulation is linear and at maximum rate only during the initial repopulation period. Analyzing only the first 7 d, the initial rates of partially deleted molecules were significantly faster (approximately five to eight times) than the rates for full-length molecules (Figure 5 and Table 2). As mentioned above, the mtDNA repopulation rates after EtBr removal did not follow a linear pattern during the entire repopulation process, particularly after 30 d, when the rate decreased substantially. At 45 d after EtBr removal (without selection for respiratory function), mtDNA levels were close to orig-

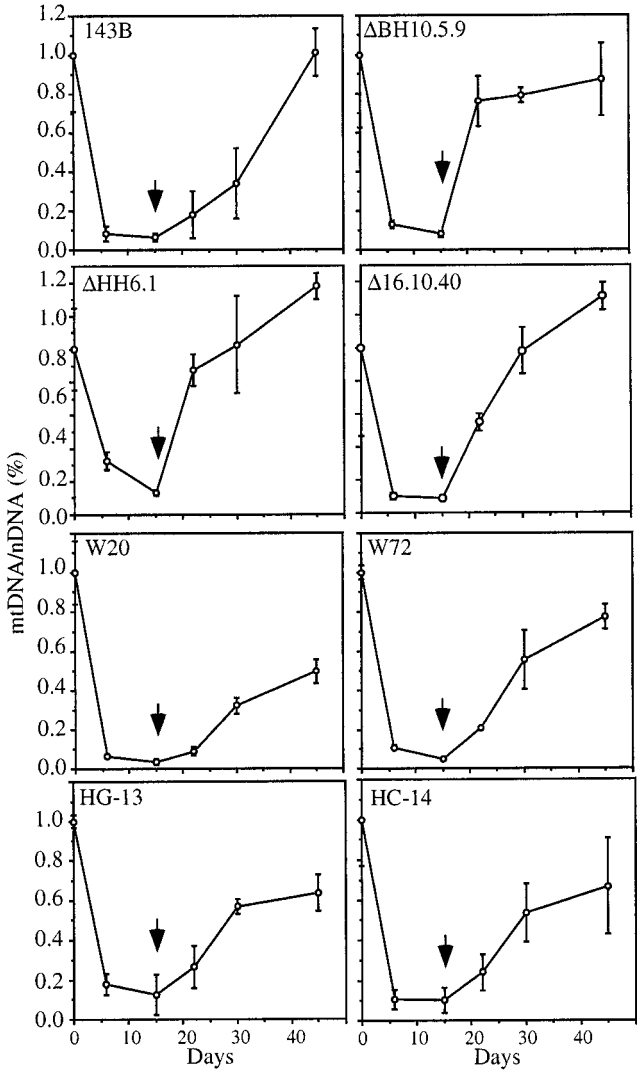

Figure 4. Partially deleted mtDNA repopulates mitochondria faster than full-length mtDNA. Different cell lines (identified in the upper left corner of each panel) harboring various mtDNA forms were treated with 50 ng/ml ethidium bromide for 15 d. After this period the drug was removed, and the cells continue to grow for an additional 30 d. DNA samples were obtained at days 0, 6, 15, 22, 30, and 45. Relative mtDNA levels were determined as described in MATERIALS AND METHODS. Note the fast recovery of mtDNA in cell lines harboring partially deleted mtDNA when compared with cells harboring full-length molecules.

inal levels in most but not all cell lines (Figure 4). The reason for these differences between the cell lines is not understood, but it may be related to differences in residual levels of mtDNA at the time EtBr was removed.

Transcriptional Regulation of mtDNA Replication Factors in Response to Changes in mtDNA Levels

The previous experiments suggested that mtDNA maintenance and copy number control could be determined by the presence of limiting levels of nuclear-coded factors. To obtain further information in this potential mechanism, we analyzed the transcriptional response of four different nuclear genes involved in mtDNA replication to mtDNA depletion, namely, mitochondrial RNA polymerase, DNA polymerase γ, mitochondrial transcription factor A, and the mitochondrial single-strand DNA-binding protein. These transcripts were analyzed before, during, and after EtBr treatment of three cell lines (143B, Δ16.10.40, and HP4). The same membrane was probed initially for one of the four mitochondrial replication factor mRNAs followed by γ-actin (Figure 6). As expected, analysis of apocytochrome *b* transcripts showed that the levels of this mRNA were undetectable after 15 d of EtBr treatment because of the severe mtDNA depletion. We found that this severe depletion of mtDNA did not induce the transcription of any of the four mtDNA replication-related genes tested. We did observe small variations during the treatment period (Figure 6) but no correlation between mtDNA levels and increase (or decrease) in the transcription of any of these four nuclear-coded genes. Transcription of these genes in the ρ° line was similar to that in the parental 143B, the only possible exception being the slightly higher expression of the mitochondrial RNA polymerase in the ρ° (Figure 6, single open squares). The levels of the different mtDNA replication proteins were not investigated, because specific antibodies were not available, and because some of these factors require the presence of mtDNA to stabilize themselves (Larsson *et al.*, 1994), making the study of protein levels during mtDNA depletion difficult to interpret.

DISCUSSION

Mechanisms of mtDNA Maintenance and Copy Number Control in Human Cells

The present work used different approaches to investigate the mechanisms controlling mtDNA maintenance and copy number. Our results showed that the human nucleus has a strong preference for self-mtDNA, even at the expense of OXPHOS function. We were not able to identify clones that maintained a nonhuman functional mtDNA coexisting with a human function-impaired mtDNA. There were two rare exceptions to these observations. 1) Two uridine-independent clones were obtained from the fusion of a human cell line containing partially deleted mtDNA with pigmy chimpanzee cytoplasts. These cybrids had very low levels of pigmy chimp mtDNA but enough to confer OXPHOS function. The percentage of pigmy chimp mtDNA did not increase during 35 d in culture, and no recombinant molecules were observed. These cells eventually went into crisis and died under selection for OXPHOS function. 2) Of nine galactose-resistant clones obtained from the fusion of a human cell harboring a mitochondrial $tRNA^{Asn}$ gene point mutation and gorilla cytoplasts, two cell lines had the human mtDNA completely replaced by the ape mtDNA, and only one clone harboring a small percentage of gorilla mtDNA coexisting with human mtDNA was identified. Considering that hundreds of clones were consistently obtained when human cytoplasts were used in similar fusions, the exceptions described above seem to constitute rare events that

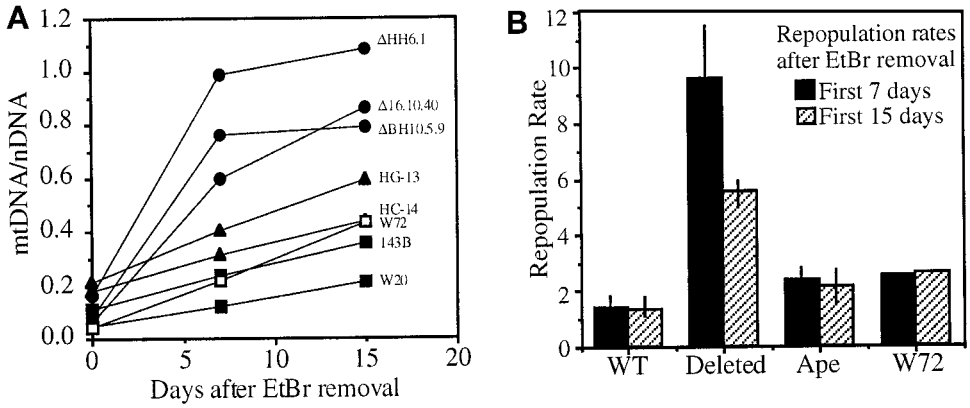

Figure 5. mtDNA repopulation rates of various haplotypes. (A) Repopulation rates of the cell lines described in Figure 3 were compared. Cell lines harboring partially deleted mtDNA increased their mtDNA content rapidly during the first 7 d after EtBr removal, slowing down during the subsequent 8 d. (B) The mean linear repopulation rates of the combined cells harboring mtDNA deletions were significantly higher than the control cell lines during the first 7 d (gray bars) or 15 d (hatched bars). Ape mtDNA (chimpanzee and gorilla) linear repopulation rates were not significantly different from those of controls during the same repopulation period. The rate in cell line W72 was also not markedly different from that in cell lines containing wild-type or ape mtDNAs. Error bars represent the range of values for the different cell lines in each group.

escaped the normal regulatory mechanisms of mtDNA maintenance. Likewise, in the absence of selection for respiratory function, hybrids of 143B and HG13 or Δ16.10.40 and HG13 maintained only human mtDNA, ruling out that the incompatibility was due to interference between heterologous mtDNA-coded factors.

MtDNA depletion and repopulation studies showed that when alone, primates' mtDNA repopulated human cells at a rate that was similar to that of wild-type human mtDNAs, and they could readily repopulate human ρ° cells. These observations suggest that competition, and not replication rate, was the key element preventing these genomes from repopulating human cells containing a defective human mtDNA. This competition also seems to operate between human mtDNAs. Even though we obtained >100 clones after fusing human cells harboring partially deleted mtDNA with human cytoplasts, this number was less than half of what was obtained using the ρ° cell as the recipient. These results may be due to the fact that partially deleted mtDNAs repopulated cells more efficiently and faster than full-length molecules.

The fact that human ρ° cells do accept and repopulate themselves with ape mtDNAs, but cells with a wild-type or mutated mtDNA do not, addresses some fundamental questions regarding mtDNA maintenance. The competition observed showed that there is a mechanism to recognize the presence of mtDNA that is independent of OXPHOS function. The simplest explanation for the competition between genomes is that human and ape mitochondria fuse, and replication factors preferentially bind to human rather than ape mtDNA (Figure 7). This putative mechanism implies that there is fusion of mitochondria, and that replication factors can "travel" and choose among mtDNA forms within the mitochondrial matrix. The lack of replication would ultimately eliminate the ape mtDNA from the cybrids after exponential cell growth ensues. Although the mtDNA control region is similar in all apes (Horai *et al.*, 1995), the few polymorphisms between the different species may be enough to confer the molecular preferences observed. In the absence of mitochondrial fusion, the competition between genomes is harder to envision. If the exogenous mitochondria remain separated from the endogenous, and both continue to receive replication factors through the mitochondrial import machinery, a signaling system between human mitochondria → human nucleus → ape mitochondria would be necessary to explain why the ape mtDNA cannot be maintained. Mitochondrial fusion with exchange of components has been reported (Hayashi *et al.*, 1994; Nunnari *et al.*, 1997; Rizzuto *et al.*, 1998), but the frequency of such events is controversial (Attardi *et al.*, 1990). It is possible that although mitochondrial fusion is a common event, the diffusion of different molecules may depend on their chemical features.

What do cells count when establishing the "normal" mtDNA copy number? They do not seem to rely heavily on any metabolic or functional cue. The relative levels of the mtDNA region preserved by the deletion (measured as a ratio to a nuclear gene) varied among the three lines harboring partially deleted mtDNA tested, and in only one line could we observe a dramatic increase in the ratio of mtDNA: 18S nuclear rDNA (doubled). It has been shown that in a mouse cell line in which the entire mtDNA population consists of head-to-tail unicircular dimers, the number of unit genomes per cell was approximately two-thirds more than that for a similar cell line with only monomeric mtDNA (Bogenhagen and Clayton, 1974). This observation suggested that neither the number of molecules nor the number of genomes is the target for copy number control (Clayton, 1982). It is also possible that cells regulate the total mtDNA mass, and have a constant rate of "mass repopulation" after EtBr depletion. However, even if normalized by mass, the partially deleted mtDNAs would still repopulate cells at least twice as fast as full-length molecules.

Our experiments showing a competition for maintenance suggest that mtDNA maintenance and copy number may be controlled by the presence of limiting levels of *trans*-acting factors involved in mtDNA replication (Figure 7). It is possible that mtDNA levels cease to increase when these factors are committed to existing molecules. In fact, a single factor could exert such control, including mtTFA (Fisher *et al.*, 1989), mtRNA polymerase (Antoshechkin and Bogenhagen, 1995; Tracy and Stern, 1995; Tiranti *et al.*, 1997; Clayton, 1998), DNA polymerase γ (Williams and Kaguni, 1995; Ye *et al.*, 1996; Graves *et al.*, 1998), RNA-processing factors (Shadel and Clayton, 1997), single-strand–binding protein (Tiranti *et*

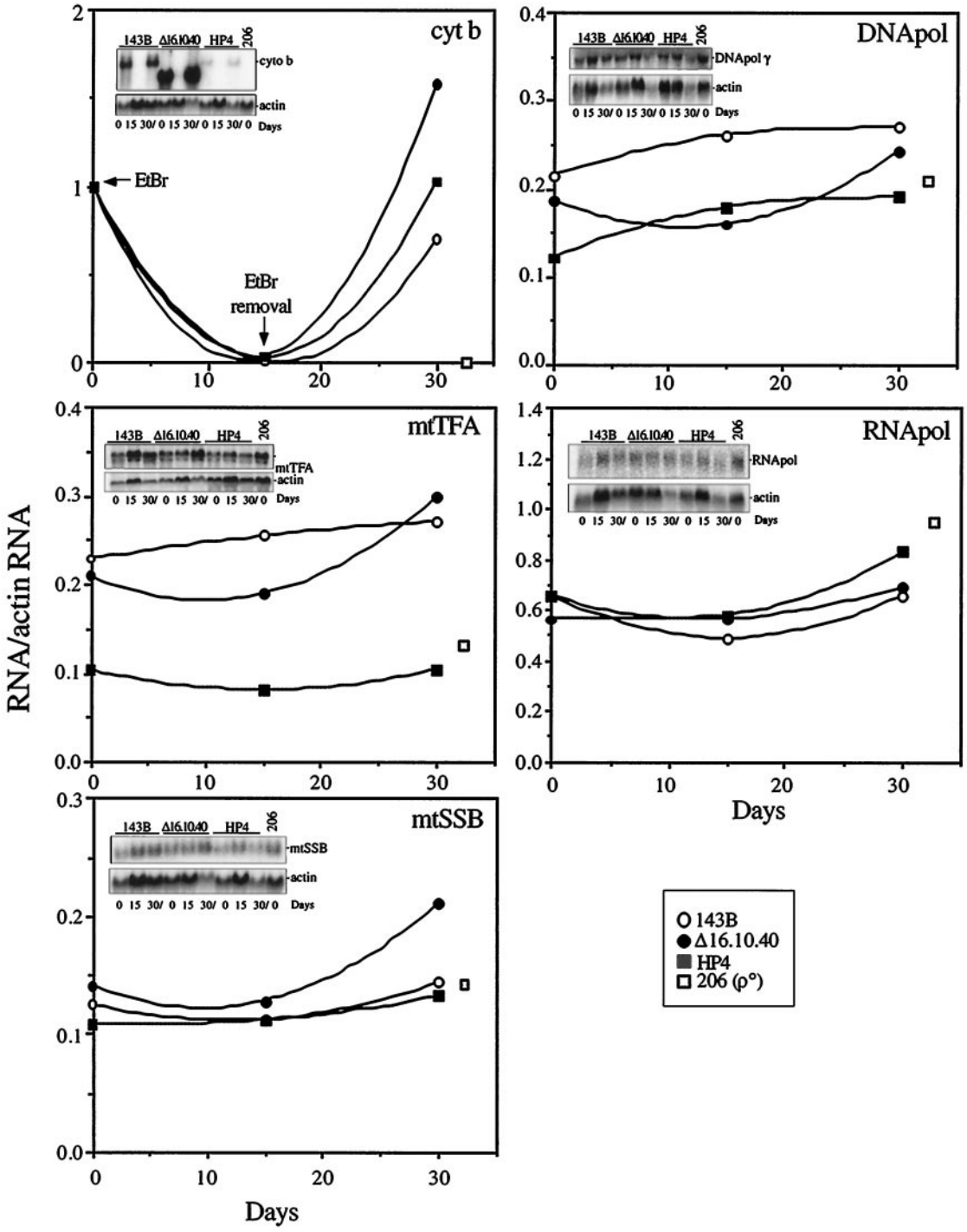

Figure 6. Effect of mtDNA depletion on transcription of mtDNA replication-associated genes. Three cell lines (143B, Δ16.10.40, and HP4) were subjected to a 15-d ethidium bromide treatment followed by an additional 15 d of growth in complete medium in the absence of ethidium bromide. Total RNA was obtained for all three cell lines at days 0, 15, and 30. These RNA samples as well as RNA from the 206 ρ° line were analyzed by Northern blots for five different transcripts (large subunit of the DNA polymerase γ, mitochondrial RNA polymerase, mitochondrial transcription factor A, mitochondrial single-strand–binding protein, and apocytochrome *b*). The graphs illustrate the variations in the ratio of the different RNAs to γ-actin (determined in the respective blots). There was no consistent up- or down-regulation of any of the nuclear-coded factors associated with the severe mtDNA depletion at day 15 or with the ρ° line (open squares). The graph for apocytochrome *b* was normalized to day 0, whereas the absolute ratios were plotted for the other RNAs. HP4 is a human xenomitochondrial cybrid harboring pigmy chimpanzee mtDNA.

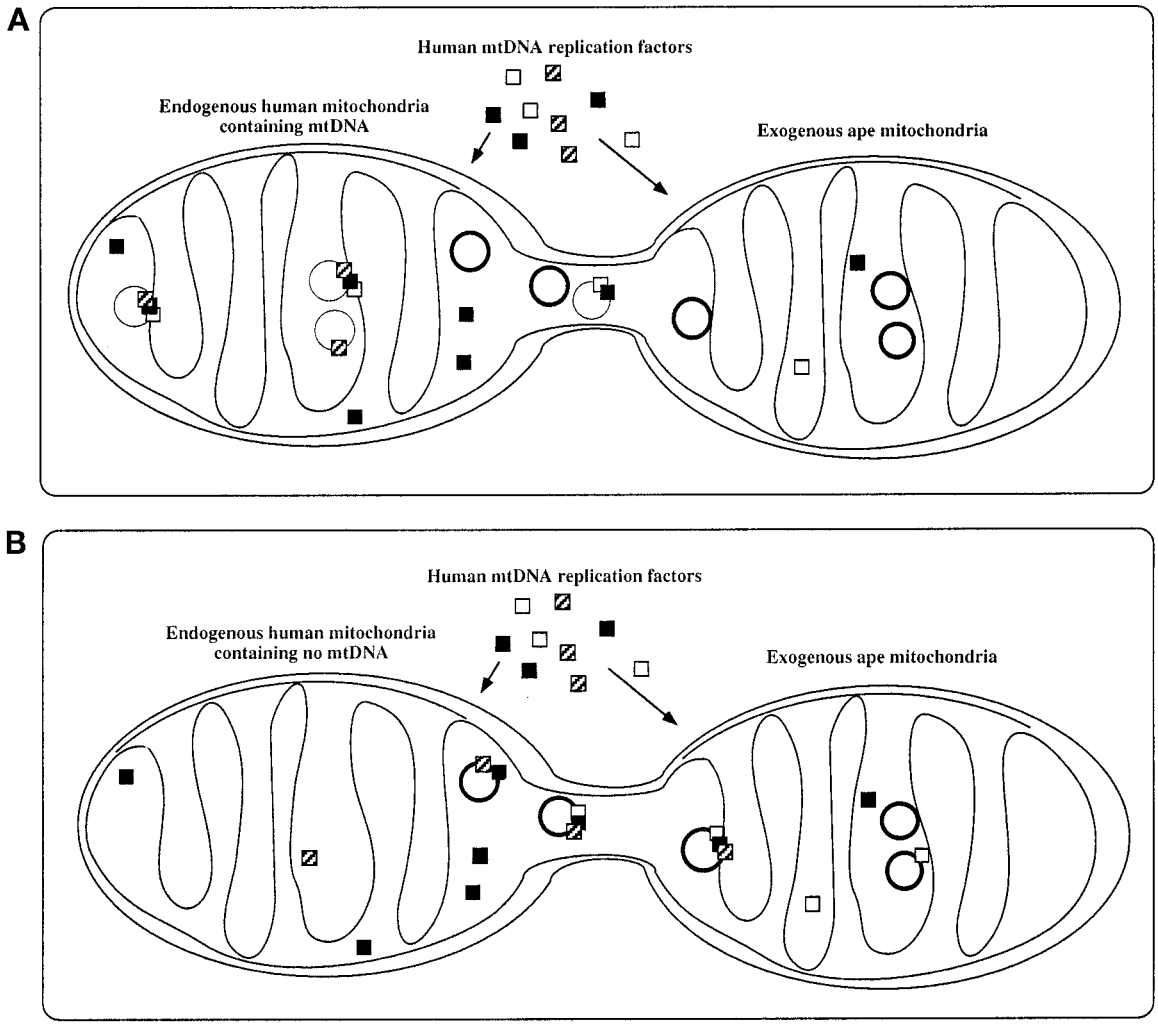

Figure 7. Model for the control of mtDNA copy number in mammals. A putative mechanism that could explain the competition observed between human and ape mtDNAs in a human nuclear background is illustrated. The model assumes that mitochondria fuse, and replication factors can "choose" their best target. Replication factors encoded by the human nucleus are represented as squares. Some of these factors are more abundant than others, and some are associated with the inner membrane (Albring *et al.*, 1977). The human mtDNA is represented as a hairline circle, whereas a thicker circle represents the ape mtDNA. (A) Fusion between ape cytoplasts and human cells harboring homoplasmic levels of a mutated mtDNA. After fusion, mitochondria fuse, but the limiting levels of certain replication factors will commit to self-mtDNA. This preference will lead to a lack of replication of the foreign mtDNA. (B) Model representing the fusion between ape cytoplasts and a human ρ° cell. Because no human mtDNA is present, replication factors can commit to ape mtDNA assuring their replication.

al., 1993), and others. All these factors seem to be required for mtDNA replication, but limiting levels of one or more would be sufficient to control mtDNA copy number. The observation that mtDNA repopulation rates are faster when the copy number is low is also compatible with the concept that replication factors are titrated out as the mtDNA copy number increases. We cannot rule out that the mtDNA copy number may be higher in some cell lines with a large deletion (as we observed in two of three lines). However, even this feature could be reconciled with our hypothesis if some factors required for replication have to bind extensive regions of mtDNA and, in the presence of deletions, may have their relative abundance increased.

Although we found that mtDNA levels do not regulate transcription of mtDNA replication-related genes, this observation per se does not establish whether the levels of these factors are influenced by a reduction of mtDNA copy number, because regulation may still occur at the protein level. Despite the fact that we do not have specific antibodies against these factors, the measurement of these proteins has a major drawback. Larsson *et al.* (1998) showed that the mitochondrial transcription factor mtTFA is required for

mtDNA maintenance, but curiously, this same factor could not be detected in cells artificially depleted of mtDNA by EtBr treatment (Larsson *et al.*, 1994; Poulton *et al.*, 1994). It seems that some mitochondrial regulatory factors have to associate with mtDNA to stabilize themselves. Therefore, the study of protein levels may be difficult to interpret because of the drastic reduction in their stability. Nevertheless, the available published information on the regulation of mtDNA replication factors is compatible with the concept that these factors do not increase when mtDNA levels decrease. Schultz *et al.* (1998) found that the mitochondrial DNA polymerase is expressed at similar levels in different tissues and does not seem to be regulated by physiological changes, whereas single-strand DNA-binding protein levels did vary between tissue types and correlated with mtDNA abundance. Davis *et al.*, (1996) showed that the mitochondrial DNA polymerase γ mRNA and protein levels in cells devoid of mtDNA were comparable with those of controls. Using a dot blot procedure, Poulton *et al.* (1994) found an increase in mRNA for mtTFA in cells depleted of mtDNA. This latter observation differs from those of Larsson *et al.* (1994) and ours, who did not find a significant alteration in mtTFA mRNA levels in cells depleted of mtDNA. Despite a few inconsistencies, the available information suggests that mtDNA replication may be regulated by the constant levels of one or more nuclear-coded mtDNA replication factors, because these factors do not seem to respond to existing mtDNA levels to adjust for their abundance. Another relevant observation is that in cultured cells, some mtDNA molecules replicate twice before others undergo any replication (Flory and Vinograd, 1973; Bogenhagen and Clayton, 1977), and close proximity to the nucleus seems to be a favored location for mtDNA replication (Davis and Clayton, 1996). These observations suggest the presence of limiting amounts of factors produced in the cytoplasmic or nuclear compartments. Functional cues, such as hormones (Brown, 1992) and metabolic defects (Heddi *et al.*, 1993), probably exert a stimulatory effect by up-regulating organelle biogenesis rather than mtDNA levels. In summary, our results demonstrate that mtDNA functionality is not the main determinant for haplotype selection in cultured cells. Selectivity for functional intraspecific mtDNA haplotypes in cybrids or hybrids has been previously reported, and all these reports point to unknown nuclear factors as the determinants in the selection (Hayashi *et al.*, 1987; Yoneda *et al.*, 1992; Dunbar *et al.*, 1995). Our studies, demonstrating that a function-less mtDNA can be preferred over a functional mtDNA, are also in agreement with experiments performed in transmitochondrial mice, which showed that mtDNA haplotype preferences vary even between tissues of the same individual, with no apparent correlation with mtDNA functionality (Jenuth *et al.*, 1997).

Implications for Aging and mtDNA Evolution

Our results provide direct evidence that, under loose copy number control, partially deleted mtDNAs have a replicative advantage over wild-type molecules. This phenomenon, previously suggested on the basis of indirect evidence (Larsson *et al.*, 1990; Chen *et al.*, 1995), has important implications for the accumulation of mtDNA deletions during the aging process (Wallace, 1997) and for the evolution of eukaryote mtDNA. It is also interesting that the repopulation rates were higher than expected by the reduction in size, suggesting that initiation may occur more frequently in partially deleted mtDNA. Similar results were observed with mouse mtDNAs, in which dimers took three times longer than monomers for strand synthesis (Bogenhagen *et al.*, 1981).

An important implication of this finding is that mtDNA deletions may occur at any time during life, but once formed, partially deleted mtDNAs would replicate faster than the wild-type counterpart. In certain tissues such as muscle, once the levels of mutated mtDNA reach a functional threshold, there is a compounding increase in defective organelles as a physiological response. Taken together, these factors would lead to an exponential increase in the levels of partially deleted mtDNA during aging. Our results also raise the possibility that the accumulation of partially deleted mtDNAs may be accelerated if metabolic or environmental factors lead to transient reduction in mtDNA levels.

The apparent selective pressure toward smaller molecules during eukaryote evolution (Kurland, 1992) can also be related to the "competitive edge" smaller molecules have during replication. This preference for smaller genomes may be exacerbated in primates, because the analysis of the D-loop region supports a progressive reduction in D-loop length within both monkey and great ape mitochondrial lineages (Blanchard and Schmidt, 1996).

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