Dynamical Change of Mitochondrial DNA Induced in the Living Cell by Perturbing the Electrochemical Gradient

Maïté Coppey-Moisan,* Anne-Christine Brunet,* Réjean Morais,# and Jacques Coppey*
*Institut Curie, Section de Recherche, 75231 Paris, France, and #Département de Biochimie, Faculté de Médecine, Université de Montréal, Québec, Canada

ABSTRACT Digital-imaging microscopy was used in conditions that allowed the native state to be preserved and hence fluorescence variations of specific probes to be followed in the real time of living mammalian cells. Ethidium bromide was shown to enter into living cells and to intercalate stably into mitochondrial DNA (mtDNA), giving rise to high fluorescence. When the membrane potential or the pH gradient across the inner membrane was abolished by specific inhibitors or ionophores, the ethidium fluorescence disappeared from all mtDNA molecules within 2 min. After removal of the inhibitors or ionophores, ethidium fluorescence rapidly reappeared in mitochondria, together with the membrane potential. The fluorescence extinction did not result from an equilibrium shift caused by leakage of free ethidium out of mitochondria when the membrane potential was abolished but was most likely due to a dynamical mtDNA change that exposed intercalated ethidium to quencher, either by weakening the ethidium binding constant or by giving access of a proton acceptor (such as water) to the interior of mtDNA. Double labeling with ethidium and with a minor groove probe (4′,6-diamino-2-phenylindole) indicated that mtDNA maintains a double-stranded structure. The two double-stranded DNA states, revealed by the fluorescence of mitochondrial ethidium, enhanced or quenched in the presence of ethidium, seem to coexist in mitochondria of unperturbed fibroblast cells, suggesting a spontaneous dynamical change of mtDNA molecules. Therefore, the ethidium fluorescence variation allows changes of DNA to be followed, a property that has to be taken into consideration when using this intercalator for in vivo as well as in vitro imaging studies.

INTRODUCTION

In vitro, DNA undergoes structural changes at several organization levels upon modification of its environment: ions (Bloomfield, 1991; Pohl and Jovin, 1972; Peck et al., 1982; Robert-Nicoud et al., 1984), water activity (Eickbush and Moudrianakis, 1978; Preisler et al., 1995), protein interaction (Werner et al., 1996), or mechanical shearing (Cluzel et al., 1996; Smith et al., 1996). Intercalated ethidium bromide has been an invaluable tool for studying the torsional dynamics of DNA (Genest and Wahl, 1978) and its perturbations upon protein binding (McMurray and van Holde, 1991; Härd and Kearns, 1990; Winzeler and Small, 1991) or superhelix density variation (Naimushin et al., 1994). In addition, measuring ethidium binding permits the probing of DNA structural changes, right- to left-handed transconformation of polynucleotides (van de Sande et al., 1982; Markovitz et al., 1985) and condensation induced by cations (Widom and Baldwin, 1983) or associated with protein binding (Angerer and Moudrianakis, 1972).

In the living state, the conformation and the dynamics of DNA are still unknown. The fact that ethidium enters living cells (Délic et al., 1992; Hayashi et al., 1994) and that the emerging fluorescence technologies in digital imaging microscopy allow the native state to be preserved (Délic et al., 1991) gave us the possibility to study the real-time dynamics of mtDNA in a single mitochondrion, directly in living cells, by analyzing the fluorescence changes of ethidium preintercalated in this DNA. The present study describes the characteristics of the ethidium fluorescence extinction that occurs in mtDNA upon abolition of the inner membrane potential by an inhibitor of respiration and/or the pH gradient by ionophores and its complete reversal upon removing the inhibitor or the ionophore. Results from double-labeling experiments with 4′,6-diamidino-2-phenylindole (DAPI, a minor groove probe) and with ethidium bromide revealed that, in living cells, DNA can adopt a double-stranded conformation in which ethidium either cannot intercalate or does not emit enhanced fluorescence.

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Address reprint requests to Dr. Maïté Coppey-Moisan, INSERM U-310 and Laboratoire de Biochimie des Acides Nucleiques, Section de Recherche, Institut Curie, 26 rue d'Ulm, 75231 Paris, Cedex 05, France.
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MATERIALS AND METHODS

Cells

Skin fibroblast cells from a normal donor were grown in minimal essential medium (Gibco, Cergy-Pontois, France) supplemented with 10% fetal calf serum and were used at low passage (<25 doublings). S2 cells, a clonal expansion from a transformed Vero monkey kidney cell line (Gao and Knipe, 1989), were grown in Dulbecco's modified minimal essential medium. S2 cells exhibited a highly proliferative potential. The experiments in S2 cells were carried out at a relatively low density, i.e., in 72 h, cultures of cells seeded at 5×10^{-4} cells per petri dish (28 cm^2). NB cells are derived from a human osteosarcoma. Their derivative deprived of mtDNA, NB ρ^0 cells, were subcultured as described elsewhere (Morais et al., 1994). NB ρ^0 cells contaminated with mycoplasma were used in one experiment. Two cycles of treatment of these contaminated cells by BM cyclin (Boehringer Mannheim, Mannheim, Germany) led, as expected, to the complete disappearance of mycoplasma.

Cell labeling with fluorophores

Cultures were preloaded with either of the following fluorophores: rhodamine 123 (rd123) at 10 μg/ml, ethidium bromide at 4 μg/ml for S2 cells and 0.4 μg/ml for fibroblast cells (unless otherwise indicated), and DAPI at 10 μg/ml diluted in growth medium and incubated for 10 min (rd123), 30 min (ethidium bromide), and 2 h (DAPI) in culture medium at 37°C. Cell cultures were then rinsed with 10 mM Hepes-buffered Hanks' balanced salt solution (HBSS) before microscopic observations. In one experiment (NB wild-type and ρ^0 cells), mitochondria with high membrane potential were visualized by the formation of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) aggregates evidenced by an orange fluorescence (Reers et al., 1991). JC-1 stock solution (1 mg/ml in dimethylsulfoxide) was diluted just before use in culture medium. Cultured cells were incubated at 37°C for 2 min with 10 μg/ml JC-1 and then rinsed with HBSS. The presence of mycoplasma was revealed directly in living cells by Hoechst fluorescence in the cytoplasm of the cell in culture (in Petriperm, Bachofer, Reutligen, Germany) after cell incubation with 1 μg/ml Hoechst 33242 for 30 min at 37°C. All fluorophores were purchased from Molecular Probes (Eugene, OR).

Fluorescence microscopy at low light level

Cells were grown in Petriperm (Bachofer), which allows direct microscopic observation and epifluorescence measurement, using an objective with high numerical aperture. All fluorescent images were acquired with very low excitation light levels as described elsewhere (Délic et al., 1991). Briefly, cells grown in Petriperm were imaged through a 100× (NA = 1.3) ultrafluor objective of an inverted microscope (Olympus IMT2). The detector was a video camera intensified with two microchannel plates (Lhesa Électronique, Cergy Pontoise, France). An electronic shutter was driven from the computer and sequential raw images were acquired, summed, and stored for further image processing. This process was repeated every 10–15 s over several minutes to get complete kinetic data. The temporal resolution was 10 s (time necessary to sum 256 video frames). For JC-1 fluorescence, λ_{exc} = 436 nm, OD = 3.6, and λ_{em} = 583 ± 3 nm. For rd123 fluorescence, λ_{exc} = 436 nm, OD = 2.6, and λ_{em} > 500 nm. For ethidium fluorescence, λ_{exc} = 540 nm, OD = 1.6, and λ_{em} > 560 nm. For DAPI fluorescence, λ_{exc} = 365 nm, OD = 2.6, and λ_{em} = 520 ± 10 nm. The excitation source was a 100-W high pressure mercury lamp. The image processing is described elsewhere (Viegas-Péquiniot et al., 1989; Coppey-Moisan et al., 1994).

Perturbation of the electrochemical gradient of mitochondria in living cells

After rinsing with HBSS, the intramitochondrial fluorescence intensity of rd123 was stable for at least 30 min in S2 cells and for several hours in fibroblast cells. A collapse of the electrochemical gradient was driven by incubating cells in the presence of inhibitors of the respiratory chain or of H^+ or H^+/K^+ ionophores, diluted in HBSS. The decrease of mitochondrial membrane potential was qualitatively followed in real time by monitoring the decrease of mitochondrial fluorescence of rd123, starting with the switch of HBSS to HBSS containing either inhibitor or ionophore, directly in the Petriperm settled on the microscope stage. In contrast to other reports (Emaus et al., 1986; Bunting et al., 1989; Farkas et al., 1989), no quenching of rd123 fluorescence was evidenced in energized mitochondria in living cells. We attributed this to the fact that, after cell incubation with 10 μg/ml rd123 for less than 10 min, there were only low levels of dye inside mitochondria. Under these conditions, there was no swelling of intracellular mitochondria, which occurs if the incubation time is longer (approximately 20 min). In addition, no residual rd123 fluorescence in mitochondria was observed in our experimental conditions when the potential was collapsed (in the presence of 10 mM sodium azide). This is probably due to the very low level of light excitation (a filter of optical density = 2.6 is present on the excitation light path; see the previous paragraph), which avoids cross-linking of rd123 with macromolecules in the mitochondrial inner membrane. Indeed, at higher levels of light excitation, some bleaching occurred and incubation of 10 mM sodium azide could not completely abolish the rd123 fluorescence in mitochondria. The effect of perturbing the electrochemical gradient on the mitochondrial ethidium fluorescence was recorded in parallel cell cultures preincubated with ethidium bromide followed by rinsing. In all types of cells, the intramitochondrial fluorescence intensity of ethidium was stable for more than 24 h without ethidium bromide in the culture medium. The reversibility was followed, using the same cells and without moving the Petriperm, by replacing HBSS containing the inhibitor or the ionophore with inhibitor/ionophore-free HBSS. The inhibitors and the ionophores were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Subcellular distribution of ethidium fluorescence in living cells

Ethidium is taken up by cells in culture (Délic et al., 1992; Hayashi et al., 1994). By using very low excitation light levels, which allow photodynamic reactions to be avoided (Délic et al., 1991), we detected, inside cycling cells, ethidium fluorescence in nucleoli and cytoplasmic organelles but not in nuclear DNA (Fig. 1 *A*) (Délic et al., 1992; Hayashi et al., 1994). The fluorescence in nucleoli is due to ethidium intercalation in double-stranded ribosomal RNA (Délic et al., 1992). The intracellular fluorescence

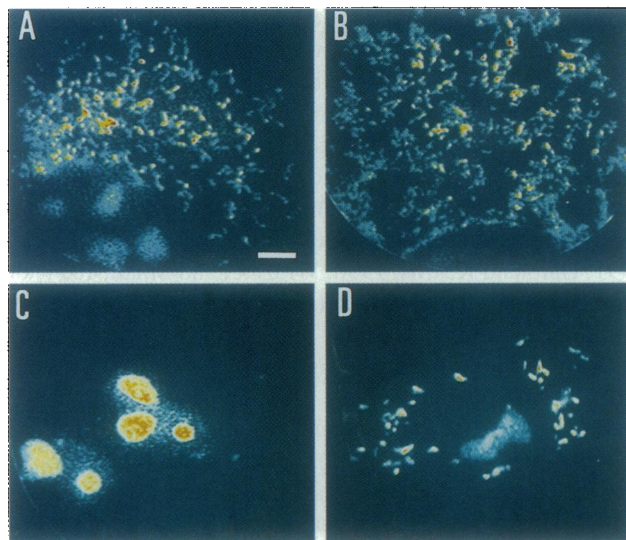

FIGURE 1 The cytoplasmic fluorescence of ethidium in living cells arises from molecules intercalated in mitochondrial DNA/RNA. Shown are the patterns (thermographic pseudo-color) of intracellular fluorescence of ethidium (*A* and *C*) and of JC-1 (*B* and *D*) in a single cell of transformed human NB cells, wild type (*A* and *B*), or their derivative deprived of mitochondrial DNA, ρ^0 cells (*C* and *D*). There was no detectable cytoplasmic ethidium fluorescence in NB ρ^0 cells, even at the highest detection sensitivity (the intensification by microchannel plates was 10 times higher in *C* than in *A*). However, these ρ^0 cells contain mitochondria with membrane potential as revealed by the cytoplasmic pattern of JC-1 orange fluorescence (*D*). In *D*, the nucleoli are visible (ethidium fluorescence) because labeling with JC-1 was carried out on cells preloaded with ethidium. See Materials and Methods for fluorescence image acquisitions. Bar in *A*, 5 μm.

intensity increased with the incubation time, temperature, and ethidium bromide concentration (not shown). The cytoplasmic labeled structures were mitochondria as 1) the corresponding fluorescence co-localized with that of mitochondrial membrane potential probe JC-1 (Fig. 1 *B*) or rd123 (not shown) and 2) cells devoid of mtDNA (ρ^0 cells) contain mitochondria (Morais et al., 1994) with a high membrane potential, as revealed by orange fluorescence of JC-1 in cytoplasmic organelles (Fig. 1 *D*) but did not exhibit any cytoplasmic ethidium fluorescence despite the high intensification used here with the microchannel plate set at a high voltage (Fig. 1 *C*), even for concentrations up to 100 μg/ml (not shown), as also reported in another ρ^0 cell line (Hayashi et al., 1994). If ethidium molecules are present in the mitochondrial matrix of ρ^0 cells, they do not emit fluorescence above background levels. Therefore, the mitochondrial fluorescence in wild-type cells results from ethidium molecules intercalated in DNA/RNA of the organelle. RNA does not, however, participate in this fluorescence; after ethidium bromide treatment (60 min at 37°C), its intensity remained stable for at least 24 h, i.e., under conditions known to block totally transcription of mtDNA (Zylber et al., 1969). Moreover, mitochondrial RNA is metabolically unstable (Gelfand and Attardi, 1981); consequently, if the fluorescence were partly due to ethidium intercalated in mitochondrial double-stranded RNA, its intensity would have decreased over 24 h, which is not the case. We deduce that the mitochondrial fluorescence is emitted from ethidium stably intercalated in mtDNA.

Relationship between membrane potential and mtDNA

In mitochondria of respiring cells, the inner membrane is impermeable to ions, particularly protons. The resulting proton gradient gives rise to an electrochemical gradient, $\Delta\mu_{H+}$, with a pH gradient (ΔpH) and an electrical potential ($\Delta\psi$) (Nicholls and Ferguson, 1992). Simultaneous abolition of $\Delta\psi$ and of ΔpH was provoked by applying inhibitors of electron transport enzymes, sodium azide or antimycin A, or a H^+ ionophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Abolition of $\Delta\psi$ only was provoked by applying valinomycin in high-K^+ medium (as in the cytoplasm), a condition shown to leave ΔpH unaffected (Nicholls and Ferguson, 1992).

Under unperturbed conditions, rd123 fluorescence selectively concentrated inside mitochondria (Fig. 2 *Aa*) in which it remained for more than 30 min after removal of the dye from the incubation medium, indicating the persistence of a high $\Delta\psi$. Addition of sodium azide (10 mM, Fig. 2, *A* and *B*, left), FCCP (0.1 μM), valinomycin (10 μg/ml), or antimycin A (10 μg/ml) rapidly abolished $\Delta\psi$, as evidenced by the decrease of the mitochondrial rd123 fluorescence. In cells preloaded with ethidium bromide (Fig. 2 *Ae*), a decrease of the mitochondrial fluorescence down to total extinction was observed, over a similar time scale, after addition of sodium azide (Fig. 2, *A* and *B*, right), antimycin A, FCCP, or valinomycin. The kinetics of decrease recorded in real time dropped, in all cases, down to background level in less than 2 min at 37°C (Fig. 2 *B* for azide). Oligomycin (up to 1 mg/ml), an inhibitor of ATP synthesis in mitochondria (Nicholls and Ferguson, 1992) did not modify ethidium fluorescence; hence, ATP production is not involved in its extinction. We have ruled out the possibility of a direct quenching of ethidium fluorescence by azide or by the ionophores by the fact that the fluorescence intensity of ethidium intercalated in mycoplasma DNA in the cytoplasm of infected ρ^0 cells did not vary in cells incubated with azide (Fig. 2 *C*) or with an ionophore.

Distinct mechanisms for the disappearance of rd123 and ethidium fluorescence upon perturbing the mitochondrial energetics

$\Delta\psi$ could be responsible for retaining high levels of ethidium (a cation) inside this organelle, in which case free ethidium would leak out of mitochondria when $\Delta\psi$ is abolished. Results with nigericin rule out this possibility as, in contrast to the other ionophores, this ionophore, which exchanges K^+ for H^+, abolishes ΔpH while leaving $\Delta\psi$ unaffected or even increased (Nicholls and Ferguson, 1992). After addition of nigericin (3 μg/ml), rd123 fluorescence persisted unchanged in mitochondria (Fig. 3, *left*) whereas mitochondrial ethidium fluorescence extinguished over a time scale similar to that observed with the other ionophores (Fig. 3, *right*). Thus, the ethidium fluorescence extinction is not due to an equilibrium shift, provoked by leakage of ethidium out of mitochondria, when $\Delta\psi$ is abolished. Other results demonstrate that the disappearance of rd123 fluorescence and that of ethidium fluorescence proceed by distinct mechanisms, diffusion out of mitochondria and the cell for rd123 and fluorescence quenching by locally available proton acceptors (such as water) for ethidium. Indeed, when rd123- or ethidium-preloaded cells were treated with azide (10 mM) for 20 min (Fig. 4, *c* and *d*), the mitochondrial ethidium fluorescence returned to the initial level, after a short lag time, without a new ethidium load (Fig. 4 *c*). In contrast, rd123 fluorescence did not reappear in mitochondria (Fig. 4 *d*) unless an additional load of rd123 was applied. This new load of rd123 was needed to show the recovery of $\Delta\psi$ after this treatment. In the absence of $\Delta\psi$, mitochondrial rd123 molecules freely diffused into the incubation medium. After switching the azide-containing medium for azide-free medium, there were not enough available rd123 molecules to detect labeled mitochondria. In contrast, for ethidium, the complete recovery of mitochondrial fluorescence without a new load strongly suggests that, during the 20 min without $\Delta\psi$, ethidium molecules stay close to mtDNA (or at least inside the cell), the cytoplasmic diffusion coefficients being likely comparable for rd123 and ethidium. As water probably quenches ethidium fluorescence by transferring a proton from ethidium to water

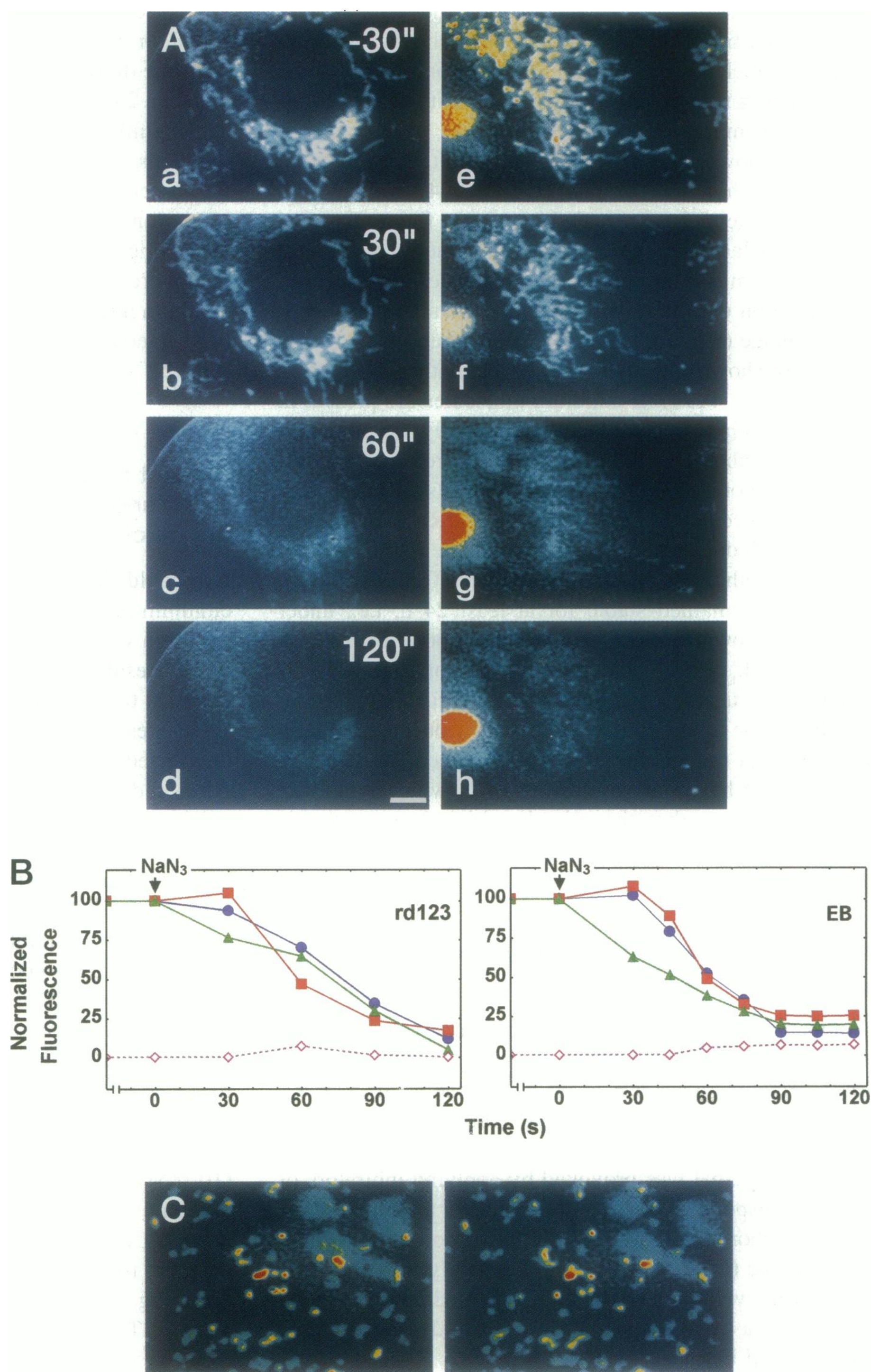

FIGURE 2 Synchronous decrease in living cells of mitochondrial membrane potential and of fluorescence of ethidium molecules stably intercalated in mitochondrial DNA upon sodium azide treatment. (*A*) rd123 (*left*) and ethidium (*right*) fluorescence images (thermographic pseudo-color) of S2 cells before and after treatment by sodium azide (10 mM). The incubation medium, HBSS supplemented with 20 mM Hepes, pH 7.4, was switched to sodium-azide-containing medium at 0 s. Shown are images taken before (*a* and *e*) and 30 (*b* and *f*), 60 (*c* and *g*), and 120 (*d* and *h*) s after sodium azide addition. Bar, 5 μm. (*B*) Time course of normalized fluorescence variations in mitochondria of cells displayed in *A* (*left*, rd123; *right*, ethidium). Each closed symbol corresponds to normalized fluorescence intensity per pixel measured in a discrete region for three mitochondria. Each open symbol corresponds to normalized fluorescence intensity per pixel measured in a cytoplasmic region close to the three mitochondria. The arrows indicate the time at which the incubation medium was replaced by azide-containing HBSS. The decays of rd123 and ethidium fluorescences shown here in two cells are representative of the results obtained in all cells examined. (*C*) Cytoplasmic fluorescence of ethidium molecules intercalated in mycoplasma DNA of ρ^0 cells harboring this microorganism (*left*). Note that the mycoplasma fluorescence was insensitive to sodium azide addition (*right*).

(Olmsted and Kearns, 1977), the fluorescence enhancement upon ethidium intercalation is due to a restricted access of water and/or other proton acceptors to the interior of the double-helix. Conversely, the fluorescence decrease (up to extinction) from preintercalated ethidium indicates a change of the environment of ethidium allowing proton transfer to occur. This means either that the ethidium binding constant toward mtDNA is reduced or that the intercalating sites are more accessible to proton acceptors (such as water). The last possibility would imply a helix deformation to give proton access to intercalated ethidium.

We cannot formally exclude the possibility that the ethidium binding constant could be changed by altering only the chemical potential of free ethidium in the mitochondrial matrix. However, if this were the case, the equilibrium should be shifted back toward the bound state by increasing the local

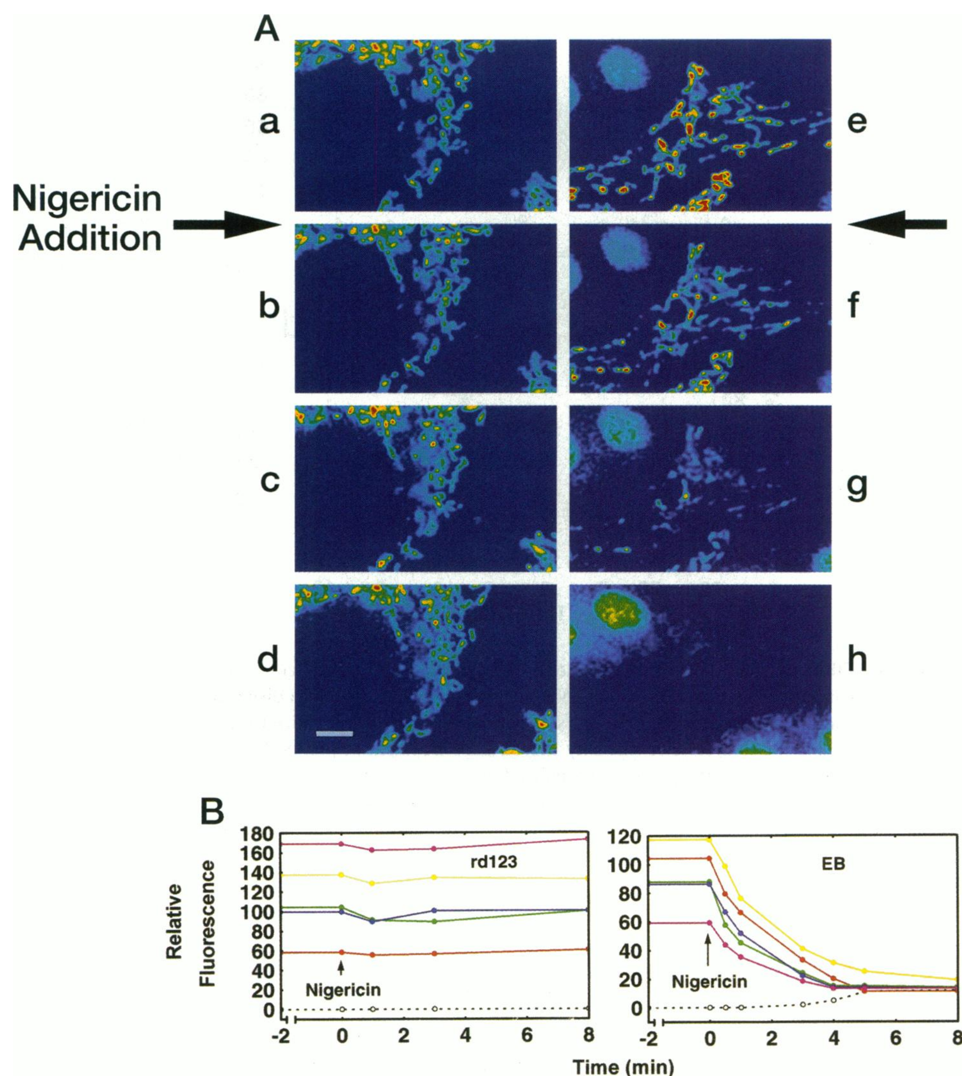

FIGURE 3 Disappearance of mitochondrial ethidium fluorescence and concomitant stability of $\Delta\psi$ in nigericin-treated cultures. (*A*) rd123 (*left*) and ethidium (*right*) fluorescence images (thermographic pseudo-color) in S2 cells before and after treatment by nigericin (3 μg/ml). The incubation medium, 130 mM KCl, 50 mM NaCl, 1 g/L glucose, 20 mM Hepes, pH 7.4, was switched to nigericin-containing medium at 0 s. Shown are images taken before (*a* and *e*) and 30 (*b* and *f*), 60 (*c* and *g*), and 120 (*d* and *h*) s after nigericin addition. Bar, 5 μm. (*B*) Time course of fluorescence variations in mitochondria of cells displayed in *A* (*left*, rd123; *right*, ethidium). Each closed symbol corresponds to the relative fluorescence intensity per pixel measured over a discrete region in five mitochondria. Each open symbol corresponds to the relative intensity per pixel measured in a cytoplasmic region close to the five mitochondria. The arrows indicate the time at which the incubation medium was replaced by nigericin-containing medium. The mitochondrial fluorescence changes were not normalized to show that, whatever the initial fluorescence intensity, there was no change of rd123 fluorescence but a complete extinction of ethidium fluorescence. The stability of rd123 fluorescence as well as the decays of ethidium fluorescence shown here in two cells are representative of the results obtained in all cells examined.

concentration of ethidium. However, experiments carried at high concentrations of ethidium bromide have ruled out this possibility; indeed, when the mitochondrial membrane potential was abolished, no ethidium fluorescence above background could be detected in mitochondria in the presence of 10^{-4} M ethidium, a 100-fold higher concentration than that used in the experiments described previously (not shown). Hence, the extinction of ethidium fluorescence, which occurred whatever the free ethidium concentration, most likely involves a dynamical change of mtDNA due to modification of its microenvironment.

mtDNA maintains double-strandedness throughout its dynamical change

To further characterize the change occurring in mtDNA, we used a fluorescent probe of the minor groove, DAPI, which has been shown to interact with mtDNA in cultured cells (Hayashi et al., 1994) (Fig. 5). In the presence of FCCP, the DAPI fluorescence remained unaltered (Fig. 5 *D*) whereas, in the same mitochondrion, ethidium fluorescence was extinguished (Fig. 5 *B*). Similar results were obtained with antimycin A, azide, valinomycin, or nigericin. We deduce that the mtDNA remained double stranded, without melting, throughout the change.

Both mtDNA states coexist inside mitochondria of unperturbed fibroblast cells

Although in all transformed or immortalized cells the ethidium fluorescence was homogeneous along whole energized mitochondrion, in human skin fibroblast cells, the mitochondrial ethidium fluorescence pattern was discontinuous, i.e., segments of ethidium fluorescence adjacent to segments of quenched ethidium fluorescence (*arrows* in Fig. 6, *A* and *C*). This pattern was different from that of DAPI fluorescence; i.e., the DAPI fluorescence in the same mitochondria was continuous and more homogeneous (Fig. 6, *B* and *D*). The mtDNA segments of quenched ethidium fluorescence are not due to quenching by DAPI, as compa-

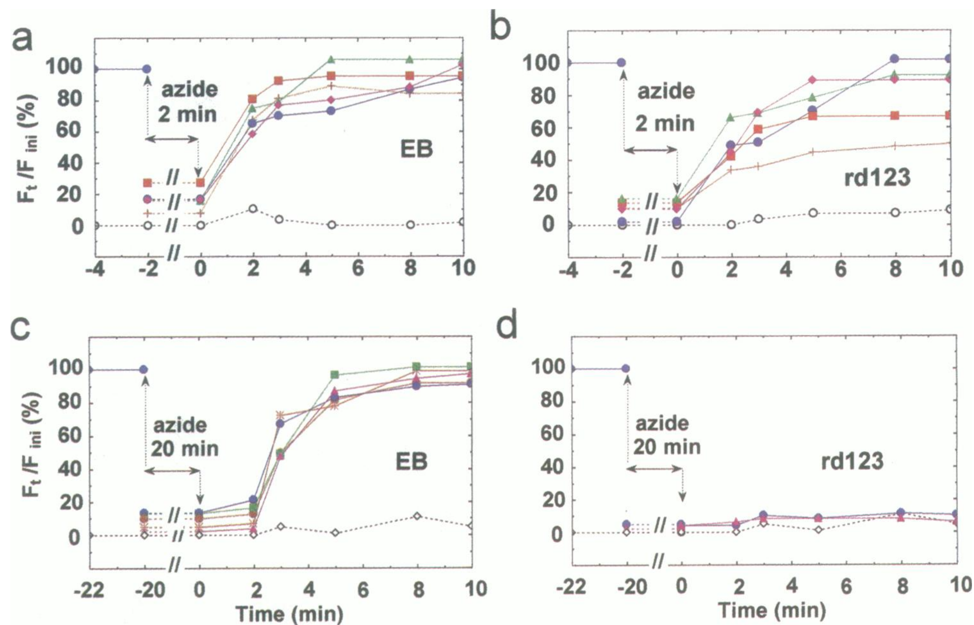

FIGURE 4 Ethidium and rd123 exhibit distinct fluorescence recovery after azide removal. Time course of normalized fluorescence variations of ethidium (EB) (*a* and *c*) and rd123 (*b* and *d*) in mitochondria of two cells from two petri dishes (one preloaded with ethidium, the other preloaded with rd123) after azide addition and then removal. Each closed symbol corresponds to normalized fluorescence intensity per pixel measured in a discrete region for five mitochondria; F_{ini} is the fluorescence intensity before azide treatment; F_t is the fluorescence intensity at different times after 10 mM azide addition and removal. Each open symbol corresponds to normalized fluorescence intensity per pixel measured in a cytoplasmic region. The bars indicate the time interval during which the incubation medium was replaced by azide-containing HBSS. After removal of azide, after a short incubation time (<2 min), the mitochondrial fluorescence of rd123 did not completely return to the starting level; after a longer incubation time with azide, the mitochondrial fluorescence of rd123 did not recover at all, although $\Delta\psi$ spontaneously reverted (not shown). In contrast, ethidium fluorescence completely recovered, even after long incubation times with azide.

rable discontinuity of the ethidium fluorescence was observed in the absence of DAPI (not shown). Thus, in the respiring fibroblast, there are some mtDNA molecules (or regions of mtDNA molecules) that are double stranded (evidenced by DAPI fluorescence) but in which ethidium does not intercalate or does not fluoresce above the background. This is reminiscent of the mtDNA state evidenced when the mitochondrial potential was collapsed. We do not know whether it is the underlying perturbation involved in the abolition of mitochondrial membrane potential that stabilizes this mtDNA state, characterized by absence of ethidium fluorescence in respiring fibroblast. It has been reported by others that the mitochondrial membrane potential exhibits heterogeneity along each mitochondrion in fibroblast cells (Smiley et al. 1991). However, by using the same dye (JC-1) we were unable to reproduce these results. Thus, we do not know whether, in our experiments, the mitochondrial membrane potential was heterogeneous or not along a single mitochondrion. The coexistence of both DNA states inside the same mitochondrion in fibroblasts

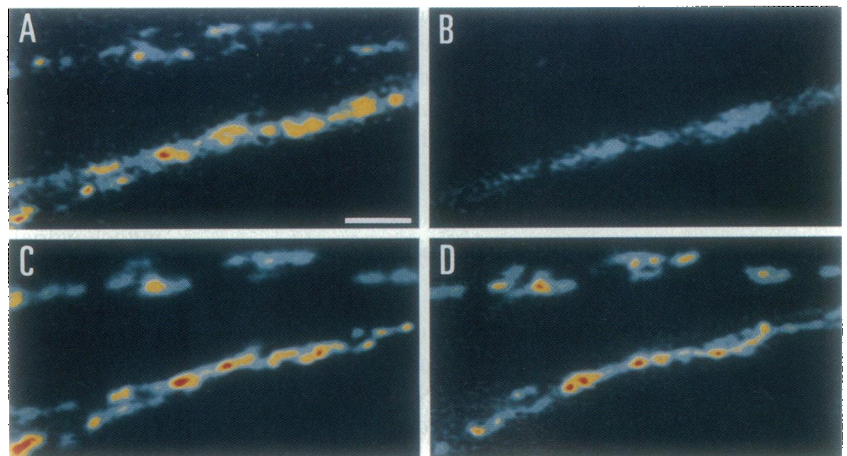

FIGURE 5 Differential effect of FCCP on the mitochondrial fluorescence of DAPI and that of ethidium. A fibroblast culture was preincubated at 37°C in presence of DAPI (10 μg/ml for 2 h) and of ethidium bromide (0.4 μg/ml for 30 min) followed by rinsing. Shown are fluorescence images (thermographic pseudo-color) of the same cytoplasmic region: fluorescence of intercalated ethidium molecules (*A*) and of DAPI molecules (*C*). Extinction of ethidium fluorescence (*B*) and persistence of DAPI fluorescence (*D*) after treatment by 0.1 μM FCCP. The fluorescent images were sequentially acquired at low light levels. For ethidium fluorescence, λ_{exc} = 540 nm, OD = 1.6, and λ_{em} > 560 nm. For DAPI fluorescence, λ_{exc} = 365 nm, OD = 2.6, and λ_{em} = 520 ± 10 nm. Bar in *A*, 5 μm.

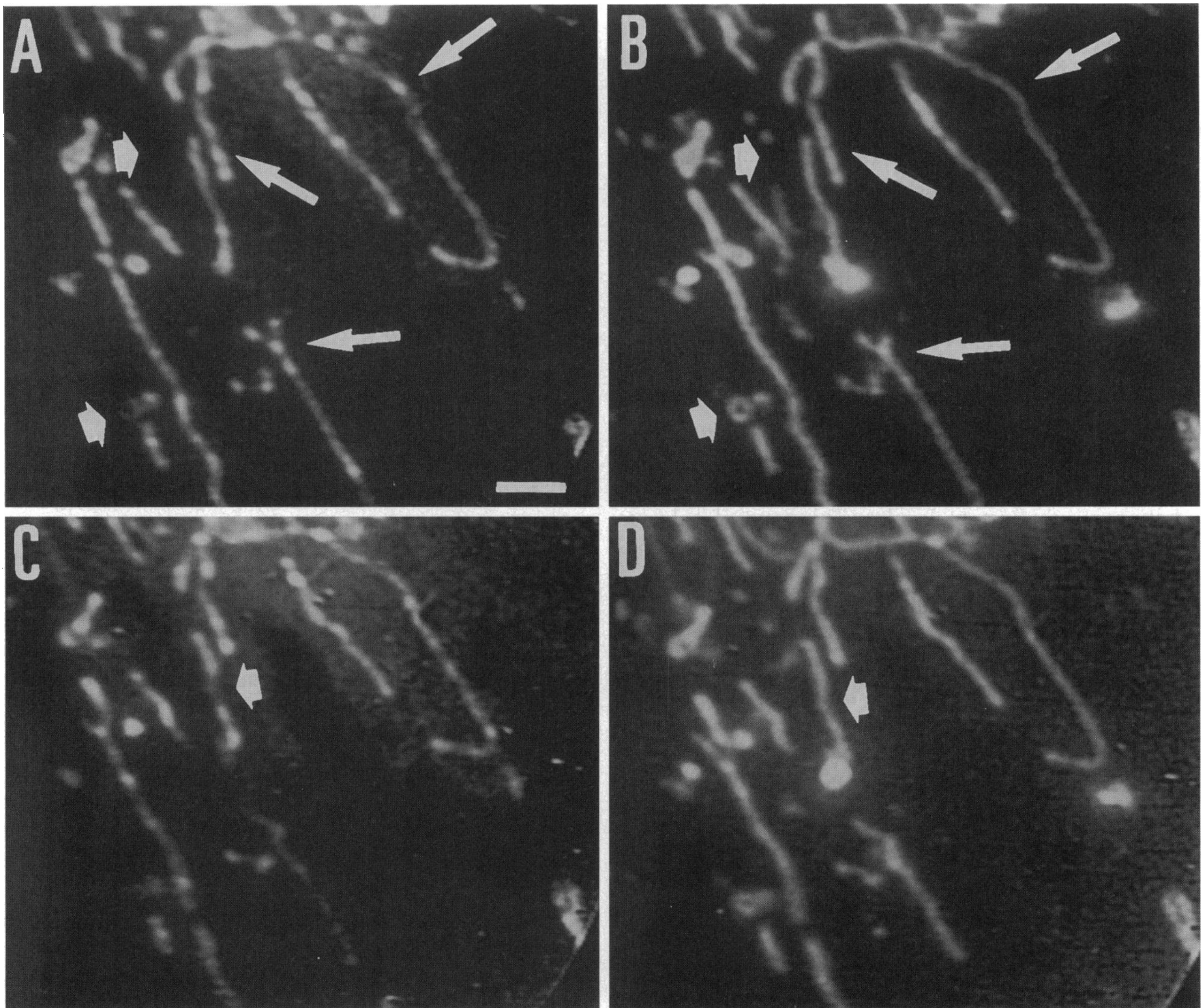

FIGURE 6 Coexistence of two DNA conformations inside a single mitochondrion in a respiring human fibroblast. The fluorescence images of ethidium (*A*) and DAPI (*B*) were sequentially acquired in the conditions of Fig. 5. The time elapse between the acquisition of the two images was 20 s. (*C* and *D*) Same as *A* and *B*, respectively, 1 min later. Thin arrows point to the mitochondrial regions where ethidium fluorescence was typically heterogeneous whereas DAPI fluorescence was homogeneous. Thick arrows show DNA-containing mitochondria (revealed by the presence of DAPI fluorescence) but no ethidium fluorescence. Bar, 3 μm.

could probably be observed because these cells have elongated mitochondria. Bereiter-Hahn and co-workers also obtained a discontinuous DNA fluorescence pattern with another DNA probe, picogreen, in single mitochondria in living cardiac epithelial cells from *Xenopus laevi* (personal communication, to be published).

DISCUSSION

We show here that 1) the mitochondrial ethidium fluorescence arises from ethidium molecules stably intercalated in double-stranded mtDNA, 2) the decrease of ethidium fluorescence upon perturbing the electrochemical gradient by inhibitor or ionophore always goes down to the background level whatever the starting amount of intercalated ethidium molecules and it is not possible to shift back the equilibrium to the ethidium-mtDNA bound state by increasing the amount of disposable ethidium, and 3) the decrease of fluorescence is not triggered by leakage of free ethidium out of the mitochondria.

Due to the fluorescence properties of ethidium bromide (Olmsted and Kearns, 1977), these results mean that ethidium molecules preintercalated in mtDNA become more accessible to a proton acceptor molecule, such as water, after the perturbation. Thus, a possibility is that the binding constant of ethidium to intercalating sites in mtDNA was strongly reduced. Another possibility is that the fluorescence of ethidium molecules still intercalated could be quenched due to a minimal deformation of the helix to permit transfer of protons from the amino group of

ethidium to an acceptor molecule. Such a deformation has not yet been described in vitro.

Under the experimental conditions required to carry out observations in living cells, diffusion of the inhibitor or the ionophore from the extracellular medium to the mitochondrial membrane is the limiting step in the kinetics of ethidium fluorescence decrease. Thus, a decrease of the binding constant could arise either from a decrease of the association rate constant, from an increase of the dissociation rate constant, or from both parameters.

It cannot be rigorously excluded that the decrease in binding constant in mtDNA could be due to chemical potential change of free ethidium molecules alone. Indeed, in alcoholic solutions, interactions of solvent with ethidium molecules are unveiled by enhancement of ethidium fluorescence as well as by the red-shifted excitation and emission fluorescence spectra. These effects correspond to a dehydration of ethidium molecules. This type of solvent interaction can indeed decrease the ethidium binding constant. However, it is not likely that a decrease of mitochondrial water activity was capable of strongly dehydrating ethidium molecules without affecting the water and/or counterion molecules bound to mtDNA. As Dickerson and co-workers have noticed, DNA is built not only from bases, sugars, and phosphate groups but also from water molecules and bound counterions (Privé et al., 1991). Thus, we therefore infer that a change occurs at the level of mtDNA.

Taken together, the present results suggest that a dynamical change of mtDNA is involved in the extinction of mitochondrial ethidium fluorescence upon perturbing the electrochemical gradient. We do not know at present the molecular nature of this change in mtDNA.

Interestingly, results from pressure-jump chemical relaxation studies suggest that the mechanism of intercalation of ethidium in DNA would involve, before the intercalation step, a DNA conformational change (McGregor et al., 1987), namely, a transient base pair opening, such as that measured by hydrogen exchange. The existence of such a dynamical conformational change of the double helix in the absence of the dye was proposed earlier by Sobell et al. (1977). A stabilization of a conformation incapable of intercalation, by modification of the microenvironment, can account for the results obtained in mtDNA containing pre-intercalated ethidium.

Whatever the nature of the physicochemical perturbations provoked inside mitochondria, we cannot exclude the participation of proteins. Indeed, it has been shown that DNA-interacting proteins can be involved in the fluorescence extinction of ethidium by modifying the intercalation sites (Angerer and Moudrianakis, 1972), by reducing the flexibility of DNA (Härd and Kearns, 1990; Winzeler and Small, 1991), or by restraining its elasticity (McMurray and van Holde, 1991). Indeed, ethidium cannot intercalate into DNA when its elasticity is restrained in nucleosomes by the histone octamer (McMurray and van Holde, 1991) and a histone fold motif is found in numerous non-histone-related proteins (Baxevanis et al., 1995). However, the only mammalian mitochondrial protein so far known to condense DNA in vitro is mtTF1 (Fisher et al., 1992). Upon binding to closed circular DNA, mtTF1 creates supercoiling and bending but only in regulatory sequences (Fisher et al., 1992). Moreover, mtDNA, when extracted, is not associated with proteins in a nucleosome-beaded fashion, except at the replication initiation site (Albring et al., 1977; DeFrancesco and Attardi, 1981).

As the extinction of the fluorescence of ethidium intercalated in mtDNA occurs upon abolition of $\Delta\psi$ or ΔpH either by inhibiting the electron transport or by applying ionophores, it is likely that there is a common underlying process. Isolated mitochondria undergo a profound ultrastructural transformation when switching from an active respiratory state toward an inactive one, a phenomenon that is reversible (Hackenbrock, 1968a,b). During the switch, water leaves the mitochondria, leading to a shrinkage of the inner space, the inner membrane staying physically linked to the matrix network (Hackenbrock, 1968a,b). When ionophores are applied, water movements across the inner membrane also occur (Nicholls and Ferguson, 1992). Thus, it is likely that a water activity decrease takes place here inside the mitochondrial inner compartment when $\Delta\psi$ or ΔpH are canceled in the living cells. A decrease in water activity associated with the shrinkage of the mitochondrial matrix produces an increase of osmotic pressure, probably similar to the osmotic stress carried out with polyethylene glycol. Moreover, it cannot be excluded that a decrease of the dielectric constant of the surrounding mtDNA takes place in addition to the increase of osmotic pressure.

Interestingly, independent in vitro studies have shown that, in the condensed state, DNA molecules undergo conformational and phase transitions, respectively, upon small decreases in the dielectric constant (Arscott et al., 1995) and small increases of osmotic pressure (Rau and Parsegian, 1992). Indeed, it was recently shown that, in the cobalt hexaammine condensed state, DNA undergoes a transition from B form to A form when the dielectric constant drops between 70 and 60 (Arscott et al., 1995). A reversible phase transition can be triggered in DNA, starting from a cobalt hexaammine condensed state, by small increase of the applied osmotic pressure (Rau and Parsegian, 1992). The transition was evidenced by an abrupt change in the spacing between adjacent helix surfaces, indicating that the hydration forces switched from a repulsive status to an attractive one (Rau and Parsegian, 1992). In this context, it is important to note that, at a yet undefined stage during the condensation of λ phage DNA by cobalt hexaammine, the fluorescence of intercalated ethidium was extinguished (Widom and Baldwin, 1983). However, as the physicochemical properties of the mitochondrial matrix are still unknown in either the collapsed or energized state, particularly in the surrounding of mtDNA, the results obtained in vitro cannot yet be extrapolated to the mechanisms underlying the change triggered in mtDNA in living cells.

The activity of DNA is mediated by the specificity of its interactions with proteins. As recently shown, water release is involved in the specificity of protein-DNA interactions (Arbuckle and Luisi, 1995; Garner and Rau, 1995; Parsegian et al., 1995). Therefore, it will be interesting to measure the activities of replication and transcription of mtDNA in relation to its dynamical change described here.

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