Environmentally induced differential amplification of mitochondrial populations

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Resistance to the drug rutamycin, an inhibitor of mitochondrial ATPase, has been shown to be cytoplasmically inherited in a mouse fibroblast line (TL) on fusion of the cytoplasm (enTL) with a nucleated recipient A9 [Lichter & Getz (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 324–328]. The cytoplasmic hybrid (cybrid) so formed may be readily grown in the presence [CY(+)] or absence [CY(–)] of rutamycin. The ATPase of TL mitochondria is similarly resistant to rutamycin whether grown in the presence or absence of antibiotic. The ATPase of CY(+) mitochondria is resistant to rutamycin, but CY(–) mitochondrial ATPase is sensitive to rutamycin. Nevertheless, CY(–) can be readily grown in rutamycin after a brief lag. The pH optima of mitochondrial ATPase are 8.0 for A9 and CY(–) cells and 7.5 for TL cells, whereas the pH optimum for CY(+) spans the optima of A9 and TL. The TL mitochondrial NADH–cytochrome *c* reductase is resistant to rotenone, whereas that of A9 mitochondria is sensitive to this agent. CY(–) and CY(+) mitochondria are sensitive and resistant respectively to rotenone. Growth of cybrids in rutamycin for 2 weeks results in a 2–3-fold increase in mitochondrial mass, measured on the basis of electron microscopic morphometry, mitochondrial membrane enzyme assays, mass of cardiolipin, and quantification of mitochondrial DNA. These data suggest that the cybrid harbours two populations of mitochondria and that the proportions of the two populations dramatically influence morphology, growth and mitochondrial phenotype in the cybrid. Selective pressure appears to induce these changes through the differential amplification of mitochondria.

INTRODUCTION

Mammalian cells are estimated to contain several hundred copies of the mitochondrial genome per cell (Bogenhagen & Clayton, 1974; Robin & Wong, 1988). Errors in replication may result in heterogeneity in mitochondrial populations. Homogeneity of mitochondrial populations in yeast is maintained by mitochondrial segregation. However, there is some clinical evidence for mitochondrial heterogeneity in disease states in humans (Darley-Usmar *et al.*, 1986; DiMauro *et al.*, 1987).

Heterogeneity in mitochondrial populations can be created experimentally by the formation of hybrids and cytoplasmic hybrids (cybrids). Hybrids, and to a lesser extent cybrids, have been used to examine the patterns of segregation or maintenance of parental mitochondrial DNA. Although the factors influencing mitochondrial segregation are not well understood, a few studies have demonstrated the maintenance of two mitochondrial populations within cloned cybrid cell lines (Bunn *et al.*, 1977; Wallace, 1981; Oliver & Wallace, 1982; Hayashi *et al.* 1984; Kearsey *et al.*, 1985). However, little attention has been paid to the potential for inducing differential expression in cells containing distinct mitochondrial populations.

A mouse fibroblast line, TL, has been described (Lichter & Getz, 1978) which is resistant to the drugs rutamycin and rotenone and is respiratory-deficient due to a defect in Complex I. Resistance to rutamycin, an inhibitor of ATPase, and the respiratory deficiency have been shown to be cytoplasmically inherited on fusion of the cytoplasm, enTL, with a nucleated mouse recipient, A9 (Lichter *et al.*, 1979). The cytoplasmic hybrid so formed may be readily grown in the presence [CY(+)] or absence [CY(–)] of rutamycin. Such a cybrid, with little or no incompatibility between nucleus and mitochondria or between mitochondria themselves, provides a system in which the in-

tracellular dynamics of mitochondrial populations can be followed. We examined the effect of prolonged culture in the presence or absence of selective pressure on mitochondrial populations in the cybrid and on the cybrid cell phenotype in order to determine whether the selective pressure differentially amplifies mitochondrial populations.

MATERIALS AND METHODS

Cells

Clone 1D (C1D) is a mouse L cell line lacking cytoplasmic thymidine kinase but retaining the mitochondrial form of that enzyme (Kit *et al.*, 1963). This deficiency provides an opportunity to mutagenize the mitochondrial genome through the selective incorporation of 5-bromodeoxyuridine into the mitochondrial DNA. Mutagenesis is enhanced by ultraviolet irradiation. Using this strategy, rutamycin-resistant cell lines were obtained from C1D cells after growth in rutamycin (Lichter & Getz, 1978). The mutant line thus obtained (TL) is resistant to rutamycin and to rotenone, and is respiratory deficient due to a defect in Complex I activity. (Lichter *et al.*, 1979) The mutant cell line TL and the cybrid enTL × A9 (CY) were subcloned using the method of limiting dilution. The experiments reported below were performed with TL clones 1, 2 and 4, and with enTL × A9 clones 1, 2 and 5.

Cells were maintained at 37 °C and 5% CO₂ in RPMI 1640 medium containing 10% fetal calf serum, 2 mM-L-glutamine, 100 units of penicillin/ml and 0.1 mg of streptomycin/ml. Medium for TL and cybrid was supplemented with 3 g of sodium bicarbonate/l. TL passaged in 1 µg of rutamycin/ml is referred to as TL(+), and CY passaged in 1 µg of rutamycin/ml is referred to as CY(+), in contrast with the same subclones maintained in

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drug-free media for at least 6 months, which are referred to as TL(-) and CY(-). Rutamycin (Eli Lilly Co.) was dissolved in dimethyl sulphoxide, and the final concentration of the solvent in growth media was 0.1%.

Plating efficiencies of A9, CY(+), CY(-), TL(+) and TL(-) were determined in normal media and in various concentrations of rutamycin. A total of 100 cells were plated in 40 mm² culture dishes. The medium was changed every 3 days. After 10 days, the dishes were rinsed with 0.85% NaCl, pH 7.5, stained with saturated Crystal Violet in 0.85% NaCl, and colonies were counted.

Preparation and characterization of mitochondria

Cells were harvested with 0.05% trypsin/EDTA solution, washed twice with medium containing serum, and homogenized in hypo-osmotic buffer (910 mM-NaCl, 1.5 mM-MgCl₂, 10 mM-Tris, pH 7.5), and the mitochondria were isolated from a discontinuous sucrose gradient (Bogenhagen & Clayton, 1974). NADH-cytochrome *c* reductase activity was measured spectrophotometrically as reduction of cytochrome *c* at 550 nm at room temperature using between 5 and 20 µg of protein. Succinate cytochrome *c* reductase activity was assessed at 37 °C, using 25–40 µg of protein, as previously described (Lichter *et al.*, 1979). In order to assess the relative contributions of the rotenone-sensitive and -insensitive NADH-cytochrome *c* reductase pathways, both intact mitochondria and submitochondrial particles were examined (Sottocasa *et al.*, 1967; Lichter *et al.*, 1979). Cytochrome *c* oxidase activity was measured spectrophotometrically at room temperature at 550 nm (Cooperstein & Lazarow, 1951). A coupled enzyme assay was employed for the determination of ATPase activity in which the ADP formed drives the oxidation of NADH and the formation of lactate (Pullman *et al.*, 1960). The oxidation of NADH was measured at 340 nm with a blue filter at room temperature. Cardiolipin mass was determined by t.l.c. of the lipids extracted from cells that had been equilibrium-labelled for 4 days in medium containing 3 µCi of ³²P_i/ml (Getz *et al.*, 1970).

Quantification of mitochondrial DNA

Escherichia coli strain c600r-m- containing the plasmid pAM1, which carries the entire mouse mitochondrial genome (Morten & Clayton, 1979), was grown in Difco antibiotic medium 3 (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 25 mg of ampicillin/l and 33 mg of kanamycin/l. The plasmid DNA was isolated by the alkaline lysis procedure (Birnboim & Doly, 1979). Purified plasmid DNA was digested with *Hae*II restriction endonuclease and the insert was separated by electrophoresis (Maniatis *et al.*, 1982). The separated DNA fragments were revealed under u.v. light and slices containing the appropriate fragments were cut out of the gel. The entire mouse mitochondrial DNA 16.295 kb fragment was electro-eluted from the gel slices and precipitated with absolute ethanol, and labelled with [α -³²P]dCTP by the method of random prime synthesis (Feinberg & Vogelstein, 1983). The labelled probe was then purified on a Bio-Gel A-0.5 m column, 200–400 mesh (Bio-Rad Laboratories, Richmond, CA, U.S.A.) to a specific radio-activity of (0.5–1.0) × 10⁸ c.p.m./µg of DNA. Labeled probe (10⁷ c.p.m.) was hybridized to a dot-blot of total DNA from the cell lines.

Total cellular DNA was isolated from cell lines, treated with RNAase A at 37 °C for 3 h and extracted with phenol and chloroform (Maniatis *et al.*, 1982). A range of concentrations of cellular DNA up to 4 µg were loaded on to a Gene Screen membrane (New England Nuclear, Boston, MA, U.S.A.) in a dot-blot apparatus (Bio-Rad) (Kafatos *et al.*, 1979). The DNA was baked on to the membrane for 2 h at 80 °C. The membrane was then prehybridized with constant agitation at 42 °C overnight

with a 10 ml solution containing 50% formamide, 0.3 M-NaCl, 30 mM-sodium citrate, 50 mM-sodium phosphate (pH 6.5), 0.02% BSA (Pentax fraction V), 0.02% Ficoll and 100 µg of denatured salmon sperm DNA/ml. This solution was replaced with 5 ml of the same solution containing the denatured radioactive 16.295 kb probe and hybridized overnight at 42 °C (Southern, 1975; Wahl *et al.*, 1979). The solution was removed and the membrane was washed, dried and autoradiographed at -70 °C with intensifying screens for 0.5–2 h.

Fig. 1. Cell growth as a function of previous selective pressure

Cell lines were plated in equal numbers in drug-free medium and then half of the flasks were changed to medium containing 0.5 µg of rutamycin/ml on day 0. ○, Cells grown in drug-free medium; ●, cells grown in drug-containing medium. Cells were harvested and counted on the days indicated. The cell number is the average number of cells in each of three flasks. The rutamycin-resistant mutant TL (c and e) and the cybrid enTL × A9 (d and f) were examined after passage in drug-free medium (c and d), and after passage in 1 µg of rutamycin/ml (e and f). Growth of the mutant and the cybrid are compared with growth of their normal respective parental cell lines C1D (a) and A9 (b).

DNA dot-blots were quantified spectrophotometrically at 500 nm after eluting the silver grains from film slices (Suijsa, 1983). Agarose-gel-purified total mouse mitochondrial DNA (Maniatis *et al.*, 1982) from pAM1 plasmid cut with *Hae*II (0.5–4.0 ng) was used to construct a standard curve for each dot-blot. These standard DNA samples were processed as described above. The plot of absorbance versus DNA was linear up to 40 ng of DNA and was used to measure cellular mitochondrial DNA.

Electron microscopy

Cells were fixed for electron microscopy in the dish on which they were grown (Lucky *et al.*, 1975). After dehydration, the cells were infiltrated with Polybed 812 (Polysciences EPON replacement; Polysciences Inc., Warrington, PA, U.S.A.). Small pieces were cut from the hardened disc, mounted on a resin cylinder and sectioned on a MT-2B microtome with a diamond knife. Sections were collected on 1 mm × 2 mm single-slot Formbar covered grids, stained for 7 min in both uranyl acetate and lead citrate and examined with a Philips 201 electron microscope at 60 kV. The mitochondrial inner membrane length per cell was determined stereologically (Weibel, 1974). A minimum of 24 cells from each cell line were selected randomly and examined at low magnification (×7320) to determine the cellular area. The mitochondrial inner membrane length per cell was then determined at high magnification (×24400 and 36600) from a minimum of 12 electron micrographs per cell line (Steer, 1981). Statistical analysis was performed using Student's *t* test.

RESULTS

Growth of cells

The response of the cybrid enTL × A9 to rutamycin was initially assessed by comparing its growth in the presence and absence of rutamycin (Fig. 1). The cybrid and the rutamycin-resistant mutant, TL, maintained either in drug-free medium (Figs. 1c and d) or with continuous selective pressure [CY(+)] and [TL(+)] (Figs. 1e and 1f) were compared. C1D, TL(−) and CY(−), the cell lines maintained in drug-free media, exhibited the fastest growth rates. Relative to these growth rates, rutamycin inhibited the growth of both the mutant and the cybrid. When CY(−) was exposed to rutamycin there was an initial lag period after which growth proceeded at a rate comparable with that of the cybrid growing in the absence of the drug (Fig. 1d). The normal cybrid parental cell line A9 (Fig. 1b), although sensitive to rutamycin, was somewhat more resistant than C1D (Fig. 1a). However, growth of A9 was markedly inhibited after more than 4 days in the presence of rutamycin (results not shown).

The plating efficiency of the cell lines was examined to determine whether the lag in growth of CY(−) when exposed to rutamycin was attributable to the selection of a subpopulation of rutamycin-resistant cybrid cells (Fig. 2). Although the colonies formed by CY(−) cells were smaller than those of CY(+), the plating curve demonstrates that CY(+) and CY(−) have virtually identical resistance to rutamycin. There was also no significant difference in the plating efficiencies of TL(+) and TL(−) (results not shown). The degree of rutamycin resistance of the cybrid was intermediate between those of A9 and TL.

Although there was no difference between CY(+) and CY(−) in plating efficiency, growth in rutamycin did induce a change in the appearance of the cybrid. A9 and CY(−) are small, slightly rounded cells, but 2 days after the application of selective pressure the cybrid had assumed the angular, elongated appearance of TL cells (Fig. 3). The appearance of TL did not change in the absence of selective pressure (results not shown).

Fig. 2. Plating efficiency in rutamycin

A total of 100 cells was plated in normal medium or medium containing various concentrations of rutamycin. Cybrid cells passaged in the absence of selective pressure [CY(−), △] were compared with cybrid cells passaged in medium containing 1 μg of rutamycin/ml [CY(+), ×] and with the cybrid parental lines A9 (■) and TL (○).

Fig. 3. Cell morphology as a function of previous selective pressure

Cells were plated at equal density and photographed after 2 days in their respective media. (a) A9, (b) TL(+), (c) CY(−), (d) CY(+). Magnification ×57.

Mitochondrial ATPase

The effects of selective pressure on isolated mitochondria from each cell line were assessed by examining the sensitivity of mitochondrial ATPase activity to rutamycin (Fig. 4). The mito-

Fig. 4. Sensitivity of ATPase activity to rutamycin

ATPase activity from isolated mitochondria was assessed spectrophotometrically. All points represent the averages of two assays done with duplicate samples. The ATPase specific activity (μmol of P_i /min per mg of protein) for the cell lines was: 0.117 ± 0.015 for TL(+), 0.132 ± 0.014 for TL(-), 0.237 ± 0.014 for A9 (Δ), 0.137 ± 0.017 for CY(+), and 0.203 ± 0.007 for CY(-) ($\blacktriangle$).

chondrial ATPase of the cybrid grown without rutamycin for many generations displayed a sensitivity to rutamycin that was almost identical to that of A9, the parent recipient in the formation of the cybrid. On the other hand, the mitochondria of the cybrid grown in the presence of rutamycin were highly resistant to the antibiotic. This suggests that the bulk of the mitochondria in the cybrid grown without antibiotic are the mitochondria of A9, but that there is a small population of rutamycin-resistant mitochondria which can relatively rapidly repopulate the cytoplasm in the presence of selective pressure. The differences in the response to rutamycin of mitochondria isolated from cybrid cells grown in the presence or absence of the drug could also be due to the carry-over of significant amounts of antibiotic during the preparation of mitochondria from cells containing rutamycin; however, the inhibition profiles, when taken together, do not appear to be consistent with the latter interpretation (Fig. 4). Growth of TL cells in the presence or absence of rutamycin made relatively little impact on the sensitivity of the TL mitochondrial ATPase to the rutamycin.

Since the likely explanation for the change in the resistance profile of the cybrid is an alteration in the mitochondrial population, we examined the mitochondria of the wild-type parents, the mutant and the cybrid in more detail. The characteristics of the mitochondrial ATPase and the mitochondrial NADH-cytochrome *c* reductase, the mitochondrial membranes, the total cellular and mitochondrial proteins, and the mitochondrial DNA were studied.

ATPase pH profile

Inhibition of ATPase activity by rutamycin was assessed at various rutamycin concentrations and at different pH values in mitochondria isolated from TL and C1D cells (Fig. 5a). Although the inhibition of ATPase activity was more pronounced in C1D than in TL cells, the inhibitory effect of rutamycin was most

Fig. 5. ATPase activity pH profiles of isolated mitochondria as a function of previous selective pressure

Profiles over selected pH ranges are shown for mitochondrial ATPase from (a) C1D (■) and TL(+) (○), (b) A9 (■) and TL(-) (○), and (c) CY(-) (■) and CY(+) (○). All points represent the averages of assays done in triplicate.

marked at the ATPase pH optimum, which is distinct for each cell line. The optimum pH for the ATPase of parental (C1D) mitochondria was 8.0, whereas that for TL mitochondria was 7.5. The pH optima were then compared over a limited range for isolated mitochondria from each of the cell lines as a function of previous rutamycin exposure. The mutant grown in the absence of drug, TL(-), exhibited the same pH optimum as when it was grown in rutamycin, although there was a smaller decline of activity with increasing pH (Fig. 5b). Mitochondria from A9 cells have the same pH maximum as those from C1D cells (Fig. 5b). The profile for CY(-) was similar to that of A9, the parent recipient (Fig. 5c). However, the profile for CY(+) resembled a combination of the pH profiles of TL and A9 mitochondria, probably reflecting a mixture of mitochondria derived from each of the parent lines.

Table 1. NADH-cytochrome *c* reductase activity in submitochondrial particles

Total activity represents the average of at least two determinations.

Cell line	NADH-cytochrome <i>c</i> reductase activity (μ mol of cytochrome <i>c</i> reduced/min per mg of protein)	
	Rotenone-insensitive	Rotenone-sensitive
C1D	6.8	4.8
TL(–)	11.0	1.6
TL(+)	10.4	2.1
A9	6.7	14.0
CY(–)	6.9	9.1
CY(+)	12.4	2.0

NADH-cytochrome *c* reductase

To demonstrate more clearly the change in the mitochondrial population in the cybrid with selective pressure, we examined the resistance of NADH-cytochrome *c* reductase activity to rotenone. In addition to its resistance to rutamycin, the mutant cell line TL is resistant to rotenone, an inhibitor of NADH-cytochrome *c* reductase, and is deficient in Complex I activity (Lichter *et al.*, 1979). Rotenone-sensitive NADH-cytochrome *c* reductase activity is a mitochondrial inner membrane enzyme of the electron transport chain, whereas rotenone-insensitive NADH-cytochrome *c* reductase activity is an outer membrane marker not associated with the respiratory chain (Sottocasa *et al.*, 1967). Therefore rotenone resistance of the inner membrane enzyme is a marker of TL mitochondria and reflects a biochemical difference which is probably not directly affected by the selective agent rutamycin.

Rotenone-sensitive and -insensitive NADH-cytochrome *c* reductase activity was determined in isolated mitochondria and submitochondrial particles from the parent cells A9 and TL, as well as from CY(–) and cybrid grown for 2 weeks in rutamycin (Table 1). The percentage of NADH-cytochrome *c* reductase activity in CY(–) that was rotenone-insensitive was 39%, which was similar to normal parent A9 cells, in which 34% of the activity was rotenone-insensitive. However, after only 2 weeks in rutamycin, the profile of NADH-cytochrome *c* reductase activity

for the cybrid had changed, and resembled the mutant parent TL: the total NADH-cytochrome *c* reductase activity was markedly decreased, and 86% of the activity was rotenone insensitive. Growth in the presence or absence of rutamycin had little effect on NADH-cytochrome *c* reductase activity in the mutant TL cell line.

Mitochondrial proteins and DNA

The total cellular proteins of C1D and TL cells were examined in the presence of graded concentrations of rutamycin. No qualitative differences were apparent between the cell lines (results not shown). Although there were differences in the relative amounts of several proteins in the two cell lines at given rutamycin concentrations, this probably reflects the varying sensitivity of the synthesis of individual proteins to deprivation of mitochondrial ATP. Labelled proteins were also isolated from gradient-purified mitochondria and examined by two-dimensional gel electrophoresis. This analysis demonstrated that the same spectrum of proteins was present in all cell lines (results not shown).

The restriction endonuclease digestion of wild-type, mutant and cybrid mitochondrial DNA revealed no differences with the enzymes *HincII*, *XbaI*, *HpaII* and *HinfI* (results not shown).

Mitochondrial mass

We examined the mitochondrial mass in the cybrid to see if the change in the proportions of cybrid mitochondria induced by selective pressure is accompanied by a change in mitochondrial mass. Several parameters of mitochondrial mass were measured: electron microscopic morphometry, mitochondrial membrane enzyme assays, mass of cardiolipin, and quantification of mitochondrial DNA (Table 2). We have previously shown that the mitochondria of TL cells are swollen and less dense than the mitochondria in A9 cells (Lichter *et al.*, 1979). CY(–) mitochondria are similar in appearance to those from A9 cells, with only an occasional swollen mitochondrion. In contrast, almost all CY(+) mitochondria resemble those seen in TL cells (Fig. 6). The cross-sectional length of the mitochondrial inner membrane (including cristae) per cell for A9 and TL was $85.4 \pm 18.2 \mu\text{m}$ and $114.8 \pm 43.5 \mu\text{m}$ respectively, whereas the length of the mitochondrial inner membrane of CY(–) was $65.0 \pm 2.3 \mu\text{m}$, compared with $210.4 \pm 44 \mu\text{m}$ for the cybrid cultured in drug for 2 weeks. Cross-sectional inner membrane length was measured rather than area so as to avoid any bias due to mitochondrial

Table 2. Estimation of mitochondrial mass as a function of selective pressure

The mitochondrial mass was estimated in the cybrid cell line, enTL $\times$ A9, passaged for a prolonged period in the presence [CY(+)] and absence [CY(–)] of selective pressure, and compared with that in the parent cell lines, TL and A9. Mass parameters for TL(+) and TL(–) did not differ significantly. The cell lines were plated at approximately equal density and harvested 14 days later for analysis. Mitochondrial mass was determined enzymically from the specific activity of the cell homogenate per cell mass/specific activity of the isolated mitochondria, from the average of four determinations from each of six separate experiments. Cardiolipin was determined in cells labelled for 4 days with $3 \mu\text{Ci}$ of $^{32}\text{P}_i/\text{ml}$. The length of the mitochondrial inner membrane/cell is the average of morphometric measurements on at least 12 electron micrographs per cell line. The values for mitochondrial DNA represent the means $\pm$ S.D. of 36 dilutions per cell line for each of three different blots.

Cell line...	CY(–)	CY(+)	TL	A9
Cytochrome oxidase (mg of mitochondrial protein/ 10^{10} cells)	550 ± 100	1137 ± 269	402 ± 158	430 ± 105
Cardiolipin (c.p.m./ 10^6 cells)	719	1074	724	755
Mitochondrial inner membrane length (μm)	75.0 ± 2.3	210.4 ± 44.0	114.8 ± 43.5	85.8 ± 18.2
Mitochondrial DNA (ng of mitochondrial DNA/ μg of total DNA)	3.03 ± 0.15	8.44 ± 0.67	5.74 ± 0.70	4.26 ± 0.24

Fig. 6. Change in mitochondrial morphology as a result of selective pressure

The cybrid cell line was grown for at least 6 months in the absence of antibiotic, then divided into portions that were grown in (a) drug-free medium and (b) 1.0 µg of rutamycin/ml for 2 weeks before fixation *in situ* for electron microscopy. The bar indicates 2 µm.

swelling. The increase in size of cybrid mitochondria in the presence but not in the absence of rutamycin indicates that the swelling of CY(+) and TL(+) mitochondria could not be an artefact of fixation. Nor could it be an artefactual swelling induced by rutamycin, since mitochondria in the mutant line TL appear swollen regardless of whether they are grown in rutamycin or not (Lichter *et al.*, 1979).

The change in mitochondrial mass was also estimated by using cytochrome *c* oxidase activity as a marker of the mitochondrial inner membrane in both the cell homogenate and the mitochondrial preparation. Since this enzyme is uniquely and completely localized to the mitochondrial inner membrane, the specific activity of the homogenate (in units/cell mass) may be divided by the specific activity of the isolated mitochondria (in units/mg of protein) to obtain an estimate of the amount of mitochondrial protein per cell. Using these enzymic parameters to estimate mitochondrial mass, the cybrid mitochondrial mass increased by more than 2-fold after exposure to rutamycin for 2 weeks. A comparable change in cybrid mitochondrial mass was demonstrated by measuring the mass of cardiolipin (Table 2), which is also localized almost exclusively in the mitochondrial inner membrane (Getz & Bartley, 1959). Cellular DNA was extracted from each cell line and the mitochondrial DNA was quantitatively assessed. Cybrid cells cultivated in the absence of rutamycin had a mitochondrial mass of 3.03 ± 0.15 ng of mito-

Fig. 7. Mitochondrial DNA as a function of selective pressure

The mitochondrial DNA from the cybrid line was determined from cells passaged in drug-free medium and after growth in 1 µg of rutamycin/ml for 2, 4 and 12 weeks.

chondrial DNA/µg of total cellular DNA. After growth in rutamycin for 2 weeks, the cybrid showed an almost 3-fold increase in mitochondrial DNA mass (8.44 ± 0.67 ng of DNA/µg of total DNA, $n = 108$) within the first 2 weeks of selective pressure. Mitochondrial DNA continued to increase a little between 2 and 12 weeks (Fig. 7). The mutant TL cells exhibited no significant difference in mitochondrial mass (as determined by enzyme assays) whether the cells were grown in rutamycin or not.

The dramatic changes in cell morphology and cellular and mitochondrial phenotype which occurred in the cybrid with selective pressure were accompanied by increases in the size of the cell and in phospholipid mass. The mean area occupied per cybrid cell grown in rutamycin for 2 weeks was $228 \mu\text{m}^2$, compared with $142 \mu\text{m}^2$ for a cell grown without drug (a 61 % increase). The total phospholipid mass per cell increased by 36 % when the cybrid was cultured in rutamycin. These modest increases occurred at the same time that mitochondrial mass increased by almost 3-fold.

DISCUSSION

In this study we examined the influence of selective pressure, in the form of rutamycin in the growth medium, upon mitochondrial populations in the rutamycin-resistant cybrid cell line enTL × A9, in which rutamycin resistance had previously been shown to be cytoplasmically transmitted. The precise molecular mechanism accounting for the rutamycin resistance of the mutant TL cell line and the derived cybrid has not yet been defined. In previous studies, it was shown that the TL mutant phenotype is pleiotropic (Lichter *et al.*, 1979), having resistance to rutamycin and rotenone, and being respiratorily impaired with a defect in Complex I. These characteristics are all reproduced in the cybrid, but only when it has been grown in rutamycin, i.e. under selective pressure. Particularly noteworthy is the fact that the mitochondria isolated from such cybrid cells contain NADH-cytochrome *c* reductase which is largely resistant to rotenone, even though these cells had not previously been exposed to this agent and rutamycin has no known influence on this reductase. These observations and the pH optimum profile of the mitochondrial ATPase suggest that the cybrid grown in rutamycin is populated by mitochondria that are predominantly of the mutant or TL type, rather than by mitochondria having the properties of

A9 cells, the parent nucleated recipient in the cybrid fusion. In contrast, the cybrid grown in drug-free medium for an extended period contains predominantly A9 mitochondria, with ATPase and NADH-cytochrome *c* reductase activities that are sensitive to inhibition by rutamycin and rotenone respectively, and with a mitochondrial ATPase pH optimum of 8.0, like the non-mutant parent. However, our studies provide strong evidence that the cybrid grown in rutamycin-free medium for extended times still retains a population of resistant or TL mitochondria that is capable of relatively rapid expansion. This is evident from the ease with which CY(–) cells are capable of growing in rutamycin, albeit after a brief lag of 2 days (Fig. 1).

The observations just discussed could be the consequence of cellular selection from a heterogeneous cell population or cellular selection following mitochondrial segregation regulated by the presence of selective pressure. For several reasons, we think these explanations are highly unlikely. The plating curves demonstrate that CY(+) and CY(–) cells have identical survival in rutamycin. In our studies, three clones of TL and three clones of cybrid behaved in strictly comparable ways. Moreover, CY(–) cells adapt to growth in rutamycin within 3 days (Fig. 1), and the mitochondria of such cells take on the predominant characteristics, described above, of TL or mutant mitochondria within 2 weeks. Both of these changes are much too rapid to be accounted for by mitochondrial segregation, even under selective pressure. Mitochondrial segregation has been reported to take place by a stochastic process (Ho & Coon, 1979; White & Bunn, 1984), but this is slow, even though it may be modestly accelerated by selective pressure (Hayashi *et al.*, 1983; Zukerman *et al.*, 1984). The explanation more likely lies with a plasticity of mitochondrial populations within a fairly uniform population of cells.

The plasticity of mitochondrial populations within cybrid cells could be accomplished either by a replacement of existing mitochondria by others having different properties or by an expansion of the cellular mitochondrial population to provide more mitochondria of an adaptive phenotype. The time course of the processes we have observed suggests that mitochondrial expansion is the basis for the phenotype adaptation. Only the cybrid cell, which by its nature contains two different classes of mitochondria, should be subject to this mitochondrial population expansion. Indeed, from our observations, this seems to be the case. The selective pressure that causes a marked shift in cybrid mitochondrial populations does not affect the parental rutamycin-resistant line TL. There is no loss of resistance to rutamycin of isolated TL mitochondria or shift in the ATPase pH profile after prolonged culture in the absence of antibiotic.

Our observations indicate that the mitochondrial mass of the cybrid cells grown in the presence of antibiotic [CY(+)] is expanded 2–3 times relative to the mitochondrial mass of the cybrid grown in the prolonged absence of antibiotic [CY(–)] and that this amplification of mitochondrial mass occurs within 2 weeks of the introduction of selective pressure. The increase in mitochondrial mass appears to be due primarily to the replication of TL mitochondria, since the mitochondria isolated from CY(+) have the properties of TL mitochondria. It is our hypothesis that the respective masses of TL and A9 mitochondrial DNA are dependent on the energy requirements of the cell. In the absence of selective pressure, the cybrid has only a small number of mutant mitochondria with the TL phenotype, because they are less energy efficient (having a defect in Complex I and lowered ability to phosphorylate ADP) compared with A9. Rutamycin inhibits the ATPase of A9 cells, but not of TL cells. Selective pressure presumably stimulates the increase in mitochondrial mass in order to increase ATP synthesis.

The transmission and maintenance of mitochondrial popula-

tions has been carefully examined in yeast, where mitochondrial segregation produces homogeneous mitochondrial populations within a single cell (Rabinowitz & Swift, 1970). Hybrid, and to a lesser extent, cybrid, cell lines have been used to study mitochondrial inheritance in mammalian cells. Mitochondrial segregation occurs in interspecific cybrid lines, whereas both parental mitochondria may be retained in intraspecific cybrids (Bunn *et al.*, 1977; Wallace *et al.*, 1977; Wallace, 1981; Hayashi *et al.*, 1984; Howell *et al.*, 1984; Kearsey *et al.*, 1985). Antibiotic selection can influence the segregation pattern, and the proportion of drug-resistant parental mitochondria determines the drug resistance of the cybrid cell (Oliver & Wallace, 1982; Walker & Shay, 1983). In contrast with the observations cited above, King & Attardi (1988) observed rapid and nearly complete replacement of host cell mitochondrial DNA by chloramphenicol-resistant mitochondria in host cells injected with chloramphenicol-resistant mitochondria. The apparent contradiction between these results and the observations of other investigators cited above may be due to the fact that King & Attardi (1988) analysed cells soon after transformation and under conditions which selected for antibiotic-resistant mitochondria.

Our study provides additional support for the concept of the maintenance of heterogeneous mitochondrial populations in mammalian cells. However, unlike previous investigations, we have also demonstrated a remarkable plasticity of expression in terms of differences in morphology and growth, in addition to the pH profile and rutamycin-resistance of mitochondrial ATPase and sensitivity of isolated mitochondria to rotenone in the cybrid. Using a chloramphenicol-resistant cybrid line, Hayashi *et al.* (1984) suggested that, whereas the proportion of parental mitochondria varied in different cybrid clones, the proportions of parental mitochondria within those clones was stable after prolonged culture in the absence of selective pressure. He also noted that, whereas predominance of one type of parental mitochondria affected resistance to chloramphenicol, it did not affect other growth characteristics or total cellular proteins. Kearsey *et al.* (1985) also observed the retention of two populations of mitochondrial DNA in a chloramphenicol-resistant cell line and noted a lower frequency of drug-resistant mitochondrial DNA in cells grown in the absence of antibiotic. However, Kearsey *et al.* (1985) concluded that changes of mitochondrial populations occurred only over very prolonged culture periods. They speculated that selection for drug resistance is made at the cellular level on the basis of the phenotypes produced as a consequence of the proportions of mitochondrial genomes due to random segregation. Our observations, like the findings of Oliver & Wallace (1982), suggest that mitochondrial expression may be a more dynamic process. Those investigators demonstrated cooperativity in the translation of different mitochondrial DNAs with selective pressure, which they postulated to be due to the fusion of mutant and normal mitochondria. Our results suggest that an alternative response to selective pressure may be the differential amplification of a mitochondrial population. This suggests that selection of mitochondrial phenotype can be exerted at both the cellular and the subcellular level in mammalian cells. Such manipulatable homologous cybrids may represent models for the study of mitochondrial biogenesis in mammalian cells and provide insight into the basis of heterogeneous mitochondrial expression in disease states.

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