Organization and dynamics of human mitochondrial DNA

Frédéric Legros, Florence Malka, Paule Frachon, Anne Lombès and Manuel Rojo*

INSERM U582 (IFR 14, UPMC) Institut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, 47, Boulevard de l'Hôpital, 75651 Paris CEDEX 13, France

*Author for correspondence (e-mail: m.rojo@myologie.chups.jussieu.fr)

Accepted 2 February 2004
Journal of Cell Science 117, 2653-2662 Published by The Company of Biologists 2004
doi:10.1242/jcs.01134

Summary

Heteroplasmic mutations of mitochondrial DNA (mtDNA) are an important source of human diseases. The mechanisms governing transmission, segregation and complementation of heteroplasmic mtDNA-mutations are unknown but depend on the nature and dynamics of the mitochondrial compartment as well as on the intramitochondrial organization and mobility of mtDNA. We show that mtDNA of human primary and immortal cells is organized in several hundreds of nucleoids that contain a mean of 2-8 mtDNA-molecules each. Nucleoids are enriched in mitochondrial transcription factor A and distributed throughout the entire mitochondrial compartment. Using cell fusion experiments, we demonstrate that nucleoids and respiratory complexes are mobile and diffuse efficiently into mitochondria previously devoid of mtDNA. In contrast, nucleoid-mobility was lower within mitochondria of mtDNA-containing cells, as differently labeled mtDNA-molecules remained spatially segregated in a significant fraction (37%) of the polykaryons. These results show that fusion-mediated exchange and intramitochondrial mobility of endogenous mitochondrial components are not rate-limiting for intermitochondrial complementation but can contribute to the segregation of mtDNA molecules and of mtDNA mutations during cell growth and division.

Key words: Mitochondria, Mitochondrial fusion, Mitochondrial complementation, Mitochondrial nucleoid

Introduction

Mitochondria are essential organelles that play a key role in fundamental cellular processes such as oxidative phosphorylation, calcium signaling and apoptosis (Kroemer and Reed, 2000; Pozzan and Rizzuto, 2000; Tzagoloff, 1982). They contain their own genome, the mitochondrial DNA (mtDNA), which encodes a limited number of essential mitochondrial proteins as well as the rRNAs and tRNAs necessary for intramitochondrial translation (Andrews et al., 1999; Foury et al., 1998). Mutations of mtDNA cause severe diseases in humans. Mutations are most often heteroplasmic, i.e. mutant and wild-type molecules coexist within cells, and the proportion of mutant molecules can vary between tissues and with age (Leonard and Schapira, 2000; Lightowlers et al., 1997). The characterization of mtDNA-organization and dynamics is necessary to understand the transmission and segregation of wild-type and mutant mtDNA between cells and tissues, as well as their transmission to the progeny of affected women (reviewed by Birky, 2001; Chinnery et al., 2000; Jacobs et al., 2000).

In protists, mtDNA is organized in a discrete number of nucleoprotein complexes (nucleoids) that are visualized by fluorescence microscopy with DNA-binding dyes (reviewed by Kuroiwa, 1982). In trypanosomes, mtDNA accumulates in a single region of the mitochondrion, the kinetoplast (Robinson and Gull, 1991), while in budding yeast mtDNA is organized in 10-20 nucleoprotein complexes called nucleoids (Stevens, 1981). The ability to visualize mtDNA nucleoids in yeast has allowed the study of nucleoid-dynamics (Azpiroz and Butow, 1993; Miyakawa et al., 1984; Nunnari et al., 1997; Okamoto et al., 1998) and the development of protocols for their purification and characterization (Miyakawa et al., 1995). In addition, mating studies have shown that mtDNA is highly mobile in crosses between haploid ρ+ and ρ0 cells, but almost immobile in crosses between ρ+ cells (Azpiroz and Butow, 1993; Nunnari et al., 1997). This, together with the nonrandom transmission of mtDNA to budding cells (Azpiroz and Butow, 1993; Okamoto, 1998), argue for the existence of an apparatus that regulates positioning and mobility of mitochondria and mtDNA as well as their active segregation into the emerging bud (reviewed by Boldogh et al., 2001). The genetic and physical organization of mtDNA differ significantly between protists (which have significant proportions of linear mtDNA-molecules of varying length) and vertebrates (which have compact and circular mtDNA-molecules of homogeneous size) (Burger et al., 2003; Williamson, 2002). Therefore, it is unclear whether and how the findings on protist-mtDNA can be extrapolated to human mtDNA.

Although vertebrate mtDNA is generally assumed to be organized in nucleoids, their putative nature, number and dynamics remain largely unknown. Studies on living cells stained with DAPI and/or ethidium bromide revealed a diffuse intramitochondrial distribution of mtDNA (Coppey-Moisan et al., 1996; Hayashi et al., 1994; Spelbrink et al., 2001), as well as its capacity to diffuse into the mitochondria of ρ0 cells (Hayashi et al., 1994). In contrast, studies on fixed cells report the accumulation of mtDNA in punctate structures (Garrido et al., 2003; Magnusson et al., 2003; Margineantu et al., 2002).

Surprisingly, the time required for functional complementation in hybrids of cells containing different mutants of mtDNA (10-14 days (Ono et al., 2001) is significantly longer than that required for mixing of small and soluble fluorescent matrix proteins by mitochondrial fusion (10-12 hours) (Legros et al., 2002). This, together with other controversies on the frequency and extent of intermitochondrial complementation (Enriquez et al., 2000), suggests that the mobility of mtDNA may be restricted within human mitochondria, as in yeast (Nunnari et al., 1997).

In this work, we reveal the organization and dynamics of mtDNA in primary and immortal human cells. Using DNA-specific antibodies, we show that human mtDNA is organized in hundreds of nucleoids that are enriched in mitochondrial transcription factor A (mtTFA) and can incorporate BrdU. Nucleoids containing a mean of 2-8 mtDNA molecules are distributed throughout the entire mitochondrial compartment. Cell fusion experiments reveal that nucleoids are mobile within ρ+ and ρ0 mitochondria. However, nucleoid mobility was reduced within ρ+ mitochondria leading to the spatial segregation of different mtDNA-molecules within a significant fraction (37%) of polykaryons.

Materials and Methods

Reagents, antibodies and standard procedures

Antibodies against DNA (clone AC-30-10) were obtained from Boehringer Mannheim Biochemica and are now distributed by Progen GmbH. The mature mtTFA protein carrying a 6His-tag at its N-terminus was expressed in *E. coli* strain C41(DE3) (Miroux and Walker, 1996), purified with Ni-NTA Agarose (Qiagen) and used to generate mtTFA-specific antibodies in rabbits. Antibodies against mitochondrial single strand binding protein (mtSSB) were a kind gift of Massimo Zeviani (Milan, Italy) and antibodies against cytochrome c oxidase subunit 2 (COX2) were previously characterized in our laboratory (Bakker et al., 2000). Rat monoclonal antibodies against 5-Bromo-2′-deoxy-uridine (BrdU) were from abcam (product code ab6326) and the BrdU-labeling and detection kit I was from Roche Applied Science (Cat. No. 1 296 736). Antibodies against mouse, rabbit and rat IgG coupled to AlexaFluor dyes 568, 488 or 350 were from Molecular Probes. Source and handling of all other reagents have been described (Legros et al., 2002; Rojo et al., 2002). Cell homogenization, subcellular fractionation, SDS-PAGE and western-blot analysis were performed as described (Rojo et al., 2002).

Nucleic acid manipulation

Expression vectors encoding DsRed or GFP targeted to the mitochondrial matrix (mtRFP, mtGFP) have been described (Legros et al., 2002). An expression vector encoding a GFP molecule targeted to the mitochondrial outer membrane by the transmembrane domain of OMP25 (GFPOM) (Nemoto and De Camilli, 1999) was provided by Pietro de Camilli (New Haven, USA). The sequence encoding human mitochondrial transcription factor A (mtTFA, accession number X64269) lacking its mitochondrial presequence was amplified by PCR from an EST (IMAGE Consortium Clone ID 785845) and cloned into the pET15b vector (Novagen). A 1603 bp fragment of human mtDNA (nucleotides 109 to 1714) (Andrews et al., 1999) was amplified by PCR using total human DNA as a template and cloned into the pGEM-T Easy Vector (Promega) to give pGEMTE-12S. The pGEMTE-12S vector included the entire 12S RNA gene and was used as a standard in quantitative PCR. For extraction of total cellular DNA, pellets of 25,000 to 100,000 cells were resuspended by vortexing in 100 μl extraction solution (0.2 mg/ml proteinase K, 0.2% SDS and 5 mM EDTA in PBS) and incubated with shaking for 2-3 hours (250 rpm, 50°C). Total DNA was then precipitated by addition of 10 μl 3 M sodium acetate (pH 5.2), 100 μl isopropanol and incubation for 20 minutes on ice. The DNA-pellet was washed once with cold 70% ethanol and resuspended in 100 μl TE buffer (1 hour at 60°C). The DNA-concentration of cellular extracts and of plasmid solutions (pGEMTE-12S) was determined with Sybr Green I (Molecular Probes) using λ/*Hin*d III DNA (Invitrogen) as a standard.

Quantitative PCR

Quantification of the mtDNA copy number was performed using real-time PCR amplification on Light Cycler (Roche Diagnostics) and Light Cycler FastStart DNA Master SYBR green I (Roche Diagnostics) following the instructions of the manufacturer. Primers were designed with the Light Cycler Probe Design™ software (Roche Diagnostics). A 211 bp fragment of the mtDNA 12S RNA gene was amplified between nucleotide 1095 and nucleotide 1305 (Andrews et al., 1999). The amplifications were simultaneously performed on 4 ng of total cellular DNA and on known amounts of the linearized pGEMTE-12S vector. Duplicates of each sample were analyzed in two independent runs. The number of mtDNA copies per ng total cellular DNA was determined after logarithmic regression of the standard samples using the Lightcycler software.

Cell culture

Maintenance of HeLa and 143B cells were performed as described (Legros et al., 2002; Rojo et al., 2002). Human skin fibroblasts derived from healthy subjects (3-year-old male and 30-year-old female) were provided by the Banque de tissus pour la recherche (AFM) of the Pitié-Salpêtrière Hospital. They were maintained in DMEM (4.5 g Glc/l) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. Confluent quiescent fibroblast cultures were trypsinized, diluted 3-6-fold and subjected to fixation and/or DNA-extraction after 2-3 days, before they reached confluency. Stable transfectants were generated by transfection with the calcium phosphate technique (Jordan et al., 1996) and selection with G418. Cells were fused with PEG as described (Legros et al., 2002) and maintained in medium containing cycloheximide (50 μg/ml) to inhibit protein synthesis.

Microscopy

Standard fixation and permeabilization of cells for immunofluorescence as well as image acquisition and processing were performed as described (Legros et al., 2002; Rojo et al., 2002). For triple labeling in cells expressing mtGFP or mtRFP, rabbit antibodies were decorated with secondary antibodies coupled to AlexaFluor 350 and mouse antibodies with secondary antibodies coupled to AlexaFluor 568 or 488. For BrdU-labeling of DNA, cells were incubated with 15 μM BrdU for 8-20 hours. For BrdU-detection, cells fixed with paraformaldehyde and permeabilized with Triton X-100 were incubated for 10 minutes with 2N HCl and extensively washed with water and PBS. For co-labeling of DNA and BrdU, fixed and permeabilized cells were first decorated with primary DNA-specific antibodies and secondary fluorescent antibodies. These antibodies were post-fixed with paraformaldehyde (3%, 20 minutes) to avoid their loss during incubation with 2N HCl. For labeling of BrdU alone, we also used the 5-Bromo-2′-deoxy-uridine (BrdU) labeling and detection kit I (Roche Applied Science), where DNA is denatured with nucleases (Magnusson et al., 2003). Mitochondrial DNA-positive structures were counted manually on negative prints of enlarged immunofluorescence images. In fibroblasts, the absence of a nuclear signal allowed easy identification (and counting) of nucleoids in the perinuclear area. The total number of nucleoids was thus determined after the merge of 2-3 focal planes (covering the entire cell volume)

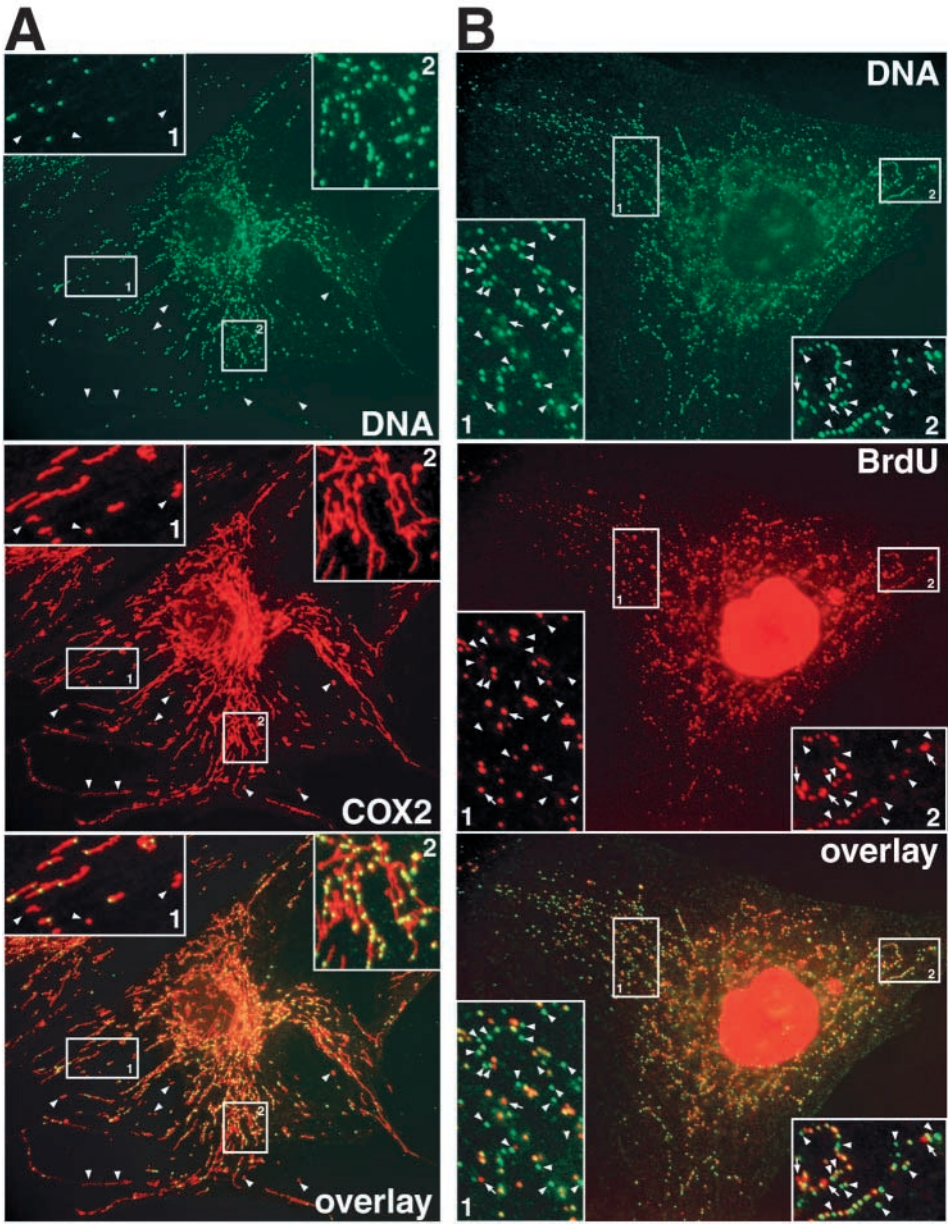

Fig. 1. Mitochondrial DNA localizes to punctate structures that are distributed throughout the mitochondrial compartment. Primary skin fibroblasts were labeled with antibodies against DNA, COX2 and/or BrdU under normal conditions (A) or after a 20 hour pulse with BrdU (B). Insets 1 and 2 depict enlargements of the boxed areas. (A) Monoclonal antibodies against DNA label punctate structures that are distributed throughout mitochondria labeled with antibodies against the inner membrane protein COX2. Only a few mitochondria appear devoid of DNA-labeling (arrowheads). (B) Antibodies against the thymidine analogue BrdU label the nucleus as well as punctate mitochondrial structures labeled with DNA-antibodies. Some of the mitochondrial DNA-positive structures are devoid of BrdU (arrowheads) and very few BrdU-positive structures escape detection with DNA-antibodies (arrows).

to a single image. In HeLa and 143B cells, the nuclear signal rendered the identification (and counting) of perinuclear nucleoids difficult. Nucleoids were thus counted on images of low focal planes that visualize most of the cell volume, but exclude for part of the perinuclear region. The numbers of nucleoids identified in these cells represent thus subestimations.

Results

Mitochondrial DNA accumulates in punctate structures that are distributed throughout the entire mitochondrial compartment

The localization and organization of mtDNA in human cells was determined with monoclonal antibodies against DNA. The DNA-specific antibodies labeled numerous punctate structures in primary skin fibroblasts (Fig. 1), as well as in HeLa and in 143B cells (Figs 3, 4). These structures are distributed throughout the entire mitochondrial compartment, as shown by double immunofluorescence with COX2, an inner membrane protein (Fig. 1A), and mtRFP, a fluorescent molecule targeted to the mitochondrial matrix (Fig. 4A). Most filamentous and elongated mitochondria contained several DNA-positive structures (Fig. 1A, Fig. 4A), whereas only a few small mitochondria appeared devoid of DNA (Fig. 1A, arrowheads). The specificity of the intramitochondrial DNA-labeling was demonstrated by the absence of such a labeling in the mitochondria of 143B-ρ0 cells devoid of mtDNA (see below, Fig. 6A). These monoclonal antibodies strongly labeled the DNA of DAPI-stained mycoplasma in contaminated cell cultures (data not shown). However, nuclear DNA was not labeled in most fibroblasts (Figs 1, 4) and only weakly in HeLa and in 143B cells (see below Figs 3, 4, 6).

To confirm the specificity of DNA-labeling within mitochondria and to investigate the efficiency of DNA-specific antibodies, fibroblasts were incubated with BrdU, a thymidine analogue that is incorporated into replicating DNA. The treatment of fixed cells with 2N HCl, which denatures DNA and renders the BrdU-epitope accessible to antibodies, significantly lowered and/or modified the posterior labeling of several antigens (data not shown). Therefore, DNA was decorated with primary and secondary antibodies before HCl-treatment and BrdU-visualization (see Materials and Methods). After a 20 hour pulse with BrdU, the majority of mitochondrial DNA-positive structures (~80%) were labeled with varying amounts of BrdU (Fig. 1B). The remaining DNA-positive structures were BrdU-negative (Fig. 1B, arrowheads), revealing that their mtDNA-molecules had not undergone replication during the time of the BrdU-pulse. The number of BrdU-positive

Table 1. Mitochondrial DNA in cultured human cells

Cells	MF	FF	HeLa	143B
Total DNA mass* (pg/cell)	8.4±1.6 (*n*=5)	10.5±0.2 (*n*=3)	12±0.9 (*n*=4)	14.7±1.6 (*n*=5)
mtDNA-copies† (per cell)	1632±201 (*n*=5)	1961±235 (*n*=3)	2637±451 (*n*=4)	4126±1077 (*n*=5)
mtDNA-mass‡ (fg/cell // % total DNA)	27.7 // 0.33	33.3 // 0.32	44.7 // 0.37	69.9 // 0.47
mtDNA-nucleoids§ (per cell)	700±281¶ (*n*=18)	807±200¶ (*n*=15)	>466±63** (*n*=6)	>553±151** (*n*=5)
mtDNA-copies (per nucleoid)	2.3±0.3	2.4±0.3	<5.7±1.0	<7.5±2.0

MF, male fibroblasts; FF, female fibroblasts. Values are mean±s.d.

*The predicted mass of a diploid human genome of 3.3×10^9 base pairs is 6.75 pg/cell.

†Measured by quantitative PCR.

‡Calculated after the predicted mass of human mtDNA (16571 bases).

§Counted in micrographs of cells labeled with DNA-antibodies.

¶2-3 focal planes covering the entire cell volume were merged to a single image before analysis.

**A focal plane that visualizes the major part of the cell (but excludes part of the signal in the perinuclear area) was analyzed.

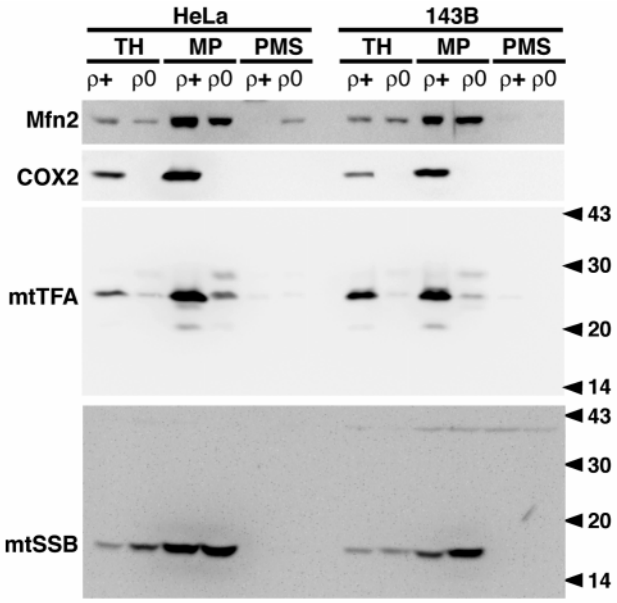

Fig. 2. Antibody characterization by subcellular fractionation and western-blot analysis. HeLa and 143B cells (normal ρ+ and mtDNA-less ρ0) were subjected to subcellular fractionation. Equal protein amounts of total homogenate (TH), mitochondrial pellet (MP) and post-mitochondrial supernatant (PMS) were separated by SDS-PAGE and transferred to membranes. Arrowheads point to the positions of marker proteins with the indicated molecular mass (kDa). The distributions of outer membrane Mfn2 and inner membrane COX2 demonstrate mitochondrial enrichment in both cell types. The ρ0 cells do not have any mtDNA-encoded COX2. The mtTFA-protein has an apparent molecular mass of 25 kDa and is strongly downregulated in ρ0 cells. The mtSSB-protein has an apparent molecular mass of 16 kDa and is present at similar levels in fractions of ρ+ and ρ0 cells.

structures that were not labeled with DNA-antibodies (Fig. 1B, arrows) was very low (~5%) showing that very few DNA-molecules escape detection with DNA-antibodies.

The structures visualized with antibodies could either represent isolated mtDNA-molecules or nucleoids containing several mtDNA-molecules. To discern between these two posibilities, we compared the numbers of mtDNA-structures and of mtDNA-molecules in various cell-types. The number of mtDNA-structures counted on immunofluorescence images of fixed cells was similar in skin fibroblasts from two different donors (Table 1, ~750) as well as in HeLa and in 143B cells (Table 1, >500). In the latter cells, the number of mtDNA-positive structures is somewhat underestimated, given the difficulty to visualize mtDNA in the mitochondria of the perinuclear region (see Materials and Methods and legend to Table 1).

The mtDNA-content of the different cells was determined by quantitative PCR of mtDNA using total cellular DNA as a template and a plasmid encoding the target sequence as a standard. In primary fibroblasts, the amount of extracted cellular DNA (Table 1, 8-10 pg/cell) was similar to the predicted mass of a diploid human genome (Table 1, 6.75 pg). In HeLa and 143B cells, the amount of extracted DNA was higher (Table 1, 12-15 pg/cell), as expected for hyper-diploid immortal cells. The number of mtDNA-copies per cell (Table 1), which was within the range of previous estimations (10^3-10^4 molecules per cell) (Lightowlers et al., 1997), was lower in primary cells (Table 1, 1600-2000 copies/cell) than in the immortal cell lines (Table 1, 2600-4100 copies/cell). In all cell types, the number of mtDNA-molecules was higher than the number of DNA-positive structures observed by immunofluorescence. This predicts the organization of mtDNA in nucleoids containing 2-8 molecules each (Table 1).

Mitochondrial DNA-positive structures represent nucleoids that are enriched in mitochondrial transcription factor A

To further characterize the nature of mtDNA-containing structures, we analyzed the relative localization of mitochondrial transcription factor A (mtTFA). The mtTFA-protein is essential for mtDNA-maintenance in animals (Larsson et al., 1998) and is the closest mammalian homologue of Abf2p, a component of yeast nucleoids (Okamoto et al., 1998). We generated antibodies against human mtTFA and investigated their specificity by western blot analysis of subcellular fractions from human cell lines. These antibodies were specific and decorated a unique band that was enriched in mitochondrial fractions and had an apparent molecular mass similar to that calculated for mature mtTFA (24 kDa, Fig. 2). These antibodies confirmed that, as described previously (Larsson et al., 1994), cells devoid of mtDNA (ρ0 cells) contain very low amounts of mtTFA (Fig. 2). Immunofluorescence microscopy revealed that the mtTFA protein was largely restricted to mtDNA-positive structures in primary human fibroblasts (Fig. 3). In HeLa and 143B cells, mtTFA displayed a wider intramitochondrial distribution and was only partially enriched in some of the DNA-positive structures (Fig. 3). The mtTFA-protein was not detectable in ρ0 cells devoid of mtDNA (see below, Fig. 6A), confirming the specificity of the immunofluorescence signal. Interestingly, the colocalization of mtTFA and mtDNA became more apparent after fusion of ρ+ and ρ0 cells, when mtDNA-structures had diffused into ρ0 mitochondria (see below, Fig. 6B,C). The restriction of

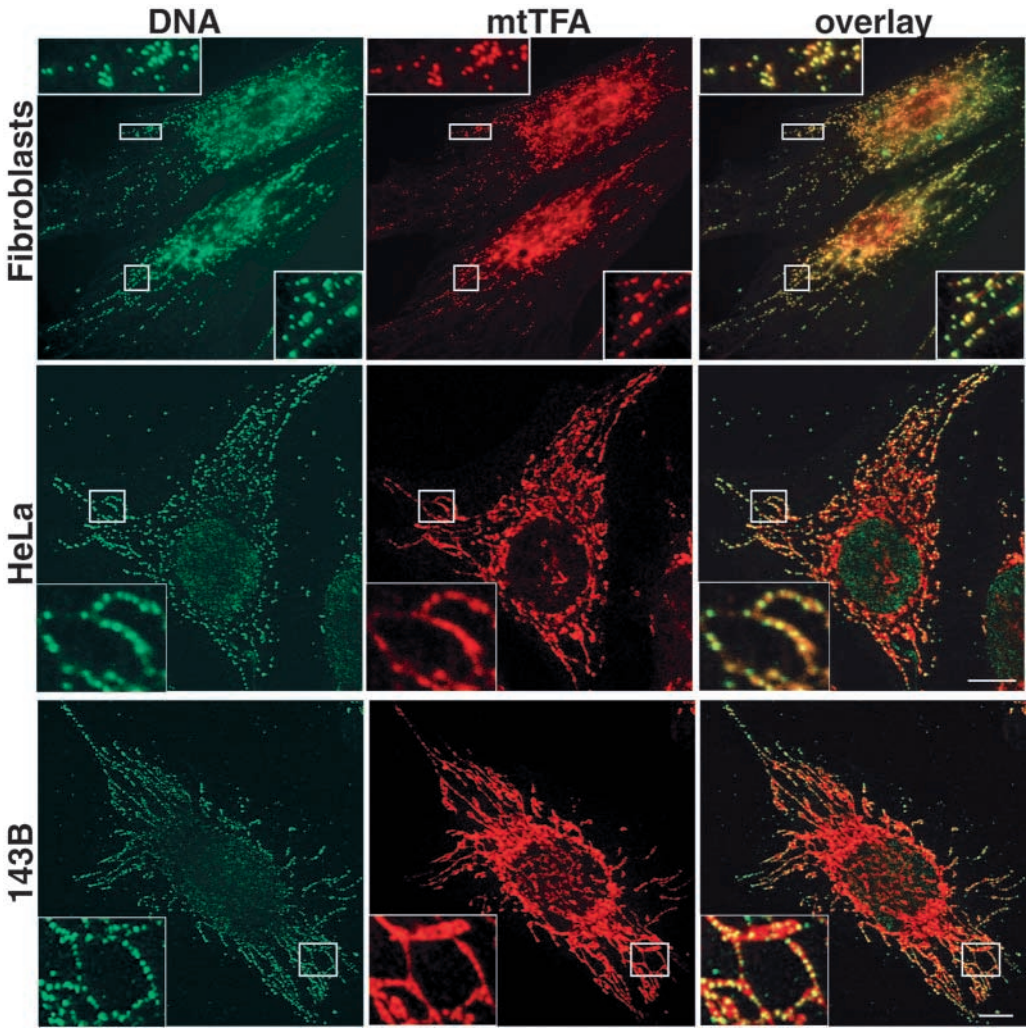

Fig. 3. Mitochondrial transcription factor A (mtTFA) is enriched in punctate DNA-positive structures. Primary skin fibroblasts, HeLa cells and 143B cells were decorated with antibodies against DNA and mitochondrial transcription factor A (mtTFA) and analyzed by conventional (Fibroblasts) or confocal (HeLa, 143B) fluorescence microscopy. The insets are enlargments of the boxed image regions. In fibroblasts, mtTFA is restricted to punctate mtDNA-positive structures. In Hela and 143B cells, mtTFA has a more homogeneous intramitochondrial distribution and is partially enriched in some mtDNA-positive structures. Bars, 10 μm.

mtDNA to punctate structures, as well as the co-enrichment of mtTFA suggests that human mtDNA is organized in nucleoprotein-complexes, homologous to the nucleoids described in protists.

Attempts to reveal the intramitochondrial localization of mitochondrial single strand binding protein (mtSSB), another protein involved in mtDNA function and structure, failed. Available antibodies against mtSSB (Tiranti et al., 1997) were highly specific in western-blots, where they decorated a single band of the expected apparent molecular mass (15 kDa) that was enriched in mitochondrial fractions (Fig. 2). In contrast to mtTFA, the levels of mtSSB were similar in normal ρ+ cells and in ρ0 cells devoid of mtDNA (Fig. 2). Both in HeLa and 143B cells, three different fixation and permeabilization conditions revealed a punctate intramitochondrial pattern (data not shown) which resembled that obtained with the same antibodies in other reports (Garrido et al., 2003; Tiranti et al., 1997). These structures colocalized poorly with mtDNA and were not detected in 143B ρ0 cells (data not shown). The discrepancy between western-blot and immunofluorescence led us to conclude that, under the chosen conditions, these mtSSB antibodies were not suited for mtSSB-localization in fixed cells.

The observation that, under normal conditions, some small mitochondria were devoid of mtDNA (Fig. 1A, arrowheads), prompted us to study the intermitochondrial distribution of mtDNA in more detail. In human cells, overall mitochondrial morphology results from the balance of antagonizing fusion and fission reactions. Accordingly, the dissipation of the inner membrane potential with cccp and the concomitant inhibition of mitochondrial fusion, provoke fragmentation of mitochondrial filaments by the endogenous fission machinery (Legros et al., 2002). To investigate mtDNA distribution between small punctate mitochondria, cells were incubated for 4 hours with cccp. As expected, this treatment led to the appearance of numerous small mitochondria, both in HeLa cells expressing mtRFP (Fig. 4B,C) and in primary skin fibroblasts (Fig. 4D,E). Analysis of such images revealed that only a fraction of the mitochondria (25% of 1262 HeLa and 352 fibroblast mitochondria) were devoid of mtDNA (Fig. 4C,E, arrowheads). This demonstrates that, even after extensive fragmentation, a majority of mitochondria remain mtDNA-positive.

Mitochondrial nucleoids and respiratory complexes are mobile and diffuse into the mitochondria of ρ0 cells

To investigate the mobility of mtDNA nucleoids we analyzed their localization after the fusion of human cells containing mtDNA (143B ρ+) with human cells devoid of mtDNA (143B

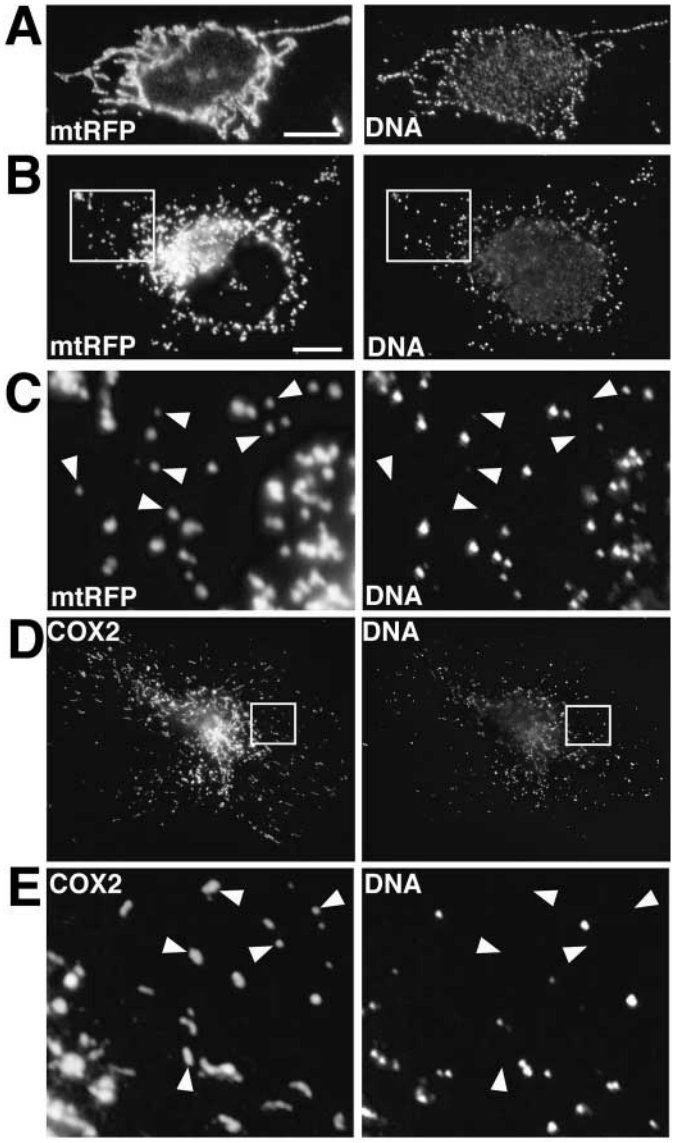

Fig. 4. The majority of mitochondria remain DNA-positive after fragmentation of mitochondrial filaments in vivo. HeLa cells expressing mtRFP (A-C) and primary skin fibroblasts (D,E) were fixed and decorated with COX2 and/or DNA-specific antibodies under control conditions (A) or after a 4-hour treatment with cccp (B-E). C and E are enlargements of the areas boxed in B and D, respectively. (A) In HeLa cells expressing mtRFP, DNA-specific antibodies label the cell nucleus and punctate intramitochondrial structures. Numerous DNA-positive structures colocalize within elongated mitochondrial filaments. (B-E) Inhibition of mitochondrial fusion with cccp leads to mitochondrial fragmentation in HeLa cells (B,C) and fibroblasts (D,E). The majority of punctate mitochondria are DNA-positive. Arrowheads point to mitochondria devoid of mtDNA. Bars, 10 μm.

ρ0 cells). To establish and characterize this cell fusion system, we first investigated the mobility of cytochrome c oxidase (COX), a large respiratory complex of the inner mitochondrial membrane. Before fusion, ρ+ cells (labeled with mtGFP) were positive for mitochondrially encoded COX2 subunit and ρ0 cells (labeled with mtRFP) were devoid of COX2 protein (Fig. 5A). Mitochondria appeared less elongated in respiratory-deficient ρ0 cells (Fig. 5A) than in respiring ρ+ cells (Figs 1, 4). Cells were fused with PEG, maintained in the presence of chloramphenicol and cycloheximide and fixed after 8 hours, a time period allowing intermixing of matrix fluorescent proteins (Legros et al., 2002). After fusion, all three markers (mtRFP, mtGFP and COX2) displayed a relatively homogeneous distribution within the mitochondrial compartment of polykaryons (Fig. 5B), confirming extensive mitochondrial fusion and demonstrating the intermitochondrial exchange of mtGFP, mtRFP and COX-complexes. The inhibition of cytoplasmic protein synthesis alone lowers the rate of mitochondrial protein synthesis and leads to rapid degradation of the residual mitochondrially translated peptides (Costantino and Attardi, 1977). Therefore, very similar results were obtained when fused cells were treated only with cycloheximide (Fig. 5C). Close analysis revealed that in some polykaryons, certain regions of the mitochondrial network were labeled more strongly with mtGFP than with COX2 (Fig. 5C, arrowheads). Within a polykaryon, the diffusion of molecules throughout the mitochondrial component is determined by (1) the mobility of mitochondria, (2) their fusion rate and/or frequency, and (3) the mobility of mitochondrial components within fused mitochondria. Factors 1 and 2 being identical for all mitochondrial components, a restricted distribution can result only from reduced intramitochondrial mobility. Our results thus indicate that inner membrane COX2 is mobile, but may diffuse with a slightly lower velocity than matrix mtGFP.

To investigate the mobility of mtDNA nucleoids, we used untransfected 143B ρ+ cells and 143B ρ0 cells expressing mtRFP. Note that in mtRFP-expressing ρ0 cells, mitochondria were also devoid of mtTFA and monoclonal antibodies against DNA only labeled the nucleus (Fig. 6A). Twelve hours after fusion, polykaryons originating from the fusion between ρ+ and ρ0 cells were identified through the simultaneous presence of mtDNA, mtTFA and mtRFP (Fig. 6B). In all these polykaryons, mitochondria displayed the filamentous morphology that is typical of respiring ρ+ mitochondria and the entire mitochondrial network contained mtRFP as well as nucleoids positive for both mtDNA and mtTFA (Fig. 6B,C). The distribution of mtDNA nucleoids to the entire mitochondrial network demonstrates that, upon fusion between ρ+ and ρ0 cells, the mobility of mtDNA nucleoids is similar to that of small soluble matrix proteins.

Mitochondrial DNA diffuses throughout the mitochondrial compartment of r+ cells

To investigate the mobility of human mtDNA within ρ+ mitochondria, the DNA of one of the cell populations destined to fuse was labeled with BrdU. For identification, the other cell line was stably transfected with GFPOM, a GFP-molecule that is anchored to the mitochondrial outer membrane via a C-terminal transmembrane domain. In HeLa cells pre-incubated with BrdU for 8-12 hours and co-plated with GFPOM-expressing cells, BrdU-labeled nucleoids are distributed throughout the entire mitochondrial compartment (Fig. 7A, BrdU), as observed previously in primary fibroblasts (Fig. 1B). The number of BrdU-positive nucleoids was always lower than that found with DNA-specific antibodies, regardless of the method used for DNA-denaturation and BrdU-visualization (see Materials and Methods). Co-plated cells were fused with

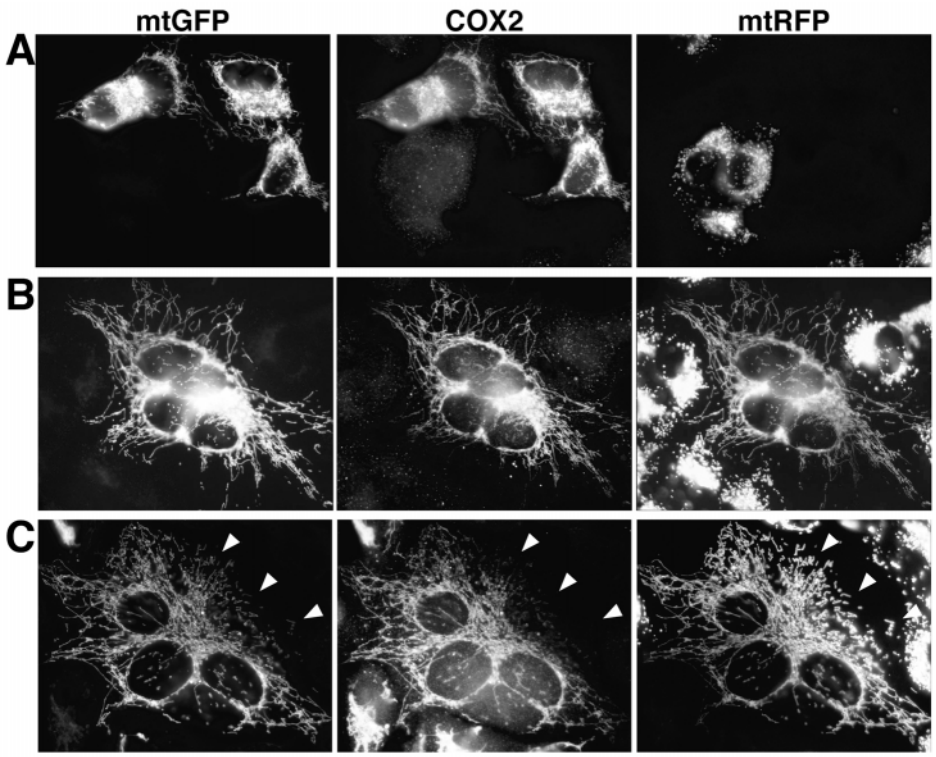

Fig. 5. Cytochrome c oxidase (COX) complexes diffuse into ρ0 mitochondria. Stably transfected 143B-ρ0 cells expressing mtRFP and 143B-ρ+ cells expressing mtGFP were co-plated and analyzed without further treatment (A), or 8 hours (B) or 12 hours (C) after PEG-mediated cell fusion. To inhibit mitochondrial (B) and/or cytosolic (B,C) protein synthesis, fused cells were treated with chloramphenicol (B) and/or cycloheximide (B,C). (A) 143B-ρ0 cells expressing mtRFP display a punctate morphology and are devoid of mitochondrially encoded COX2. 143B-ρ+ cells expressing mtGFP are filamentous and contain mitochondrially encoded COX2. Eight (B) and twelve (C) hours after PEG-mediated cell fusion, polykaryons derived from the fusion of ρ+ and ρ0 cells depict filamentous mitochondria that are positive for mtRFP, mtGFP and COX2. In some polykaryons, the amount of COX2 is lower than that of mtGFP in certain regions of the mitochondrial network (arrowheads), revealing a lower mobility of COX2.

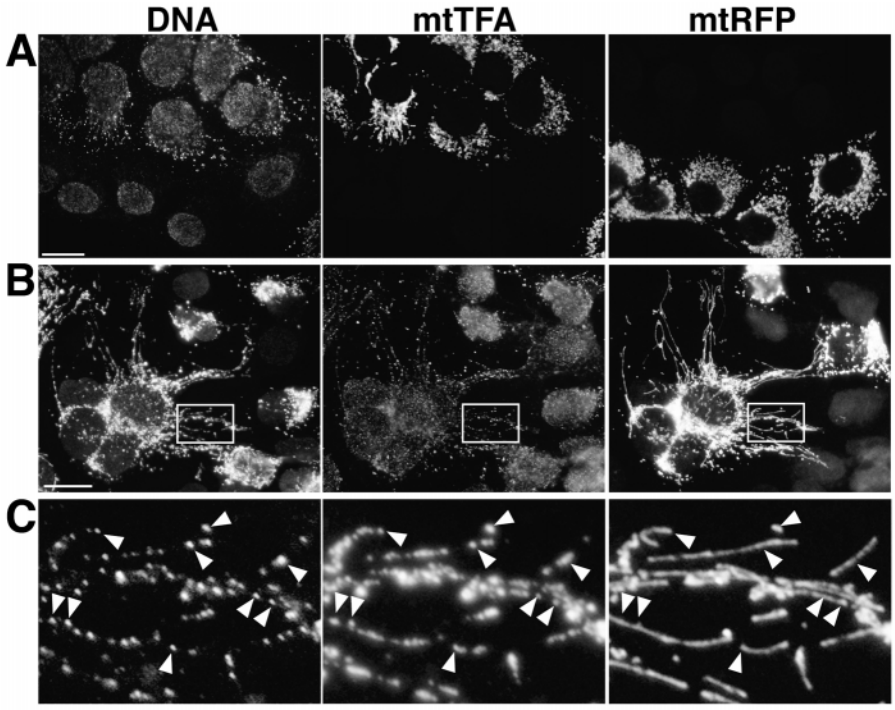

Fig. 6. Mitochondrial nucleoids are mobile and diffuse into ρ0-mitochondria. Stably transfected 143B-ρ0 cells expressing mtRFP and untransfected 143B-ρ+ cells were co-plated and analyzed without further treatment (A) or 12 hours after PEG-mediated cell fusion (B,C). (A) 143B-ρ0 cells expressing mtRFP have punctate mitochondria that are devoid of mtDNA and of mtTFA. The mitochondria of 143B-ρ+ cells contain mtDNA and mtTFA. (B) Twelve hours after PEG-mediated fusion, the polykaryons derived from the fusion of ρ+ and ρ0 cells depict filamentous mitochondria that contain mtRFP, mtDNA and mtTFA. Nucleoids and mtRFP are distributed throughout the entire mitochondrial compartment. (C) An enlargement of the area boxed in B shows that mtDNA nucleoids are enriched in mtTFA. The position of some mtDNA nucleoids is indicated with arrowheads. Bars, 20 μm.

PEG and fixed after a further 12-14 hours. In all polykaryons containing BrdU and GFPOM, GFPOM is distributed throughout the entire mitochondrial network (Fig. 7B,C), revealing that the mobility of such tail-anchored proteins is similar to that of soluble proteins targeted to the mitochondrial matrix (Legros et al., 2002). Twelve hours after fusion, the majority of polykaryons (63% of 371 polykaryons) had BrdU-labeled nucleoids that were distributed throughout the entire mitochondrial network (Fig. 7C). However, BrdU-labeled nucleoids were absent from important sub-regions of the mitochondrial network (Fig. 7B) in a significant fraction (37% of 371) of polykaryons. At later time-points (24-48 hours), all polykaryons depicted BrdU-labeled nucleoids that were distributed throughout the entire mitochondrial network (Fig.

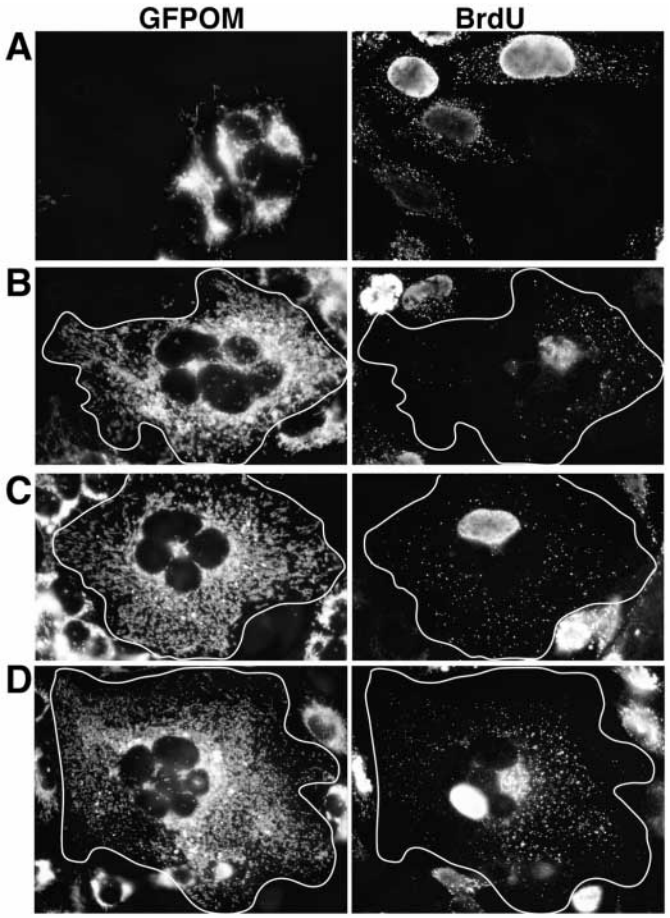

Fig. 7. Mitochondrial DNA nucleoids are mobile within ρ+ mitochondria. Stably transfected human 143B cells expressing GFPOM were co-plated with human HeLa cells that had been pre-incubated with BrdU. Co-plated cells were analyzed without further treatment (A), or 12 hours (B,C) or 24 hours (D) after PEG-mediated cell fusion. (A) The nuclear and mitochondrial DNA of untransfected HeLa cells is labeled with BrdU, and GFPOM-transfected cells are devoid of BrdU-labeling. (B-D) The polykaryons derived from the fusion of HeLa and 143B cells are positive for GFPOM and BrdU. The GFPOM-protein is distributed throughout the entire mitochondrial network (B-D). Twelve hours after fusion, BrdU-labeled nucleoids are absent from some regions of the mitochondrial network (B) or distribute to the entire mitochondrial compartment (C). Twenty-four hours after fusion, BrdU-labeled nucleoids are seen throughout the entire mitochondrial compartment. The outlines of the polykaryons are depicted with a white line.

7D). These finding show that mtDNA is mobile and can diffuse throughout the mitochondria compartment of fused ρ+ mitochondria. The absence of BrdU from some mitochondrial sub-regions after 12-14 hours indicates that, within ρ+ mitochondria, the mobility of nucleoids is somewhat lower than that of GFPOM.

Discussion

Organization of mtDNA

In this work we show that human mtDNA is organized in hundreds of punctate structures that contain 2-8 mtDNA molecules each and that are distributed throughout the mitochondrial compartment. The comparison of DNA-specific and BrdU-specific labeling after a long pulse with BrdU revealed that only a small minority of mtDNA-molecules localize outside these structures (and escape detection with DNA-specific antibodies). It is not clear why monoclonal antibodies against DNA label nuclear DNA less efficiently than mitochondrial DNA. It is possible that the association of nuclear DNA with histones and/or the diffuse distribution of chromatin throughout the nucleus contribute to lower the signal of anti-DNA antibodies. The organization of mtDNA in punctate structures differs from that observed in living cells incubated with DAPI and/or ethidium bromide, where mtDNA-distribution appeared significantly more diffuse (Coppey-Moisan et al., 1996; Hayashi et al., 1994). We infer that the latter findings resulted from the weak labeling efficiency achieved in living cells and from the use of dyes that, like ethidium bromide, label both DNA and RNA. In contrast, the distribution of mtDNA to punctate structures agrees with findings on fixed cells by in situ hybridization (Margineantu et al., 2002) and after BrdU-incorporation (Garrido et al., 2003; Magnusson et al., 2003). We show that the number of mtDNA-structures was similar in primary fibroblasts (~750/cell) and in immortal human cell lines (> 500/cell), and that the number of mtDNA-molecules (per cell and per nucleoid) is 2-3 times higher in immortal cell lines.

In contrast to primary fibroblasts, where mtTFA molecules were restricted to mtDNA nucleoids, immortal cell lines contained mtTFA molecules that did not localize to mtDNA nucleoids. This is in agreement with the large excess of mtTFA in these cells (Takamatsu et al., 2002). Regardless of their abundance and steady-state distribution, it is tempting to speculate that all mtTFA-molecules belong to a single pool of molecules that interact reversibly with mtDNA. This would also prevent degradation of (excess) mtTFA in immortal cell lines. Garrido and co-workers have further reported co-localization of mtSSB with BrdU-labeled mtDNA as well as the presence of detectable mtSSB-levels in the ρ0 mitochondria of fixed cells (Garrido et al., 2003). Further work will be necessary to elucidate why we obtained the opposite results (minor colocalization of mtSSB with mtDNA in ρ+ cells and failure to detect mtSSB in fixed ρ0 cells) with the same antibody preparation.

The co-localization of mtDNA with endogenous mtTFA (this work) as well as with tagged forms of Twinkle (Garrido et al., 2003), a putative helicase, suggests that DNA-positive structures represent nucleoprotein complexes homologous to the mtDNA nucleoids of protists. The first report on the enrichment of human nucleoids has shown that, with the exception of mtTFA, several human proteins involved in mtDNA-function do not accumulate in mtDNA-enriched fractions (Garrido et al., 2003). Another study revealed the presence of four abundant mitochondrial proteins, apparently unrelated to mtDNA-function (such as adenine nucleotide translocator and subunits of pyruvate dehydrogenase), among the components of *Xenopus* oocyte nucleoids (Bogenhagen et al., 2003). Interestingly, similar findings have been reported in yeast (Kaufman et al., 2000). These results emphasize that the molecular characterization of mitochondrial nucleoids remains a challenging task. Given the differences between protists and mammals in the organization of the mitochondrial genome

(Burger et al., 2003; Williamson, 2002), as well as in the number and distribution of nucleoids, it is possible that their nucleoids also differ significantly in nature, composition and/or structure. In this respect, it is important to note that, in contrast to some protists, human nucleoids are predicted to contain very small amounts (2-8 molecules) of mtDNA.

Mitochondrial nucleoids localized all along mitochondrial filaments and did not accumulate at the tips of mitochondria, in contrast to findings reported elsewhere (Garrido et al., 2003; Margineantu et al., 2002). The majority of mitochondria were positive for mtDNA, even after extensive mitochondrial division by the endogenous fission machinery. The presence of a mtDNA nucleoid in 75% of the small mitochondria of cccp-treated cells revealed similarities between the total number of nucleoids and the maximal number of mitochondria. This may only reflect the homogeneous distribution of mtDNA nucleoids within the mitochondrial compartment. However, it is also possible that the mitochondrial division apparatus has the ability to recognize sub-domains of mitochondria surrounding a single nucleoid. Further work will be necessary to elucidate the mechanisms that may regulate and/or coordinate nucleoid distribution and mitochondrial division.

The presence of a single nucleoid in the small mitochondria of cccp-treated fibroblasts predicts the presence of a mean of 2.3 mtDNA molecules in each of these mitochondrial particles. This number is similar to the number of nucleoids found in the mitochondrial particles of homogenized fibroblasts (2 mtDNA-molecules per particle) (Cavelier et al., 2000). Although mitochondria were probably fragmented during homogenization and sorting, the proportion of mtDNA-less mitochondria found in the latter work ($\leqslant$40%) was similar to that found upon extensive mitochondrial division in vivo (~25%).

Molecular exchanges between mitochondria

We demonstrate that mtDNA nucleoids as well as respiratory complexes (COX) are mobile mitochondrial components. Upon fusion of ρ+ and ρ0 cells, nucleoids diffused into ρ0 mitochondria with kinetics similar to those of matrix mtRFP, whereas inner membrane respiratory complex IV (COX) displayed a slightly reduced mobility. Nucleoids also diffused within ρ+ mitochondria, but with a somewhat lower mobility. Indeed, BrdU-labeled mtDNA molecules were often absent from some regions of the polykaryons, after time periods (12-14 hours) that allow equilibration of matrix and outer membrane proteins throughout the mitochondrial compartment. It is interesting to note that the mobility of mtDNA is also restricted and/or regulated in yeast (Nunnari et al., 1997; Okamoto et al., 1998) where molecules which could participate in these processes are being identified (Hobbs et al., 2001). The factors that control the distribution and mobility of human mtDNA remain unknown. Our observations on mtDNA mobility and intracellular distribution may have implications for the transmission and segregation of mtDNA mutations in heteroplasmic cells. A homogeneous distribution of different mtDNA molecules throughout the cell ensures faithful transmission of heteroplasmy to daughter cells. In contrast, spatial restriction of mtDNA could favor the accumulation of nucleoids with different mtDNA species in different cell areas and lead to their segregation during cytokinesis.

Our results further show that intermitochondrial fusion and intramitochondrial mobility of endogenous nucleoids and respiratory complexes can ensure functional complementation in normal cells and in heteroplasmic cells containing mutant mtDNA. However, the time required for equilibration through the mitochondrial compartment of polykaryons (12-14 hours) is significantly smaller than the time required for functional complementation between the mitochondria of fused cells containing different mtDNA mutations (10-14 days) (Ono et al., 2001). This suggests the existence of a rate-limiting step other than the exchange of complementing molecules. We hypothesize that the assembly of functional respiratory complexes may be rate-limiting in such cell fusion experiments. Transcription/translation of nuclear and/or mitochondrial genes could be inhibited in cells carrying high concentrations of mutant mtDNA (before fusion) and may require time to be restored. Assembly may be further delayed if the formation of functional respiratory complexes depends on the assembly of newly synthesized subunits (encoded by nuclear and by mitochondrial DNA) and not on the addition of ‘missing’ subunits (encoded by mtDNA) to ‘incomplete’ complexes assembled before fusion.

We are grateful to Ana Ferreiro, Gillian S. Butler-Browne and Marc Fiszman for critical reading of the manuscript. We thank Maite Coppey-Moisan and Catherine Godinot for the HeLa ρ0 cells, John Walker and Bruno Miroux for bacterial strain C41, Pietro de Camilli for an expression plasmid encoding GFPOM, Massimo Zeviani for the antiserum against mtSSB and the UK HGMP Resource Center for an IMAGE consortium cDNA clone encoding the open reading frame of mtTFA. M.R. is an investigator of the Centre National de la Recherche Scientifique (CNRS). This work was supported by the Institut National de la Santé et la Recherche Médicale (INSERM) and by grants from the Association Française contre les Myopathies (AFM).

References

Andrews, R. M., Kubacka, I., Chinnery, P. F., Lightowlers, R. N., Turnbull, D. M. and Howell, N. (1999). Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat. Genet.* **23**, 147.

Azpiroz, R. and Butow, R. A. (1993). Patterns of mitochondrial sorting in yeast zygotes. *Mol. Biol. Cell* **4**, 21-36.

Bakker, A., Barthelemy, C., Frachon, P., Chateau, D., Sternberg, D., Mazat, J. P. and Lombes, A. (2000). Functional mitochondrial heterogeneity in heteroplasmic cells carrying the mitochondrial DNA mutation associated with the MELAS syndrome (mitochondrial encephalopathy, lactic acidosis, and strokelike episodes). *Pediatr. Res.* **48**, 143-150.

Birky, C. W., Jr (2001). The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu. Rev. Genet.* **35**, 125-148.

Bogenhagen, D. F., Wang, Y., Shen, E. L. and Kobayashi, R. (2003). Protein components of mitochondrial DNA nucleoids in higher eukaryotes. *Mol. Cell. Proteomics* **2**, 1205-1216.

Boldogh, I. R., Yang, H. C. and Pon, L. A. (2001). Mitochondrial inheritance in budding yeast. *Traffic* **2**, 368-374.

Burger, G., Forget, L., Zhu, Y., Gray, M. W. and Lang, B. F. (2003). Unique mitochondrial genome architecture in unicellular relatives of animals. *Proc. Natl. Acad. Sci. USA* **100**, 892-897.

Cavelier, L., Johannisson, A. and Gyllensten, U. (2000). Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. *Exp. Cell Res.* **259**, 79-85.

Chinnery, P. F., Thorburn, D. R., Samuels, D. C., White, S. L., Dahl, H. M., Turnbull, D. M., Lightowlers, R. N. and Howell, N. (2000). The inheritance of mitochondrial DNA heteroplasmy: random drift, selection or both? *Trends Genet.* **16**, 500-505.

Coppey-Moisan, M., Brunet, A. C., Morais, R. and Coppey, J. (1996).

Dynamical change of mitochondrial DNA induced in the living cell by perturbing the electrochemical gradient. *Biophys. J.* **71**, 2319-2328.

Costantino, P. and Attardi, G. (1977). Metabolic properties of the products of mitochondrial protein synthesis in HeLa cells. *J. Biol. Chem.* **252**, 1702-1711.

Enriquez, J. A., Cabezas-Herrera, J., Bayona-Bafaluy, M. P. and Attardi, G. (2000). Very rare complementation between mitochondria carrying different mitochondrial DNA mutations points to intrinsic genetic autonomy of the organelles in cultured human cells. *J. Biol. Chem.* **275**, 11207-11215.

Foury, F., Roganti, T., Lecrenier, N. and Purnelle, B. (1998). The complete sequence of the mitochondrial genome of Saccharomyces cerevisiae. *FEBS Lett.* **440**, 325-331.

Garrido, N., Griparic, L., Jokitalo, E., Wartiovaara, J., van Der Bliek, A. M. and Spelbrink, J. N. (2003). Composition and dynamics of human mitochondrial nucleoids. *Mol. Biol. Cell* **14**, 1583-1596.

Hayashi, J., Takemitsu, M., Goto, Y. and Nonaka, I. (1994). Human mitochondria and mitochondrial genome function as a single dynamic cellular unit. *J. Cell Biol.* **125**, 43-50.

Hobbs, A. E., Srinivasan, M., McCaffery, J. M. and Jensen, R. E. (2001). Mmm1p, a mitochondrial outer membrane protein, is connected to mitochondrial DNA (mtDNA) nucleoids and required for mtDNA stability. *J. Cell Biol.* **152**, 401-410.

Jacobs, H. T., Lehtinen, S. K. and Spelbrink, J. N. (2000). No sex please, we're mitochondria: a hypothesis on the somatic unit of inheritance of mammalian mtDNA. *Bioessays* **22**, 564-572.

Jordan, M., Schallhorn, A. and Wurm, F. M. (1996). Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* **24**, 596-601.

Kaufman, B. A., Newman, S. M., Hallberg, R. L., Slaughter, C. A., Perlman, P. S. and Butow, R. A. (2000). In organello formaldehyde crosslinking of proteins to mtDNA: identification of bifunctional proteins. *Proc. Natl. Acad. Sci. USA* **97**, 7772-7777.

Kroemer, G. and Reed, J. C. (2000). Mitochondrial control of cell death. *Nat. Med.* **6**, 513-519.

Kuroiwa, T. (1982). Mitochondrial nuclei. *Int. Rev. Cytol.* **75**, 1-59.

Larsson, N. G., Oldfors, A., Holme, E. and Clayton, D. A. (1994). Low levels of mitochondrial transcription factor A in mitochondrial DNA depletion. *Biochem. Biophys. Res. Commun.* **200**, 1374-1381.

Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S. and Clayton, D. A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.* **18**, 231-236.

Legros, F., Lombes, A., Frachon, P. and Rojo, M. (2002). Mitochondrial fusion in human cells is efficient, requires the inner membrane potential and is mediated by mitofusins. *Mol. Biol. Cell* **13**, 4343-4354.

Leonard, J. V. and Schapira, A. H. (2000). Mitochondrial respiratory chain disorders I: mitochondrial DNA defects. *Lancet* **355**, 299-304.

Lightowlers, R. N., Chinnery, P. F., Turnbull, D. M. and Howell, N. (1997). Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. *Trends Genet.* **13**, 450-455.

Magnusson, J., Orth, M., Lestienne, P. and Taanman, J. W. (2003). Replication of mitochondrial DNA occurs throughout the mitochondria of cultured human cells. *Exp. Cell Res.* **289**, 133-142.

Margineantu, D. H., Cox, W. G., Sundell, L., Sherwood, S. W., Beechem, J. M. and Capaldi, R. A. (2002). Cell cycle dependent morphology changes and associated mitochondrial DNA redistribution in mitochondria of human cell lines. *Mitochondrion* **1**, 425-435.

Miroux, B. and Walker, J. E. (1996). Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260**, 289-298.

Miyakawa, I., Aoi, H., Sando, N. and Kuroiwa, T. (1984). Fluorescence microscopic studies of mitochondrial nucleoids during meiosis and sporulation in the yeast, Saccharomyces cerevisiae. *J. Cell Sci.* **66**, 21-38.

Miyakawa, I., Fumoto, S., Kuroiwa, T. and Sando, N. (1995). Characterization of DNA-binding proteins involved in the assembly of mitochondrial nucleoids in the yeast Saccharomyces cerevisiae. *Plant Cell Physiol.* **36**, 1179-1188.

Nemoto, Y. and de Camilli, P. (1999). Recruitment of an alternatively spliced form of synaptojanin 2 to mitochondria by the interaction with the PDZ domain of a mitochondrial outer membrane protein. *EMBO J.* **18**, 2991-3006.

Nunnari, J., Marshall, W. F., Straight, A., Murray, A., Sedat, J. W. and Walter, P. (1997). Mitochondrial transmission during mating in Saccharomyces cerevisiae is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol. Biol. Cell* **8**, 1233-1242.

Okamoto, K., Perlman, P. S. and Butow, R. A. (1998). The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud. *J. Cell Biol.* **142**, 613-623.

Ono, T., Isobe, K., Nakada, K. and Hayashi, J. I. (2001). Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria. *Nat. Genet.* **28**, 272-275.

Pozzan, T. and Rizzuto, R. (2000). High tide of calcium in mitochondria. *Nat. Cell Biol.* **2**, E25-E27.

Robinson, D. R. and Gull, K. (1991). Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle. *Nature* **352**, 731-733.

Rojo, M., Legros, F., Chateau, D. and Lombes, A. (2002). Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. *J. Cell Sci.* **115**, 1663-1674.

Spelbrink, J. N., Li, F. Y., Tiranti, V., Nikali, K., Yuan, Q. P., Tariq, M., Wanrooij, S., Garrido, N., Comi, G., Morandi, L. et al. (2001). Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.* **28**, 223-231.

Stevens, B. (1981). Mitochondrial structure. In *The Molecular Biology of the Yeast Saccharomyces, Vol. 1: Life Cycle and Inheritance* (ed. J. N. Strathern, E. W. Jones and J. R. Broach), pp. 471-504. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Takamatsu, C., Umeda, S., Ohsato, T., Ohno, T., Abe, Y., Fukuoh, A., Shinagawa, H., Hamasaki, N. and Kang, D. (2002). Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep.* **3**, 451-456.

Tiranti, V., Savoia, A., Forti, F., D'Apolito, M. F., Centra, M., Rocchi, M. and Zeviani, M. (1997). Identification of the gene encoding the human mitochondrial RNA polymerase (h-mtRPOL) by cyberscreening of the Expressed Sequence Tags database. *Hum. Mol. Genet.* **6**, 615-625.

Tzagoloff, A. (1982). *Mitochondria*. New York, NY: Plenum Press.

Williamson, D. (2002). The curious history of yeast mitochondrial DNA. *Nat. Rev. Genet.* **3**, 475-481.