Functional F1-ATPase Essential in Maintaining Growth and Membrane Potential of Human Mitochondrial DNA-depleted ρ° Cells

(Received for publication, April 9, 1998, and in revised form, June 19, 1998)

Karine Buchet‡ and Catherine Godinot§
From the Centre de Génétique Moléculaire et Cellulaire, UMR 5534, CNRS, Université Claude Bernard de Lyon I, 69622 Villeurbanne cedex, France

F1-ATPase assembly has been studied in human ρ° cells devoid of mitochondrial DNA (mtDNA). Since, in these cells, oxidative phosphorylation cannot provide ATP, their growth relies on glycolysis. Despite the absence of the mtDNA-coded F0 subunits 6 and 8, ρ° cells possessed normal levels of F1-ATPase α and β subunits. This F1-ATPase was functional and azide- or aurovertin-insensitive but oligomycin-insensitive. In addition, aurovertin decreased cell growth in ρ° cells and also reduced their mitochondrial membrane potential, as measured by rhodamine 123 fluorescence. Therefore, a functional F1-ATPase was important to maintain the mitochondrial membrane potential and the growth of these ρ° cells. Bongkrekic acid, a specific adenine nucleotide translocator (ANT) inhibitor, also reduced ρ° cell growth and mitochondrial membrane potential. In conclusion, ρ° cells need both a functional F1-ATPase and a functional ANT to maintain their mitochondrial membrane potential, which is necessary for their growth. ATP hydrolysis catalyzed by F1 must provide ADP3− at a sufficient rate to maintain a rapid exchange with the glycolytic ATP4+ by ANT, this electrogenic exchange inducing a mitochondrial membrane potential efficient enough to sustain cell growth. However, since the effects of bongkrekic acid and of aurovertin were additive, other electrogenic pumps should cooperate with this pathway.

The biogenesis of mitochondrial proteins is controlled by both nuclear and mitochondrial genomes. The proteins coded by the mtDNA are subunits of enzyme complexes involved in oxidative phosphorylation. Since all these complexes also contain proteins coded by the nuclear genome, mechanisms regulating the coordinated expression and assembly of the subunits of nuclear and mitochondrial origin must exist. In yeast cells, nuclear DNA-coded components continue to be synthesized and imported into mitochondria, even when the synthesis of mtDNA-coded subunits is blocked (cf. for review, Refs. 1 and 2). The groups of Schatz and co-workers (3) and Neupert (4) have shown that a mitochondrial membrane potential is a key requirement for protein import into mitochondria (5).

* This work was supported by grants from the CNRS, the French Ministry of Education and Scientific Research (MERS), the Association Française contre les Myopathies (AFM), and the Région Rhône-Alpes. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by the French Ministry of Education and Scientific Research.

§ To whom correspondence should be addressed. Tel.: 33 04 72 44 83 56; Fax: 33 04 72 44 05 55; E-mail: godinot@univ-lyon1.fr.

The JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 36, Issue of September 4, pp. 22983–22989, 1998
Printed in U.S.A.

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available on line at http://www.jbc.org

22983

Materials—Cell culture reagents were from Life Technology Inc. except uridine, which was from Sigma. The ρ° HeLa S3 cells devoid of
Functional F1-ATPase in Mitochondrial DNA-depleted ρ° Cells

mtDNA were purified by ethidium bromide treatment of HeLa S3 cells (ρ°) (ATCC CCL2.2) by Dr. J. L. Vaysseire. The ρ° 143B TK cells were obtained from 143B TK osteosarcoma (ρ°) (ATCC CRL8305) (13). The ρ° and ρ° cells were kindly provided to us by Dr. Vaysseire and Dr. Morais. The bongkrekic acid was a generous gift of Prof. P. V. Vignais. The antisense oligonucleotides to COX II and COX III were used as previously described (14). The anti-F1-ATPase α and β subunits were purified from the clones 20D6 and 14D5 (14), and the anti-cytochrome oxidase subunit II and subunit IV were prepared from the clones: 12C4-F12 and 10G8-C12-D12, respectively, kindly provided by Dr. Taanman (15). The fluorescent molecular probe NAO was obtained from Molecular Probes. The nitrocellulose membranes were from Schleicher and Schuell. All other reagents used were of the highest quality available.

Mitochondrial COX Activity Measurement — Cells were sonicated and the protein concentrations were measured as described for immunological studies. The rate of ATP hydrolysis was measured at 37 °C according to Pullman et al. (18) by adding the cell homogenate (25 to 100 μg of proteins) to 660 μl of reaction buffer containing 50 mM Tris-Hepes, pH 8.0, 3.3 mM MgSO4, 4 mM phosphoenolpyruvate, 0.33 mM NADH, 3.3 mM ATP, 10 μg of lactate dehydrogenase, 50 μg of pyruvate kinase, and 1 μg of rotenone (to inhibit NADH oxidation by the mitochondrial NADH-ubiquinone oxidoreductase). The assay was performed in the presence or absence of one of the mitochondrial ATPase inhibitors, 3.5 μM oligomycin, 60 μM aurovertin B, or 2 mM sodium vanadate. The amount of ATPase activity that was obtained in the absence of ATPase inhibitor was defined as the activity that was related to the F1 or F0F1 complex in the cell homogenate.

Cell Growth Assays — To determine the effects of aurovertin and bongkrekic acid on cellular growth, the cells were incubated into 24-well culture plates at a density varying between 2.5 × 104 to 104 cells/well. After 24 or 48 h, the culture medium was changed, and the tested drugs were added to 60 μM aurovertin and/or 2–10 μM bongkrekic acid or 0.1–0.5 μM FCCP. The cells were grown for 5 days with a culture medium change after 3 days, when indicated. The cells released by trypsin treatment were counted by trypan blue exclusion.

Determination of Lactate Production in Culture Medium — The culture medium was collected after 5 days of cell treatment with bongkrekic acid. Proteins were precipitated with 7.5% trichloroacetic acid. The assays were centrifuged for 7 min at 1,000 × g. The supernatant fraction was extracted four times with an equal volume of diethyl ether. The lactate concentration in the deproteinized samples was estimated spectrophotometrically at 340 nm by using lactate dehydrogenase (19). Fluorescence of NAO and R123 in ρ° and ρ° Cells — The fluorescence of NAO, which is reputed to be proportional to the amount of cardiolipin and independent of the mitochondrial membrane potential, was tested in both types of cells (20). R123 fluorescence was used to estimate the mitochondrial membrane potential (21). Each cell type was distributed into 96-well culture plates at a density of 1 to 5 × 104 cells/well and incubated 24 to 48 h before fluorescence measurement. Triplicates of each cell dilution were treated with or without 30 μM aurovertin and/or 10 μM bongkrekic acid for 30 min to 24 h before R123 fluorescence measurement. FCCP (0.1 mM) was used in some experiments to determine the residual fluorescence intensity when the mitochondrial membrane potential was collapsed. The cells were washed once with Hank’s balanced salt solution and incubated for 30 min at 37 °C (without CO2) with 0.1 ml of 6.3 μM NAO or at 37 °C in a humidified atmosphere containing 5% CO2 with 1 or 10 μM R123. The cells were then washed twice with Hank’s balanced salt solution, and the medium was removed. NAO and R123 fluorescence were rapidly measured in a microplate fluorescence reader (Victor) with excitation at 485 nm and emission at 535 nm (21). It was checked that, under the tested conditions, R123 fluorescence was not modified by the presence of aurovertin, bongkrekic acid, or FCCP. A linear correlation made between the number of cells counted by trypan blue exclusion and crystal violet staining (22) permitted an estimate of the number of cells in all wells.

RESULTS

Characterization of ρ° Cells — ρ° HeLa S3 cell growth was dependent on the presence of pyruvate and uridine, as shown previously for 143B cells (13). The doubling times for ρ° and ρ° cells were 51 and 25 h, respectively, and that of ρ° and ρ° cells were 29 and 20 h. In the ρ° cells, no full-size mtDNA could be detected by Southern blotting. However, although the cytochrome b fragment could not be amplified by PCR, a fragment corresponding to the 12 S RNA could be amplified in the two ρ° cell types. This came from sequences integrated into the nuclear DNA, as shown previously (23). The ρ° cells were devoid of functional mtDNA since no mitochondrial mRNA could be revealed by reverse transcription-PCR (data not shown). The immunoblot obtained after SDS-polyacrylamide gel electrophoresis of cellular proteins transferred to nitrocellulose, incubated with antibodies, and stained (Fig. 1A) demonstrates that, as expected, the mitochondrially encoded COX II was absent from the two types of ρ° cells and present in ρ° cells. On the contrary, the nuclearly encoded COX IV and F1-ATPase β subunits were expressed both in ρ° and ρ° cells. The expression of COX IV was slightly lower in ρ° than in ρ° cells, whereas that of the F1-ATPase β subunit was similar in both types of ρ° and ρ° cells. In a parallel experiment, it was shown that the F1-ATPase α subunit was also expressed similarly in all cell types (data not shown).

ATPase Activity in ρ° Cells — The ATPase activity was tested...
in \(\rho^-\) and \(\rho^+\) cell homogenates. To differentiate the part of this activity that was due to the mitochondria from that originated from other cellular ATPases, inhibitors specific to the mitochondrial ATPase were added. The difference between the total activity and that obtained in the presence of the inhibitors corresponds to the mitochondrial F\(_0\)F\(_1\) activity. Fig. 1B shows that oligomycin, which binds to F\(_0\) (24), did not inhibit the ATPase activity of \(\rho^-\) cells devoid of the mitochondrially coded F\(_0\) subunits 6 and 8. However, oligomycin inhibited the ATPase activity of \(\rho^+\) HeLa S3 cells by 35% and that of \(\rho^+\) 143B cells by 50%. On the contrary, inhibitors such as azide (25) and aurovertin (26), which bind to F\(_1\), decreased the rate of ATP hydrolysis both in \(\rho^-\) and \(\rho^+\) cell extracts. Aurovertin was the most efficient inhibitor, decreasing ATP hydrolysis by 55% in \(\rho^+\) HeLa S3 cells and by 65% in \(\rho^-\) and \(\rho^+\) 143B cells. In addition, to test whether this aurovertin-sensitive F\(_1\)-ATPase activity depended on soluble F\(_1\) or on partly assembled F\(_0\)F\(_1\) complex, its cold sensitivity was studied. Fig. 1C and D, show that the \(\rho^-\) cell ATPase activity decreased much more rapidly at 4 °C than that of \(\rho^+\) cells and than that studied after incubation of \(\rho^+\) or \(\rho^-\) cells at 30 °C. Within 1 h at 4 °C, 50 or 60% of this activity was lost for the \(\rho^+\) HeLa S3 or \(\rho^+\) 143B cells, respectively, whereas at 30 °C, it decreased by about 10% for the \(\rho^-\) HeLa S3 cells and was almost stable for the \(\rho^-\) 143B cells. The ATPase activity was also stable for the \(\rho^+\) 143B cells at both temperatures, whereas it decreased for the first h by 25 and 10% in \(\rho^-\) HeLa S3 cells at 4 and 30 °C, respectively.

Effects of Aurovertin on \(\rho^-\) and \(\rho^+\) Cell Growth—To determine whether the functional F\(_1\)-ATPase activity could play a role in the survival of \(\rho^-\) cells, the effects of 3 nM–30 \(\mu\)M aurovertin B were tested on the growth of \(\rho^-\) and \(\rho^+\) cells. The percentage of cell growth after 5 days of aurovertin treatment was compared with control cells. The sensitivity to aurovertin was higher in \(\rho^+\) than in \(\rho^-\) cells, since 50% of cell survival was obtained at about 50 nM aurovertin for \(\rho^+\) HeLa S3 cells and about 30 \(\mu\)M for \(\rho^-\) HeLa S3 cells or between 5 and 10 \(\mu\)M for \(\rho^+\) 143B cells and above 30 \(\mu\)M for \(\rho^-\) 143B cells (not shown). Therefore, the aurovertin-induced inhibition of the mitochondrial F\(_1\)-ATPase activity decreased cell growth in \(\rho^-\) cells as well as in the parental \(\rho^+\) cells, although the aurovertin concentration exhibiting the same effect was higher in \(\rho^+\) than in \(\rho^-\) cells. In addition, the aurovertin concentration necessary to inhibit the 143B cell growth was higher than that inhibiting the HeLa S3 cells.

Mitochondrial Membrane Potential in \(\rho^-\) and \(\rho^+\) Cells—The green fluorescence of R123 was measured on both \(\rho^-\) and \(\rho^+\) cell
types to compare their mitochondrial membrane potential. The green fluorescence of NAO was measured in parallel assays to estimate the amount of mitochondrial membranes. Fig. 2 shows that the NAO fluorescence was similar in ρ° and ρ+ HeLa S3 cells (Fig. 2A) and was slightly lower in ρ° 143B than in ρ+ 143B cells (Fig. 2B). R123 fluorescence intensity shows that the mitochondrial membrane potential was of the same order of magnitude in ρ° HeLa S3 and ρ+ HeLa S3 cells (Fig. 2A). In ρ° 143B cells, it was much lower than in ρ° 143B cells, but the difference was much more important than that for NAO (Fig. 2B). In the case of HeLa S3 cells, a 30-min treatment with 0.1 μM FCCP strongly decreased the mitochondrial membrane potential of both ρ° and ρ+ cells to a similar basal level. In the case of 143B cells, FCCP strongly decreased ρ° mitochondrial membrane potential, but its effect was less important with ρ° 143B cells. However, the R123 fluorescence observed in the presence of FCCP reached a similar low level in ρ° and ρ° 143B cells. Therefore, in the absence of mitochondrial membrane potential, the fluorescence intensity obtained with the uncoupler FCCP seems to correspond to the basal fluorescence of R123 in the cells. Increasing the R123 concentration from 1 to 10 μM did not change the relative fluorescence intensity observed in any cell type. In conclusion, the mitochondrial membrane potential, as estimated by R123 fluorescence, is similar in ρ° and ρ+ HeLa cells, whereas that observed in ρ° 143B cells is only 10 to 20% that of the ρ° 143B cells. Because of the low mitochondrial membrane potential of ρ° 143B cells, the following studies involving mitochondrial membrane potential were conducted with HeLa S3 cells.

Effect of Aurovertin on ρ° and ρ° HeLa S3 Cell Mitochondrial Membrane Potential—The effects of 30 μM aurovertin were tested on the mitochondrial membrane potential of ρ° and ρ° HeLa S3 cells, as measured by R123 fluorescence. Fig. 3 shows that in ρ° and ρ° HeLa S3, a significant decrease of mitochondrial membrane potential was observed after a 30-min treatment. Similar results were obtained with a 6-h treatment (not shown). Since the cell number was not modified after a 30-min or a 6-h aurovertin treatment, the aurovertin effect on mitochondrial membrane potential is not due to a lower cell number.

Effects of Bongkrekic Acid on Cell Growth and Mitochondrial Membrane Potential—Since the respiratory chain is not functional in ρ° cells, the membrane potential must be set up by a mechanism independent of this chain. The ANT, which exchanges ADP3 against ATP4 between the two faces of the inner membrane, is likely to participate in the maintenance of this potential, as suggested for ρ° yeast cells (9). To determine whether the role played by the F1-ATPase could be mediated via the ANT, the effect of bongkrekic acid was tested on the growth and the mitochondrial membrane potential of both ρ° and ρ° cells. Fig. 4A shows that bongkrekic acid modified neither the ρ° 143B cells growth nor that of the ρ° 143B cells after 5 days of treatment at even the highest tested concentration (10 μM). On the contrary, an inhibition of about 50% ρ° HeLa S3 cell growth was observed after 5 days of treatment with 10 μM bongkrekic acid. Moreover, the inhibition could already be detected after a 3-day treatment (data not shown). In the case of the ρ° HeLa S3 cells, no effect was observed before 5 days. At that time, 10 μM bongkrekic acid inhibited the cell growth by about 30%, but a culture medium acidification was observed. If this medium was replaced after 3 days, the growth inhibition was limited to 15%. Since, in the presence of bongkrekic acid, the ATP produced by oxidative phosphorylation cannot reach the cytoplasm, the ρ° cell must then reconstitute its ATP pool via glycolysis. In this case, the NADH produced during glycolysis must be re-oxidized, and lactate should be accumulated. To verify this hypothesis, the lactate concentration was measured in ρ° and ρ° HeLa S3 cells (Fig. 4B). In the absence of bongkrekic acid, the lactate concentration (calculated per cell) was 6 times higher in ρ° than in ρ° cells, as expected since in ρ° cells the ATP production completely relies on glycolysis. The lactate concentration strongly increased in ρ° HeLa S3 cells treated with 10 μM bongkrekic acid, even if the number of cells was reduced. On the contrary, the lactate concentration decreased in ρ° HeLa S3 cells treated with 10 μM bongkrekic acid. However, the number of ρ° cells was simultaneously reduced. If the lactate amount was compared with the number of cells, it increased about 300% in ρ° cells and only 30% in ρ° cells.
brane potential was reduced after a 30-min treatment as well as after a 22-h treatment.

**Cooperative Effects of Aurovertin and Bongkrekic Acid Treatments on \( \rho^+ \) HeLa S3 Growth and Mitochondrial Membrane Potential**—To verify whether F1-ATPase and ANT act together or in two independent pathways to maintain the \( \rho^+ \) HeLa S3 cell growth and mitochondrial membrane potential, the effects of aurovertin and bongkrekic acid were tested simultaneously (Fig. 6). The presence of either 30 \( \mu M \) aurovertin or 10 \( \mu M \) bongkrekic acid decreased the cell growth by about 50\% after a 5-day treatment, whereas the simultaneous addition of bongkrekic acid and aurovertin decreased it by up to 85\% that of the control value. The \( \rho^+ \) cell growth decrease observed with aurovertin or bongkrekic acid was less extensive than that observed with 0.1 or 0.5 \( \mu M \) FCCP (decrease of 92\% of the control value). Similar to the effects observed on cell growth, the mitochondrial membrane potential was decreased more extensively in the presence of aurovertin and bongkrekic acid than when each drug was tested separately. When aurovertin concentration was increased up to 60 \( \mu M \), no additional effect was observed either on cell growth or on mitochondrial membrane potential.

![Figure 4](image1.png)

**FIG. 4.** Effects of bongkrekic acid on \( \rho^+ \) and \( \rho^0 \) cell growth. \( \rho^+ \) and \( \rho^0 \) cells were plated in 24-well plates at a density of \( 3 \times 10^4 \) and \( 10^4 \) cells/well, respectively. Each assay was performed in triplicate, and the experiments were repeated three times. The data are given as the mean of three experiments ± S.E. A, percentage of cell growth after 5 days of treatment. The number of cells present in the wells after incubation for 5 days in the presence of 2 \( \mu M \) (black bars) or of 10 \( \mu M \) (white bars) bongkrekic acid was counted and compared with that observed in the absence of inhibitor. For the \( \rho^0 \) HeLa S3 cells marked with a *, the cell culture medium was changed after 3 days of treatment. B, number of cells (bars) and lactate concentration in the culture medium of \( \rho^+ \) (gray bars) and \( \rho^0 \) (hatched bars) HeLa S3 cells after 5 days of bongkrekic acid treatment without medium change.

![Figure 5](image2.png)

**FIG. 5.** Effect of bongkrekic acid on the mitochondrial membrane potential of \( \rho^0 \) and \( \rho^+ \) HeLa S3 cells, as measured by R123 fluorescence. The cells were treated with 1 \( \mu M \) R123 for 30 min in the presence (black square) or absence (white square) of 10 \( \mu M \) bongkrekic acid. The results were expressed as a mean of three assays ± S.E. Similar data were obtained when the cells were treated with bongkrekic acid for 30 min, for 6 h, or for 24 h.

![Figure 6](image3.png)

**FIG. 6.** Effects of a simultaneous treatment with aurovertin and bongkrekic acid on \( \rho^+ \) HeLa S3 cell growth and mitochondrial membrane potential. A, percentage of \( \rho^+ \) HeLa S3 cell survival after 5 days of treatment. The percentage of cells surviving after a 5-days treatment in the presence of 30 \( \mu M \) aurovertin and/or 10 \( \mu M \) bongkrekic acid or 0.5 \( \mu M \) FCCP (hatched bars) was calculated by comparison with untreated cells. B, \( \rho^0 \) HeLa S3 mitochondrial membrane potential. The cells (\( 5 \times 10^4 \)) plated 48 h before the experiment were treated for 30 min with 30 \( \mu M \) aurovertin and/or 10 \( \mu M \) bongkrekic acid in the presence of 1 \( \mu M \) R123. The R123 fluorescence intensity was compared between cells treated with the inhibitors or cells treated with the same amount of ethanol as that present in the aurovertin solution. Data are given as the mean of three assays ± S.E.
First, our data demonstrate that \( \rho^\circ \) cells devoid of the FOF1-ATPase subunits 6 and 8 contain an active F1-ATPase essential for their growth. The size and amount of F1 \( \alpha \) and \( \beta \) subunits were similar in \( \rho^\alpha \) and \( \rho^\circ \) cells. Therefore, the \( \alpha \) and \( \beta \) subunit precursors were imported in \( \rho^\circ \) cells, and their signal sequences were normally processed in the mitochondria. Previous studies have already shown that the \( \rho^\circ \) 143B cells contained the same amount of F1 \( \beta \) subunit mRNA as that of the parent \( \rho^\alpha \) cells, whereas the relative abundance of some other transcripts encoding mitochondrial proteins such as cytochrome c, cytochrome oxidase subunit IV and VILA, and ANT 2 and ANT 3 were slightly more abundant (27). Doxycycline-induced inhibition of mitochondrial protein synthesis in human leukemia cells also decreased the contents of complex III or IV subunits of nuclear origin without modifying that of the F1-ATPase \( \alpha \) and \( \beta \) subunits during two culture generations (10). The authors suggest that respiratory chain complex subunits of nuclear origin were rapidly degraded when they were not assembled. In the case of complex III subunits, the partial assembly of sub-complexes has been demonstrated in yeast mitochondria (28). In the two human \( \rho^\circ \) cell lines studied here, the cytochrome oxidase subunit IV amount was only slightly decreased after many culture generations. This suggests that at least some of the cytochrome oxidase subunits of nuclear origin can be imported without being degraded. In the case of the ATPase, the normal amount of the \( \alpha \) and \( \beta \) subunits suggested that F1 could be assembled and remain stable.

The ATPase activity measured in the human \( \rho^\circ \) cells was due to this mitochondrial F1-ATPase. Indeed, it was aurovertin-sensitive (26). On the contrary, since oligomycin sensitivity (24) depends on mtDNA-encoded subunit 6 (29), only \( \rho^\circ \) cells were inhibited by oligomycin. Therefore, F1 was assembled and active in human \( \rho^\circ \) cells despite the absence of subunits 6 and 8. It was, however, not efficiently associated with the subunits constituting the stalk linking F1 to F0 since the ATPase activity was cold-labile. Indeed, it has been shown in beef heart F1 that the binding of isolated F1 to OSCP (30, 12) or to other subunits constituting the stalk such as subunits b, F6, and d (12) decreases F1 sensitivity to cold exposure. Therefore, the cold-sensitivity of the F1-ATPase in human \( \rho^\circ \) cells indicates that at least some of the stalk subunits are deficient. However, F1 might be bound to the membrane. Indeed, a binding site for F1 on F0 in the absence of F6 and OSCP have been disclosed in the beef enzyme (31).

A similar assembly of the F1 subunits in a cold-labile and oligomycin-insensitive H\(^{-}\)-ATPase has been reported in yeast \( \rho^\circ \) cells. In addition, this F1 H\(^{-}\)-ATPase activity was modulated by the insaturation degree of the mitochondrial membrane phospholipids, and therefore F1 was membrane-bound (32). In yeast, the mtDNA codes not only for the subunits 6 and 8 as in human but also for the DCCD-binding protein. Therefore, a fortiori, if F1 was membrane-bound in yeast, this must also be true in human \( \rho^\circ \) cells that might contain the DCCD-binding protein. Although the pathway of the F0F1 assembly has not been extensively studied in mammals, many experiments have been performed using yeast mutants lacking one or several F0F1 subunits. It was reported that, in yeast, subunit 6 was not essential for the binding of F1 subunits to components of the F0 factor (33). The assembly of F0F1 subunits involved the sequential addition of subunits 9 (DCCD-binding protein), 8, and 6 to a membrane-bound F1, and two other proteins, subunits b and OSCP, were not found in the complex when the F0 sector was not properly assembled (34, 35). However, the b subunit could be bound to a subunit 6-deficient mutant (36), and a tight binding of F1 to the membrane required the presence of the d subunit (37). If the same assembly pathway is true for human \( \rho^\circ \) cells, F1 should bind to the membrane after insertion of the d subunit, and the presence of F1 would permit the insertion of the DCCD-binding protein and eventually that of the b subunit. OSCP, if present, might not be efficiently associated with the membrane since the subunit 6 is absent. This would explain the cold sensitivity of the human \( \rho^\circ \) cell ATPase activity.

Interestingly, this ATPase activity was essential to maintain the human \( \rho^\circ \) cell growth. Indeed, aurovertin-induced inhibition of the F1-ATPase activity strongly decreased \( \rho^\circ \) cell growth. The effect was, however, lesser for the 143B cell lines than for the HeLa S3 cell lines, whereas the inhibition of the ATPase activity by aurovertin was of the same order of magnitude in both cell lines. This is probably related to metabolic adaptation of these two types of cancer cells. It cannot be excluded that, in the 143B cells, some multidrug resistance could have been developed (cf. Ref. 38, for review). This could reduce the intracellular steady-state levels of various drugs added to the extracellular medium. Indeed, the cell growth sensitivity to aurovertin or to bongkrekic acid was lower in 143B than in HeLa S3 cells. However, aurovertin decreased cell growth in both \( \rho^\circ \) cell lines. The sensitivity to aurovertin was higher in \( \rho^\circ \) than in \( \rho^\circ \) cells. This is probably due to the fact that, during the ethidium bromide treatment used to transform the \( \rho^\circ \) into \( \rho^\circ \) cells, the cells must have induced an alternative pathway to improve their ATP production via the glycolysis. This is demonstrated by the higher lactate production observed in \( \rho^\circ \) than in \( \rho^\circ \) cells in the absence of inhibitor. In \( \rho^\circ \) cells, oxidative phosphorylation cannot produce ATP to sustain cell growth, and therefore, the glycolytic flux must be increased to provide the ATP necessary to meet the cell energy demands. Simultaneously, the NADH produced during glycolysis must be reoxidized via, for example, the lactate dehydrogenase, which increases lactate concentration.

In \( \rho^\circ \) HeLa S3 cells, the mitochondrial membrane potential, as measured by R123 fluorescence, reached the same level as that of \( \rho^\circ \) HeLa S3 cells. The existence of a normal mitochondrial membrane potential in other \( \rho^\circ \) cells has already been observed previously. Rat hepatoma \( \rho^\circ \) cells (9) as well as human \( \rho^\circ \) HeLa cells (39) took up R123 to the same extent as the parent cells. On the contrary, \( \rho^\circ \) 143B cells show R123 fluorescence intensity, which is only 10 to 20% that of \( \rho^\circ \) 143B cells. This effect could be related to an adaptive difference between \( \rho^\circ \) HeLa S3 cells and \( \rho^\circ \) 143B cells. It is possible that the \( \rho^\circ \) 143B cells effectively possess only a low mitochondrial membrane potential, which would, however, be sufficient for their growth. In such a case, a mitochondrial membrane potential corresponding to only 10 to 20% of the normal mitochondrial membrane potential would be large enough to maintain cell viability and, hence, to sustain protein import into the mitochondria. The low mitochondrial membrane potential could explain why the \( \rho^\circ \) 143B cells are easily damaged; for example, contrary to the \( \rho^\circ \) HeLa S3 cells, which are as resistant as the \( \rho^\circ \) cells, \( \rho^\circ \) 143B cells cannot survive a mild centrifugation. One cannot however exclude that the 143B cells developed a secondary resistance to drugs during the ethidium bromide treatment used to produce the \( \rho^\circ \) cells from the \( \rho^\circ \) ones. It would mean that the mitochondrial membrane potential measured in these \( \rho^\circ \) 143B cells excluding R123 could be artificially lower than it is in reality.

Aurovertin simultaneously reduced the \( \rho^\circ \) HeLa S3 cell growth rate and their mitochondrial membrane potential estimated by R123 fluorescence, as it does for \( \rho^\circ \) HeLa S3 cells. The mitochondrial membrane potential, as well as the growth of \( \rho^\circ \) HeLa S3 cells was also reduced by bongkrekic acid. On the
contrary, bongkrekic acid did not modify the ρ° HeLa S3 mitochondrial membrane potential and barely decreased their growth under the tested conditions. However, bongkrekic acid efficiently inhibited ANT in the ρ° cells since bongkrekic acid induced lactate overproduction and therefore shifted energy production from oxidative phosphorylation to glycolysis. The simplest explanation for the simultaneous sensitivity of the ρ° HeLa S3 to bongkrekic acid and aurovertin is that, in ρ° cells, the mitochondrial membrane potential was generated by the ANT exchanging ATP4° for ADP3° produced by an active F1. The presence of aurovertin reduced the rate of ADP3° growth under the tested conditions. However, bongkrekic acid did not modify the mitochondrial membrane potential was generated by the ANT was inhibited, other electrogenic pumps, such as that involving PPi (40), could in part maintain this membrane potential (41). Our observations are consistent with the import of mitochondrial proteins of nuclear origin (3). When the ANT was inhibited, ATP could be hydrolyzed by other mitochondrial ATP-consuming processes, as for example, those involved in the import of mitochondrial proteins of nuclear origin (3). When the ANT was inhibited, other electrogenic pumps, such as that involving PPi (40), could in part maintain this membrane potential whether or not calcium movements also participate in this mechanism (41). Our observations are consistent with those reported by Giraud and Velours (42) showing that ρ° Saccharomyces cerevisiae cells lacking the F₁ δ subunit exhibit a slow growth phenotype and a membrane potential decrease comparable with that described here for aurovertin-treated ρ° cells. In conclusion, an active F₁-ATPase is mandatory for the maintenance of mitochondrial membrane potential essential for human ρ° cell growth.

Acknowledgments—The authors thank Dr. Vayssière and Dr. Morais for providing the ρ° and ρ° cells and Dr. Taanman for his generous gift of monoclonal antibodies against the cytochrome oxidase subunits II and IV.

REFERENCES

1. Tzagoloff, A., and Myers, A. M. (1986) Annu. Rev. Biochem. 55, 249–285
2. Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333
3. Suzuki, C. K., Rep, M., Maarten van Dijl, J., Suda, K., Grivell, L. A., and Schatz, G. (1997) Trends Biochem. Sci. 22, 118–123
4. Neupert, W. (1997) Annu. Rev. Biochem. 66, 863–917
5. Gasser, S. M., Daum, G., and Schatz, G. (1982) J. Biol. Chem. 257, 13034–13041
6. Anderson, S., Banquier, A. T., Barrel, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Rose, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981) Nature 290, 457–465
7. Herzberg, N. H., Middelkoop, E., Ador, M., Dekker, H. L., Van Galen, M. J., Van den Berg, M., Bolhuis, P. A., and Van den Bogert, C. (1993) Eur. J. Cell Biol. 61, 405–408
8. Kolarov, J., and Klingenberg, M. (1974) FEBS Lett. 45, 320–323
9. Martinus, R. D., Garth, G. P., Webster, T. L., Cartwright, P., Naylor, D. J., Hoj, P. B., and Hogenraad, N. J. (1986) Eur. J. Biochem. 240, 88–103
10. Nijtmans, L. G. J., Speckens, M. A. M., Zonneveld, P., and Van den Bogert, C. (1995) Biochim. Biophys. Acta 1265, 117–126
11. Boyer, P. D. (1977) Annu. Rev. Biochem. 46, 717–749
12. Collinson, I. R., van Raai, M. J., Runswick, M. J., Pearnley, I. M., Skehel, J. M., Orris, G. L., Miroux, B., and Walker, J. E. (1994) J. Mol. Biol. 242, 408–421
13. Morais, R., Zinkiewicz-Peotti, K., Parent, M., Wang, H., Babai F., and Zollinger, M. (1994) Cancer Res. 54, 3889–3896
14. Moradi-Améli, M., and Godinot, C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6167–6171
15. Taanman, J. W., Burton, M. D., Marusich, M. F., Kennaway, N. G., and Julien, R. (1997) Annu. Rev. Biochem. 66, 1099–1126
16. Bouzidi, M. F., Carrier, H., and Godinot, C. (1996) Biochim. Biophys. Acta 1317, 199–209
17. Kojuri, S., and Godinot, C. (1997) Mol. Cell. Biochem. 171, 113–125
18. Pullman, M. E., Penefsky, H. S., Datta, A., and Raaber, E. (1960) J. Biol. Chem. 235, 3322–3329
19. Bergmeyer, H. U. (1973) Methods of Enzymatic Analysis, pp. 1464–1468, Springer Verlag, Berlin
20. Petit, J. M., Huet, O., Gallet, P. F., Maftah, A., Ratinaud, M. H., and Julien, R. (1994) Eur. J. Biochem. 220, 871–879
21. Chen, L. B. (1989) Methods Cell Biol. 29, 103–123
22. Saito, K., Oka, T., Ata, N., Miyashiro, H., Hattori, M., and Saiki, I. (1997) Biol. Pharm. Bull. 20, 345–348
23. Tsvankin, T., Nomiyama, H., Setoyama, C., Maeda, S., and Shimada, K. (1983) Gene 25, 223–229
24. Lardy, H. A. (1969) Annu. Rev. Biochem. 38, 991–1034
25. Linnett, P. E., and Beechey, R. B. (1979) Methods Enzymol. 55, 472–518
26. Li, K., Neuffer, P. D., and Williams, R. S. (1995) J. Am. J. Physiol. 269, C1265–C1270
27. Gough, L. (1989) Eur. J. Biochem. 182, 477–493
28. Avner, P. R., and Griffiths, D. E. (1973) Eur. J. Biochem. 32, 312–321
29. Hundal, T., Norling, B., and Ernst, L. (1984) J. Bioenerg. Biomembr. 16, 535–550
30. Sandri, G., Wojtczak, L., and Ernst, L. (1985) Arch. Biochem. Biophys. 229, 595–602
31. Orian, J. M., Hadikusumo, R. G., Marruzi, S., and Linnane, A. W. (1984) J. Bioenerg. Biomembr. 16, 223–231
32. Choo, W. M., Hadikusumo, R. G., and Marruzi, S. (1985) Biochim. Biophys. Acta 806, 290–304
33. Hadikusumo, R. G., Metzler, S., Choo, W. M., Jean-François, M. J., Linnane, A. W., and Marruzi, S. (1988) Biochim. Biophys. Acta 933, 212–222
34. Marruzi, S., Watkins, L. C., and Choo, W. M. (1989) Biochim. Biophys. Acta 975, 222–230
35. Paul, M. F., Velours, J., Arselin de Chaeteboudeau, G., Aigle, M., and Guérin, B. (1989) Eur. J. Biochem. 185, 163–171
36. Norais, N., Prone, D., and Velours, J. (1991) J. Biol. Chem. 266, 16541–16549
37. Gottschalk, M. M., Pastan, I., and Ambudkar, S. V. (1996) Curr. Opin. Genet. Dev. 6, 610–617
38. Skowronek, P., Haferkamp, O., and Rödel, G. (1992) Biochem. Biophys. Res. Commun. 187, 991–996
39. Pereira-da-Silva, L., Sherman, M., Lundin, M., and Balschweifsky, H. (1993) Arch. Biochem. Biophys. 304, 310–313
40. Griffiths, E. J., and Halestrap, A. P. (1993) Biochem. J. 290, 489–495
41. Giraud, M., and Velours, J. (1997) Eur. J. Biochem. 245, 813–818