A novel system for assigning the mode of inheritance in mitochondrial disorders using cybrids and rhodamine 6G

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When normal human cultured skin fibroblasts were treated with the fluorescent dye rhodamine 6G (R6G), there was a drastic reduction in numbers of intact mitochondria and electron transport chain enzyme activities, despite the fact that mitochondrial DNA (mtDNA) was still present in treated cells. We used this observation to develop a novel system for generating cybrids. When cultured skin fibroblast cells from a patient with the mitochondrial encephalopathy and ragged-red fibers (MERRF) syndrome harboring the A8344G mtDNA mutation and which showed a severe reduction in cytochrome c oxidase activity were treated with R6G and fused to enucleated HeLaCOT cells, the resulting cybrid clones showed recovery of cytochrome c oxidase activity, and were shown to have mtDNA derived solely from the HeLaCOT cell line. R6G has significant advantages over ethidium bromide in removing the mitochondrial elements from cultured cells, and the results reported here demonstrate that this strategy can be used to determine the origin of the genetic defect in patients with electron transport chain abnormalities.

INTRODUCTION

The oxidative phosphorylation system, physically located at the inner mitochondrial membrane, plays a critical part in the biosynthesis of ATP and is composed of five enzyme complexes (1). These enzyme complexes are each composed of multiple polypeptides, totaling over 80 different gene products (2). In addition, there are a number of essential proteins which are involved in subunit assembly or regulation of DNA transcription. Thus, normal respiratory chain (RC) function necessitates the co-ordinated expression of greater than 100 different gene products. Most of the genes encoding respiratory chain subunits are encoded by the nuclear genome, but some are encoded by the mitochondrial genome (1).

Clinically, defects of the mitochondrial respiratory chain show marked phenotypic variability, both between and within families (3). Genetic counseling for mitochondrial disorders is extremely challenging. Inheritance patterns which have been described include autosomal (recessive or dominant) or maternal (mitochondrial), whereas other disorders appear to be de novo events (4). Consequently, where there appears to be only a single member of a family affected and no mutation is identified, it is impossible to predict with total confidence the likely recurrence risk.

Recently, ρ° cell lines, immortal cell lines that have been rendered devoid of mitochondrial DNA (mtDNA-less), have been developed (5). Cells with deficient or defective mtDNA are auxotrophic for pyruvate and uridine (5,6), allowing the use of selective media to generate an extremely valuable tool for evaluating the biochemical and molecular pathogenicity of mtDNA deletions (7) and putative mtDNA point mutations (8,9). Furthermore, such techniques have led to the development of complementation systems allowing tentative assignment of the genetic defect to the nuclear or mitochondrial genomes (10–13). However, generation of these cell lines is difficult, necessitating the use of an immortalization process followed by long-term low dose ethidium bromide treatment and does not ensure that all mtDNA has been removed from the cell line. Moreover, as ethidium bromide does not target mtDNA exclusively, it is possible that ethidium bromide could introduce random mutations into nuclear encoded RC genes, potentially making this strategy invalid.

Rhodamine 6G (R6G) is a fluorescent dye that binds tightly to the inner mitochondrial membrane and is a potent inhibitor of oxidative phosphorylation (14). It has been used to remove the mitochondrial influence from one parent cell line prior to construction of cybrids or hybrids using hamster (15) and mouse (16) cells. Cybrid genotyping and survival of the fusion product in selective media have been employed as proof of the removal of mtDNA from the parent cell line treated with R6G.

Here we report the effect of R6G on cultured human skin fibroblasts by measurement of mitochondrial enzyme function and molecular analysis of the mitochondrial genome, and demonstrate a dramatic reduction in the numbers of intact mitochondria.
and consequent severe reductions in RC activities. Interestingly, in these R6G-treated cells, we could show that mtDNA was still present after short-term culture, as judged by PCR of the ATPase 6 gene and Southern analysis of the entire mitochondrial genome. However, protein expression studies revealed that mtDNA-encoded polypeptides were either not synthesized or were unstable. From these findings, we reasoned that it would be possible to generate cybrids which would allow us to determine whether functional defects of the RC were due to a mutation in a nuclear or mitochondrial encoded gene. We studied cybrids generated by the fusion of R6G-treated fibroblast cells homoplasmic for the A8344G mitochondrial encephalopathy and ragged-red fibers (MERRF) mutation and enucleated HeLaCOT cells. We showed that following R6G treatment of patient cells and culture of cybrids in selective medium, mtDNA from the patient cells was replaced by that from the enucleated HeLaCOT cell line, and assays of all cybrids studied showed recovery of RC activity. Our results demonstrate that this novel strategy can be used to identify the likely mode of inheritance in patients with RC defects where the defect is expressed in cultured skin fibroblasts.

RESULTS

Growth characteristics of R6G-treated cells

Cells cultured in R6G-containing culture medium showed poor growth, with very few cell divisions occurring. Most cells exhibited a ‘halo’ appearance, with ragged edges due to lifting of the cell edges away from the culture surface. After 8 days in medium containing the higher concentration of R6G, the cells developed a shrunken appearance, although still maintaining their fibroblast shape. When these cells subsequently were cultured in high glucose medium (HGM), they developed a rounded shape, with less than 1 in $10^5$ cells undergoing division. We performed titration experiments with various amounts of R6G and found that the optimal concentration of R6G was 2.5 $\mu$g/ml. At higher concentrations of R6G, there were too few viable cells remaining for fusion studies.

Electron microscopy

Electron microscopy was performed on cell lines treated with both low and high doses of R6G, as well as untreated control lines from two of these.

The control cells (Fig. 1A) were elongated and their cytoplasm contained prominent cisternae of rough surfaced endoplasmic reticulum, numerous, often elongated, mitochondria and conspicuous juxtanuclear Golgi zones. Autophagic vacuoles were present in small numbers in occasional cells.

Cells treated with low doses of R6G (1.2 $\mu$g/ml) (Fig. 1B and C) displayed some rounding up. No normal mitochondria are present ($\times$9000). (C) Low dose R6G (1 $\mu$g/ml). High power electron micrograph showing myelinoid bodies sometimes containing degenerate mitochondrial remnants (arrowhead). Several abnormal mitochondria, with accumulation of dense material in their intramembranous and intracistral spaces, are also present (arrow). The adjacent cisternae of rough surfaced endoplasmic reticulum are dilated ($\times$30 000). (D) Cells treated with high doses of R6G (2.5 $\mu$g/ml) showing myelinoid bodies, enlarged, ring-shaped nucleolus and margined chromatin ($\times$22 500).
Enzyme and protein assays of cultured fibroblasts

Individual cell strains were assayed in triplicate and the mean reductions in enzyme activities were determined. For each of the cell strains treated with low dose R6G, the reduction in the activities of complexes II + III, IV and the mitochondrial matrix enzyme citrate synthase (CS) was dramatic, with the variance being small. This reduction in activities was even more dramatic for the cell strains treated with high dose R6G (Table 1).

DNA analysis of R6G-treated cells

The ATPase 6 gene was still present in cells 1 week after treatment with R6G (Fig. 2a). Densitometry of PCR products revealed no significant differences between R6G-treated (regardless of concentration used) and untreated cell strains (data not shown), suggesting that the reduction in RC enzyme activities was not due to depletion or loss of mtDNA in the R6G-treated cells. In order to confirm that the entire mitochondrial genome was present and not fragmented, we used Southern analysis to show that the intact mitochondrial genome was present and not fragmented, we used South ern analysis to show that the intact mitochondrial genome was not due to loss of the mitochondrial genome. –, no DNA negative control; MW, 123 bp DNA ladder (Gibco BRL). (b) Southern analysis of mtDNA from R6G-treated cells. Two normal control cell strains were cultured in medium containing high dose R6G (2.5 μg/ml) for 5 days, and total DNA was extracted for Southern analysis. The probe used was a labeled PCR product encompassing the ATPase 6 gene, which demonstrates the presence of the intact 16.6 kb mitochondrial genome in the R6G-treated (T) cells, with no obvious reduction in the relative amount in the untreated (U) cells.

Enzyme assayed | Low dose R6G<sup>a</sup> | High dose R6G<sup>b</sup> |
--- | --- | --- |
Complex II (nM/min/mg protein) | 20 (5) | 18 (8) |
Complex II + III (nM/min/mg protein) | 5 (5) | 1 (1) |
Complex IV (rate/mg protein) | 21 (3) | 8 (2) |
Citrate synthase (nM/min/mg protein) | 19 (4) | 9 (1) |

The results shown are pooled data for different normal control cell strains cultured in HGM with R6G at the concentrations indicated, with the assays performed in triplicate for each cell strain. Results for each R6G-treated cell strain are expressed as a proportion of the activity in the same cell strain grown in HGM without R6G. The numbers in parentheses are the standard deviations.

<sup>a</sup>Cells treated with 1.2 μg/ml R6G for 3 days (21 cell strains used).

<sup>b</sup>Cells treated with 2.5 μg/ml R6G for 8 days (6 cell strains used).

Protein expression studies

Control cells cultured with [35S]methionine showed a smear of labeled proteins on a 15% polyacrylamide gel (Fig. 3, lane 2). When these cells were co-cultured with emetine (inhibits cytoplasmic protein synthesis), only proteins derived from the mitochondrial genome were produced (lane 3), whilst with emetine and chloramphenicol (inhibits mitochondrial protein synthesis) no proteins were produced (lane 4). R6G-treated cells, on the other hand, showed only cytoplasmically synthesized proteins (lane 5) and, with the addition of emetine, no proteins were produced (lane 6). These results show that even though the mitochondrial genome is present in the cytoplasm of R6G-treated cells, functional proteins cannot be synthesized.

mtDNA analysis of cybrids

Total DNA was extracted from all of the cybrids and amplified in the 108 bp region containing the common MERRF mutation. DNA from the parent HeLaCOT cells and all 19 cybrids present in two cell lines treated with 2.5 μg/ml R6G for 5 days (Fig. 2b).
showed only the uncut 108 bp PCR product, whilst DNA from the patient’s cells showed only the 66 and 42 bp fragments (Fig. 4). Repeated amplification of the fragment containing the MERRF mutation in the cultured skin fibroblasts established from this patient showed no evidence of wild-type mtDNA. Taken together, these results suggest that the patient’s fibroblast DNA was virtually homoplasmic for the A8344G mutation, whilst the mtDNA from all 19 cybrid clones was derived from the parent HeLaCOT cell line.

Enzyme assays of cybrid clones

Cytochrome c oxidase (COX) activity was measured in the ‘parent’ cells and the cybrids. The COX/CS ratio for the HeLaCOT cell line was 17.9 (SD 2.3) and for the patient fibroblasts was 1.3 (SD 0.9), whilst the mean for the 19 cybrid clones was 17.1 (SD 3.8). Significant recovery was seen in all 19 clones, ranging from 7.5- to 19.4-fold higher than the patient fibroblast activity, with mean recovery of activity being ~13-fold higher than that seen in the patient fibroblasts (Fig. 5).

DISCUSSION

We have shown that R6G can be used to generate human cell strains which are functionally deficient in respiratory chain and other mitochondrial enzyme activities, as evidenced by the failure of R6G-treated cells to survive, and the marked reduction in enzyme activities of cells grown in medium supplemented with pyruvate and uridine. In addition, cells growing in R6G underwent fewer cell divisions than untreated cells and assumed a different morphology, becoming flatter and smaller with time in R6G-containing culture medium.

The activity of complex II + III was reduced in all cell strains, the mean activity being <5% of controls in all but two of the cell strains at the lower R6G concentration, and almost totally deficient in those cell strains exposed to the higher concentration of R6G. Complex II activity was also low, although to a lesser extent (Table I). Mean complex IV activity was reduced to <21% of that of untreated controls in all the cell strains with low dose R6G treatment, and was only 8% of that of untreated cells in 2.5 µg/ml R6G. Complex I activity was not measured in these cells as R6G treatment destroyed most of the cells, leaving too few for isolation of the necessary number of mitochondria to permit accurate analysis of complex I activity.

In cells treated with low dose R6G, levels of CS, an enzyme found exclusively in the matrix of the mitochondria, were on average only 19% of those of the untreated cell strains, and fell further to a mean of only 9% with high dose R6G, supporting the notion that R6G causes the collapse of the functional integrity of the mitochondrial membrane (16). On the other hand, rhodamine 123 (17) and other cationic lipophilic agents such as tetraphenylphosphonium salts (18) are known to cause major ultrastructural alterations specifically affecting mitochondria, and it may be that the profound reductions of RC enzyme activities and CS were due to loss of mitochondrial membrane structural integrity.

The low levels of apparent mitochondrial enzyme activity in the R6G-treated cells are probably due to the presence of other organelles in the mitochondrial-rich extracts which have non-specific cross-reactive activity with the RC enzymes (19).

Amplification of the ATPase 6 gene and Southern analysis of the entire mitochondrial genome showed that the mitochondrial genome was present in cells immediately after treatment with R6G, whilst our cybrid studies demonstrated that the mtDNA derived from the patient fibroblasts treated with R6G was lost during the process of cybrid generation. These results are consistent with those of others in murine cells (16,20). We suggest that short-term culture of cells with R6G, which leads to total disruption of mitochondria, results in the mitochondrial genome being released into the cytoplasmic pool. However, the now cytoplasmic mtDNA pool cannot be transcribed or translated to produce stable polypeptide subunits (and therefore functional enzymes), as evidenced by the protein expression studies (Fig. 3). It is possible that the process of cybrid generation and subsequent culture may result in the loss of this mtDNA, which is not ‘safely’ compartmentalized within mitochondria, with the consequence that the mtDNA in these cybrids is derived from the ‘parent’ HeLaCOT cell line.

We have shown that it is possible to render human skin fibroblast cells with defective RC mitochondria-less with R6G treatment, which potentially could allow us to identify the genetic origin of defect. By generating cybrid clones with enu-
cleaved immortal (HeLaCOT) cells, we have developed a system which would enable the assignment of the defect in oxidative phosphorylation to the nuclear or mitochondrial genomes. This has been achieved successfully by employing fibroblast cybrid studies (11), but this strategy is complex, long and cumbersome because it is necessary to first immortalize patient fibroblasts, and follow this with ethidium bromide treatment to render cells mtDNA-less.

Isobe et al. (13) recently used a p° hybrid system to show that in fibroblasts from a patient with combined complex II and IV deficiencies, the genetic etiology was likely to be due to a nuclear encoded defect. Our method has several advantages over their method. Firstly, our method is not dependent on the use of p° cells. Because these cell lines have been generated by long-term culture in ethidium bromide, there is a theoretical risk that random mutations could be created in genes for nuclear encoded RC subunits. Secondly, by creating cybrids, we are able to evaluate the impact of only one complement of nuclear genes. Thirdly, p° cell lines tend to have large numbers of mitochondria and, when screening clones of p°-patient cybrids, the RC enzymes cannot be normalized against CS (21) as they can when R6G is used. Fourthly, whilst p° cell lines are often resistant to bromodeoxyuridine, allowing this drug to be used to remove unfused patient cells, the necessity to remove uridine from the media (8) can cause loss of cultured cells harboring mtDNA mutations (22), thus restricting the usefulness of this system. In addition, Isobe et al. only selected three hybrid clones in their studies. To strengthen our data set, we selected 19 clones, which were cybrids rather than hybrids, for our studies.

Our novel strategy enables the rapid generation of cybrids where one of the cell strains used contributes only nuclear encoded respiratory chain elements, without the need for immortalization and prolonged treatment with ethidium bromide, whilst the other cell strain contributes its mitochondrial genome. It should permit the relatively rapid and accurate assignment of the likely genetic origin of RC defects in patients where there are no other clinical, biochemical or pathological clues as to the likely mode of inheritance.

**MATERIALS AND METHODS**

**Tissue culture in R6G**

Fibroblasts were established from skin biopsies of normal individuals and initially were grown in T75 culture flasks using Ham F12 (Trace Biosciences, Sydney) with penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Multicel, Sydney) supplemented with 10% fetal bovine serum (Trace Biosciences) and 200 µM uridine (Sigma, Sydney) (23). Subsequently, 1.5–2 × 10^6 fibroblast cells of each cell strain were plated into each of four T75 culture dishes. All cell strains were then cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Sydney), supplemented to give final concentrations of the following reagents: glucose (11 mM), glutamine (0.3 mM), uridine (200 µM), pyruvate (1 mM), sodium bicarbonate (4 mM) (Sigma) and fetal calf serum (10%), and is designated HGM.

Between 1.5 and 2 × 10^6 cells were plated into HGM with either 1.2 µg/ml R6G (Sigma) for 3 days or 2.5 µg/ml R6G for 8 days. After this time, extracts rich in mitochondria were isolated from the treated and untreated cells for enzymology analysis and an aliquot of the cells treated at the lower amount of R6G was plated into HGM. These cells were cultured for a further 21 days and their growth characteristics and morphology observed.

**Construction of cybrids**

Cultured skin fibroblasts with defective RC function from a patient with the MERRF syndrome harboring the A8344G mutation were treated with 2.5 µg/ml R6G for 5 days as detailed above. HeLaCOT cells were enucleated by the addition of 10 mg/ml cytochalasin B (Sigma) to Nunc (Medos, Sydney) culture vessels and centrifugation to remove the nuclei (10 500 g at 37°C for 30 min). After removal of the enucleation medium, Ham F12 with 10% fetal calf serum was added to the flask and the cells allowed to recover for 30–60 min. Staining one of the tubes with 2% Giemsa (Selby, Sydney) in methanol revealed a >95% enucleation rate. Next, an equal number of R6G-treated patient fibroblasts was plated on top of the enucleated HeLaCOT cells and left for 1 h. Then, all the medium was removed and 1 ml of fusion solution containing 50% polyethylene glycol (mol. wt 1450) and 10% dimethylsulfoxide (Sigma) was applied to the flasks for 1 min at 37°C. The cells were then washed twice with 10 ml of Ham F12 and incubated overnight at 37°C in 2 ml of Ham F12. The next day, the mixture of parent cells and cybrids was plated (4 × 10^6 cells per T75 culture flask) into Ham F12 containing hypoxanthine (1 × 10^{-6}), aminopterin (4 × 10^{-5}) and thymidine (16 × 10^{-5}) (Sigma). After 30 days in culture, 19 clones were ring isolated and grown in Ham F12.

To confirm that the culture selection strategy would permit only cybrids to survive, we cultured two additional flasks each of parent HeLaCOT cells and R6G-treated patient fibroblasts. One of each of these flasks was treated identically to the experimental flasks (including exposure to polyethylene glycol), while the other flasks were not exposed to polyethylene glycol. None of these cells survived.

**Chromosome analysis**

The chromosome content of cybrids was analyzed by flow cytometry (25). Cultured cells (1 × 10^6) were removed from the culture flask and resuspended in 2 ml of staining solution containing Ham F12:phosphate-buffered saline (PBS) (4:1), 0.4% Triton X-100 (BDH, Sydney) and 100 µg/ml propidium iodide (Sigma). RNase (ScimaR, Sydney) solution (50 mg ml\(^{-1}\)) was added and the tube incubated at 4°C for 15 min. The tube was then gently mixed and fluorescence quantitated by flow cytometry using a Becton Dickinson flow cytometer equipped with Cell Quest software. The amount of relative fluorescence of the G2/M peak was compared between clones and parent cells.

**Electron microscopy**

Cells from T75 flasks were washed with PBS and then fixed in situ in 10 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h at 4°C. Cells were removed from the plate with a cell scraper, washed in cacodylate buffer and a pellet was produced by centrifugation in 10% bovine serum albumin (BSA) (26), followed by 4 h gelling time in glutaraldehyde. The cell pellets were post-osmicated, stained en bloc with uranyl acetate,
dehydrated through a graded series of alcohols, infiltrated and embedded in Spurr's epoxy resin. Toluidine blue-stained semithin sections were examined by light microscopy and representative areas selected for ultramicrotomy. Ultrathin sections were collected on copper grids, contrast stained with uranyl acetate and lead citrate and examined in a Philips 410LS electron microscope.

**DNA preparation**

Fibroblasts, HeLaCOT cells or cybrids from 2–4 confluent 75 cm² culture flasks were suspended in 1 ml of Tris/EDTA buffer, pH 8.0, followed by DNA extraction (27).

**Amplification of the ATPase 6 gene**

A 745 bp region of the mitochondrial genome encompassing the ATPase 6 gene was amplified by the PCR using primers derived from the Cambridge sequence (28), corresponding to nucleotides 8466–8486 (5'-ACCTACCTCTCCTACAAAAGC-3') and 9210–9190 (5'-GACGTGGCTGGTGATTACCT-3'). The 100 µl reaction contained 250 ng of genomic DNA, 60 pmol of the primers, 2.5 U of Taq polymerase (Boehringer Mannheim), 100 µM dNTPs (Boehringer Mannheim) and 10 µl of 10× PCR buffer (Boehringer Mannheim), giving final concentrations in the PCR mixture as follows: 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂. Hot start PCR (29) was used, with the amplification conditions being 35 cycles consisting of denaturation for 60 s at 94°C, annealing for 90 s at 50°C and extension for 90 s at 72°C using a Perkin Elmer 480 thermal cycler. Ten microliters of the PCR product led to the creation of a 2% agarose gel and visualized with ethidium bromide (1 µg/ml staining solution) under UV light.

**Detection of the A8344G mutation**

A 108 bp fragment of mtDNA encompassing the region of the A8344G mutation was amplified using the PCR using primers based on the Cambridge sequence (28), corresponding to nucleotides 8278–8297 (5'-CTACCCCCCTCAGGCCCAC-3') and 8385–8354 (5'-GATGAATTTAGTTGGGGCATTTCACTGTAAAGCCGTGT-8297 (5'-CTACCCCCTCTAGAGCCCAC-3')) and 8385–8354 (5'-GATGAATTTAGTTGGGGCATTTCACTGTAAAGCCGTGT-8297 (5'-CTACCCCCTCTAGAGCCCAC-3')). The 50 µl PCR reaction contained 100 ng of DNA, PCR buffer, 2.5 U of Taq polymerase, 200 µM dNTPs and 0.4 µM primers. The amplification conditions were as follows: one cycle at 94°C for 5 min, then 29 cycles of 94°C for 30 s (denaturation), 55°C for 45 s (annealing) and 72°C for 2 min (extension). Using the above primers, the PCR product generated led to the creation of a BglII site in the presence of the A8344G mutation, giving rise to 66 and 42 bp fragments. Ten microliters of the PCR product was digested in a reaction mixture consisting of 10 U of BglII (Boehringer Mannheim), 2 µl of buffer, 0.25 µl of BSA (10 ng/ml solution) and 7.25 µl of de-ionized water. The reaction mix was incubated at 37°C overnight then the entire mixture was run on a 3% agarose gel and stained with ethidium bromide.

**Southern blotting**

Southern blotting of DNA from normal control human cultured skin fibroblasts, either treated or not treated with R6G, was performed (30), with the ATPase 6 PCR product labeled with [³²P]dATP using the Gigaprime DNA labeling kit (Bresatec, Adelaide) as the probe.

**Mitochondrial enzyme assays in cultured cells**

Mitochondria-rich extracts were isolated from cultured cells using a method based on that of Zurrendonk and Tager (19) and Brown et al. (24). Cultured cells (1–2 × 10⁶) were suspended in homogenization buffer containing 20 mM MOPS, 3 mM EDTA, 0.25 M sucrose, pH 7.2. After a 35 s treatment with digitonin at 4°C and subsequent washing with HB to ensure mitochondrial enrichment of the extract, the mitochondria were disrupted by three freeze–thaw cycles in a dry ice–methanol bath. The cell extracts were sonicated individually (Branson sonicator, six pulses, setting 3, 30% duty cycle) and analyzed immediately.

Complex IV (cytochrome c oxidase, COX) was measured by following the rate of oxidation of reduced cytochrome c at 550 nm after addition of sample (31). An aliquot of the cell homogenate was added to a reaction mix containing 50 mM potassium phosphate pH 7.4 and 15 µM reduced cytochrome c. Potassium ferricyanide (1 mM) was added to stop the reaction. COX activity was expressed as the pseudo first-order rate constant relative to the amount of protein and citrate synthase in the sample.

Complex II (succinate-ubiquinone oxidoreductase) was analyzed as previously reported (12). The reduction of dichloro-phenolindophenol (DCPIP) was measured at 600 nm after the addition of cell homogenate to a reaction mixture of 50 mM potassium phosphate pH 7.4, 20 mM succinate, 2 µg/ml antimycin A, 2 µg/ml rotenone, 2 mM potassium cyanide and 50 µM DCPIP.

Complex II + III (succinate cytochrome c oxidoreductase) was analyzed as previously reported (12). The reduction of ferricytochrome c was measured at 550 nm after the addition of cell homogenate to a reaction mixture consisting of 40 mM potassium phosphate pH 7.4, 0.5 M sodium EDTA, 2 mM potassium cyanide, 20 mM succinate and 30 µM cytochrome c.

Citrate synthase (CS) was measured using a well established method (32). The cell homogenate was added to a reaction mixture containing 50 mM potassium phosphate pH 7.4, 100 µM dithionitrobenzoic acid and 100 µM acetyl-CoA. The reaction was started by the addition of oxaloacetic acid (100 µM), with the rate of rise in absorbance at 405 nm measured, and activity expressed per milligram of cell homogenate protein.

**Protein expression studies**

Cultured skin fibroblasts were cultured in Ham F12 supplemented with 10% fetal bovine serum and R6G (2.5 µg/ml) until cell division was no longer detectable by visual inspection. After trypsinization, 1–2 × 10⁶ cells were transferred to each of 35 mm wells containing either PBS (0.9%), emetine (200 µg/ml) or emetine and chloramphenicol (each at 200 µg/ml). After labeling with 400 µCi of [³⁵S]methionine per well (2 ml) in methionine-free media supplemented with 10% fetal bovine serum for 4 h (37°C/5% CO₂), the cells were washed twice in cold PBS, trypsinized and lysed (45 mM Tris–HCl pH 6.8, 1% SDS, 0.2 mM phenyl-methylsulphonyl fluoride) (33,34). Protein extracts (6 mg) were applied to a denaturing one-dimensional SDS–polyacrylamide gel (15% Tris–glycine gel. ¹⁴C-labeled molecular weight markers were also run in parallel. The SDS–polyacrylamide gel was dried and exposed for 5–7 days on X-ray film (BioMax MR, Kodak).
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ABBREVIATIONS

COX, cytochrome c oxidase; CS, citrate synthase; HGM, high
glucose medium; MERRF mitochondrial encephalopathy and
ragged-red fibers; mtDNA, mitochondrial DNA; RC, respira-
ry chain; R6G, rhodamine 6G.