Complex I deficiency is associated with 3243G:C mitochondrial DNA in osteosarcoma cell cybrids

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143B.206 ρ0 cells were repopulated with mitochondria from a MELAS patient harbouring a mixture of 3243G:C and 3243A:T mitochondrial DNA. A number of biochemical assays were performed on selected cybrids with various proportions of the two types of mitochondrial DNA. These assays revealed a marked decrease in oxygen consumption with pyruvate, a complex I substrate, in cybrids containing 60% to 90% 3243G:C mitochondrial DNA. Moreover, these cybrids showed decreased synthesis of a number of polypeptides in a mitochondrial in vitro translation assay. A cybrid line with a very high level of 3243G:C mitochondrial DNA (95%) had additional deficiencies in complexes III and IV and there was a marked generalised decrease in mitochondrial translation in this cybrid. The observation of complex I deficiency is consistent with previously reported enzymatic measurements of muscle homogenates from MELAS patients with the 3243G:C mutation.

INTRODUCTION
Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) has been recognised as a clinical syndrome for 10 years (1). In 1990, a G:C for A:T substitution at nucleotide position 3243 of the human mitochondrial genome was discovered to be present in the majority of cases of MELAS (2). Like partial mitochondrial DNA deletions (3), and point mutations associated with NARP (4) and MERRF (5), the mutant population co-existed with apparently wild type mitochondrial DNA (mtDNA) (2).

The generation of human cells devoid of mtDNA (6) has allowed patient mtDNAs that carry disease-associated mutations to be analysed in a control nuclear background. The MELAS 3243G:C mitochondrial genotype has been extensively studied in an osteosarcoma ρ0 cell line (143B.206 TK−) (7–10). These studies are taken to imply a causal role for 3243G:C mtDNA in the MELAS syndrome. The authors of related studies that analysed 143B.206 cybrids with the MERRF 8344A:G mutation (11,12) or with a tRNALeu(UUR) mutation at nucleotide pair 3260 (13) drew similar conclusions. Partial mtDNA deletions have also been introduced into a ρ0 cell line, although in this case the recipient ρ0 cells were derived from HeLa cells (14). The partial mtDNA deletion markedly reduced in vitro translation of mitochondrial proteins at a relatively low level of mutant mtDNA (67%).

Despite identification of pathogenic mtDNA mutations, such as that most commonly associated with the MELAS syndrome, it is not yet known by what mechanism(s) they exert their pathophysiological effects. Individuals with MELAS tend to have a higher proportion of 3243G:C mtDNA than their asymptomatic maternal relatives, however, there is no clear correlation between the proportion of mutant mtDNA and disease severity (15). The 3243G:C MELAS mutation lies in a tridecamer sequence that acts as a transcription termination signal (16). Although it has been shown to affect transcription termination in vitro (17,18), there is, as yet, no evidence of such an effect in vivo (18). However, a possible defect in RNA processing was implied by the detection of increased levels of a precursor-like RNA (RNA 19), which contains the tRNALeu(UUR) gene, in cybrids harbouring high levels of 3243G:C mtDNA (7).

In this study, we analyse the biochemical properties of cybrid lines that contain various proportions of wild-type and 3243G:C mtDNA.

RESULTS
Mitochondria from a patient with MELAS were transferred by cytoplast fusion to cells lacking mtDNA. Several of the resulting cybrid clones, termed 206.3243, that maintained a stable relative level of 3243G:C and 3243A:T mtDNA, were analysed using a number of biochemical assays. Measurements of cytochrome c oxidase (COX) revealed similar activities in cybrids with 0 and 50% 3243G:C mtDNA. Where the proportion was between 60 and 90% 3243G:C mtDNA, there was a decrease in COX activity of approximately one third and at 95% 3243G:C mtDNA, COX activity was approximately one third of the value of those cybrids with no 3243G:C mtDNA (Fig. 1B). Succinate-cytochrome c

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Figure 1. Cytochrome c oxidase activity (COX) relative to citrate synthase activity (CS) in 143B osteosarcoma cells and mitochondrially repopulated 143B.206 cells. 
(A) 143B cells a, b, c and d were mass cultures (not clonal lines) passaged for different lengths of time in our laboratories, 143B a cells had been passaged the least, the 143B d cells the most (see text for details). (143B).206.con cybrids were repopulated with mitochondria from a normal control subject. 
(B) 206.3243 cybrid clones contained mitochondria from a patient with MELAS (3243G:C mtDNA). The COX/CS ratio was arbitrarily defined as 1 in the 206.con(trol) 1 cybrid and the remainder of the values standardized against this number. Bars are means, ± standard deviation, n = 5–10 measurements. There was no detectable COX activity in 143B.206ρ− cells.

Figure 2. Intact cell oxygen consumption rates in (A) 143B cells and (143B).206.con cybrids and (B) 206.3243 cybrids (as per Fig. 1). Each value was based on a minimum of six assays, bars are means ± standard deviation. There was no discernible rate of oxygen consumption in the 143B.206ρ− cells (<2% of control values). The level of mtDNA relative to 18S ribosomal (nuclear) DNA is indicated beneath the proportion of 3243G:C mtDNA. The ratio of the mitochondrial to nuclear DNA signals from the 206.con 1 clone was arbitrarily designated as one hundred and the other values expressed as a percentage of this number.

Intact cell oxygen consumption measurements on 206.3243 cybrids suggested that as little as 50% 3243G:C mtDNA could have a marked impact on respiratory function (Fig. 2B). Moreover, one cybrid line with 60% 3243G:C mtDNA had an oxygen consumption rate similar to a cybrid with 95% 3243G:C mtDNA; a rate that was lower than that of the three cybrids with reductase (SCCR) activity was measured in each of the cybrids shown in Figure 2 and found to be similar in all (data not shown).
Figure 3. Rate of oxygen consumption in digitonin-permeabilised cells with pyruvate, succinate and ascorbate/TMPD as substrates. Each rate was calculated from the mean of at least five runs, errors are standard deviations. The sample to sample variation for this assay was large, in the patient derived cybrids, particularly for complex II and complex IV substrates. However, the rate of oxygen consumption with the complex I substrate, pyruvate, closely correlated with the overall respiratory capacity as determined by intact cell oxygen consumption rates (Fig. 2B) for the 206.3243 cybrids.

70, 85 and 90% 3243G:C mtDNA. Although the level of mtDNA relative to nuclear DNA was relatively low in the cybrid with 60% 3243G:C mtDNA (Fig. 2B) the relationship between mtDNA copy number and respiratory function was not a simple one, as the two clones with 50% 3243G:C mtDNA had similar levels of mtDNA, but quite different oxygen consumption rates (Figs 2B and 3B).

By first permeabilising cells with digitonin it is possible to measure oxygen consumption rates with substrates that donate electrons to specific respiratory chain enzymes (19). In preliminary experiments, the rate of oxygen consumption was shown to be the same with pyruvate as with glutamate plus malate (data not shown). Therefore, only the former substrate was used routinely in the subsequent detailed study. Based on this assay we infer that 60–90% 3243G:C mtDNA results in a specific deficiency of complex I of the respiratory chain as pyruvate-driven, but not succinate-driven respiration, was decreased in cybrids with 60, 70, 85 and 90% 3243G:C mtDNA (Fig. 3B). In vitro mitochondrial translation was assessed, in the cybrid lines with 0, 50, 60, 85, 90 and 95% 3243G:C mtDNA, by labelling protein with 35S-methionine in the presence of emetine, an inhibitor of cytosolic translation. By inspection, the majority of mitochondrial translation products decreased with increasing levels of 3243G:C mtDNA (Fig. 4). However, cybrids with greater than 50% 3243G:C mtDNA synthesised little or no ND6 (Fig. 4B). There was, therefore, a correlation between the loss of the putative ND6 polypeptide and a specific deficiency of complex I. An unidentified band migrating more slowly than ND6 appeared in all samples, including the parental 143B (a) cells, interestingly this product was more intense in the cybrids with high levels of 3243G:C mtDNA (Fig. 4B). At 95% 3243G:C mtDNA there was a marked decrease in all the mitochondrial translation products (Fig. 4A) and this was associated with generalised respiratory deficiency, measurable oxygen consumption with complex I and complex II substrates being largely abolished (Fig. 3B).

Although there was little or no oxygen consumption with pyruvate and succinate as substrates in the permeabilised cybrid line with 95% 3243G:C mtDNA, the intact cells had 20% of the oxygen consumption rate found in 206.3243 cybrids with 0% 3243G:C mtDNA (Fig. 2). Moreover, it appears that the cybrid line with 95% 3243G:C mtDNA retained some respiratory capacity because it grew normally in 90% DMEM, 10% dialyzed FBS without uridine, whereas the 143B.206 p+ cells could not grow under these conditions (data not shown).

Comparison of the production of lactate and pyruvate (L/P) in cultured cybrids with various levels of 3243G:C mtDNA has been reported previously (Fig. 5B; ref. 18). These results suggested a simple relationship between the level of 3243G:C mtDNA and the dependence on glycolysis for energy production. However, the assay failed to differentiate 206.3243 cybrids with 90 and 95% 3243G:C mtDNA. Repeating the assay at 24 h, instead of 48 h, had little or no effect on the L/P ratio of cell lines, 143B b and c, but the ratio was higher in the cells with 95% 3243G:C mtDNA as compared to the 206.3243 cybrid line with 90% 3243G:C mtDNA (Fig. 5C). Moreover, the L/P ratio in the 95% 3243G:C
cybrid was higher at 24 h than 48 h. The 143B 206 ρ0 cells had a L/P ratio considerably higher than any of the 206.3243 cybrids, 743 ± 5 at 24 h, and 1849 ± 793 at 24 h. The differences in L/P ratio at 24 and 48 h for the cybrid with 95% 3243G:C mtDNA and the ρ0 cells suggest that severely respiratory deficient cells are unable to tolerate the culture conditions used in the assay for 48 h.

Respiratory deficiency in 143B.TK− cells

Assays of COX activity, and of lactate to pyruvate production, in 143B (d) cells that had been passaged almost continuously for 3 years indicated that the cells were less respiratory competent than cybrids containing control and ‘non-mutant’ patient-derived mtDNA (Fig. 1A and 5A). Oxygen consumption rates of intact cells and of permeabilised cells confirmed these differences (Figs 2A and 3A). Frozen stocks of 143B TK− cells (c) that were approximately 18 months younger than the 143B (d) cells were thawed and passaged. They also showed low respiratory capacity relative to cybrids containing control mtDNA; however, the deficiency was less marked (Figs 2A and 3A). 143B TK− cells (b) grown in another laboratory also had a decreased intact cell oxygen consumption rate relative to the value obtained 2 years earlier (13). We therefore obtained another sample of 143B cells (a) from the original source, these cells had a considerably higher respiratory capacity (Figs 2A and 3A). At present we have no explanation for this apparent progressive respiratory decline in the 143B.TK− cells. However, it is interesting that the level of mtDNA to nuclear DNA was higher in the 143B b, c and d cells compared to the 143B a cells. Furthermore, the respiratory capacity, following repopulation of the 143B.206 ρ0 cells with wild type mtDNA (206.con 1 and 2, 206.3243 0% 3243G:C mtDNA, Fig. 2) was higher than that for the 143B b, c and d cells, suggesting that the 143B parental mtDNA may be compromised. The high L/P ratio in the 143B d cells, compared to 143B cells a, b and c, may mean that the decrease in respiratory chain function has reached a critical level in these cells. Consistent with this hypothesis the 143B d cells had the lowest intact cell oxygen consumption rate.

DISCUSSION

The 3243G:C mtDNA mutation associated with the MELAS syndrome has previously been shown to result in respiratory deficiency at levels above 90% and this was attributed to the generalised decrease in in vitro mitochondrial translation (7,8). The cybrid analyses reported here suggest that the primary effect of the mitochondrial tRNALeu(UUR) gene 3243G:C mutation in 143B osteosarcoma cells is to decrease complex I activity. This could be a consequence of the decreased synthesis of a number of complex I polypeptides (Fig. 4A), or ND 6 in particular (Fig. 4B), if these changes were reflected in a decrease in steady state levels of mitochondrial polypeptides in vivo.

In 206.3243 cybrids with greater than 50% 3243G:C mtDNA, but less than 95% 3243G:C mtDNA, complex I activity was specifically affected. At a level of 95% 3243G:C mtDNA there were, in addition, measurable decreases in respiratory rates with complex II and complex IV substrates (Fig. 3B), however, these decreases could be a consequence of a marked generalised reduction in mitochondrial translation, as observed in vitro in this cybrid (Fig. 4A). Taken together, our biochemical findings closely parallel those of Zeviani and colleagues in muscle homogenates of MELAS patients (26). Thus, muscle from MELAS patients had the same respiratory deficiency as heteroplasmic 206.3243 cybrids that contained 60–90% 3243G:C mtDNA. MELAS and other mtDNA disorders are associated with mitochondrial proliferation in muscle fibres, detected histochemically as ragged red fibres (RRF). In situ hybridization studies (18, 27) have shown that the mitochondria in these regions contain a high proportion of mutant genomes. Thus respiratory failure in ragged red regions was recognised as a possible
Figure 5. Comparison of the ratio of lactate to pyruvate production in 143B cells and cybrids. The ratios for (A) and (B) were obtained from 48 h incubations, whereas in (C) the incubation time was 24 h. The 143B 206 ρ- cells had a L/P ratio of 743±254, n = 14, after 48 h incubation, and 1849±793, n = 8 after 24 h.

explanation of the observed muscle pathology. However, our demonstration of decreased respiratory function at levels as low as 60% 3243G:C mtDNA suggests that the focal accumulation of 3243G:C mtDNA, and possibly other mutant mitochondrial genomes, may not be the only important factor contributing to the disease phenotype. This finding may also explain why there is only a loose correlation between the proportion of RRF and disease severity in mitochondrial diseases (A. E. Harding and J. A. Morgan-Hughes, personal communication).

Phenotypic differences in 206.3243 cybrids with various levels of 3243G:C mtDNA were most clearly evident in comparing oxygen consumption rates, whether in intact or permeabilised cells. There was also a clear correlation between intact cell, and pyruvate supplemented permeabilised cell oxygen consumption rates (complex I activity). This implies that flux through complex I is the rate limiting step in the respiratory chain in cultured cells. Although COX activity was a poor indicator of overall respiratory capacity in the 206.3243 cybrids, it did show a step-wise drop with increasing levels of 3243G:C mtDNA. The decreased rate of cytochrome c oxidation (COX activity) was greater than the decrease in complex IV activity as measured by oxygen consumption in the presence of ascorbate and TMPD, implying that basal COX activity was not a limiting step in respiration, at least in this particular cybrid type.

Attardi and colleagues noted a marked protective effect of as little as 5% 3243A:T (i.e. wild type) mtDNA in 206.3243 cybrid studies (8,9). Although the oxygen consumption rates with pyruvate reported here indicate that the situation is more complex, three results contained in this study are in agreement with their observations. Firstly, a marked generalised decrease in labelled mitochondrial translation products was observed in the cybrid line with 95% 3243G:C mtDNA (Fig. 4). Secondly, COX activity in this clonal cybrid line was half that of clones with 60–90% 3243G:C mtDNA (Fig. 1B). Thirdly, only at 95% 3243G:C mtDNA was a decrease in respiratory rate for complexes II to IV seen (Fig. 3B). These observations are also compatible with findings for MELAS patient muscle (26) where the lowest COX activity belonged to the patient with the highest proportion of 3243G:C mtDNA (96%), whereas most patient muscle samples had COX activities within the normal range. This suggests that in our hands at least, the osteosarcoma cybrid system is modelling the situation in patient muscle, harbouring 3243G:C mutant mtDNA.

Enzymatic measurement of SCCR is held to be a measure of complex II plus III activity; however, it appears to be an insensitive measure of effective complex III activity in cells, as no deficiency was detected in the cybrid with 95% 3243G:C mtDNA, whereas this cybrid showed a large decrease in succinate-driven respiration in permeabilised cells (Fig. 3B). The subunits of complex II are nuclearily encoded and succinate dehydrogenase activity (28) was similar in cybrids with 0% or with 95% 3243G:C mtDNA and with ρ- cells (data not shown). Presumably then, measured SCCR activity is normal unless complex III activity is all but abolished. The possibility that our SCCR assay was erroneously detecting solely complex II activity can be excluded, as SCCR activity was not detectable in the ρ- cells. Therefore measurement of SCCR appears not to be a reliable indicator of flux through respiratory complexes II and III.

The finding that respiratory function may decrease over time in 143B osteosarcoma cells means that caution should be exercised in comparing data on 143B cybrids from different laboratories. In particular it demonstrates the importance of generating control cybrid lines that contain mtDNA derived from a healthy subject.

Several possible pathogenic mechanisms have been proposed for the MELAS 3243G:C mtDNA mutation. The 3243G:C mtDNA mutation lies in a tridecamer sequence that acts as a transcription termination signal (16). Although it has been shown to affect transcription termination in vitro (8,17) there is, as yet, no evidence of such an effect being of pathological relevance in vivo (7,8,18). A possible defect in RNA processing was implied
by the detection, in cybrids harbouring high levels of 3243G:C mtDNA, of increased levels of a precursor-like RNA (RNA 19) that contains the tRNA\textsuperscript{Leu\textunderscore UUR} gene (7). Abnormal processing of the ND1 precursor transcript in muscle has also been proposed as the mechanism by which a mutation at nucleotide position 3302, in the tRNA\textsuperscript{Leu\textunderscore UUR} gene, may exert its effects, including decreased complex I activity (29). A direct failure of translation due to the mutant form of tRNA\textsuperscript{Leu\textunderscore UUR} associated with MERRF was suggested by the experiments of Enriquez et al. (30). They demonstrated that mitochondrial protein synthesis was impaired due to premature termination of translation in cybrids with high levels of 8344G:C mtDNA mutation. Our data are not consistent with a direct effect of mutant tRNA\textsuperscript{Leu\textunderscore UUR} on mitochondrial translation. Although ND6 has a high number of leucine residues specified by tRNA\textsuperscript{Leu\textunderscore UUR} (14 of 174 amino acids), ND3, which did not show a marked decrease in translation (Fig. 4B), also contains a high proportion of leucine residues specified by tRNA\textsuperscript{Leu\textunderscore UUR} (9%). Moreover, the distribution of leucine residues specified by tRNA\textsuperscript{Leu\textunderscore UUR} is similar in the two polypeptides. Thus, it is unlikely that the mutant tRNA is causing failure of translation in the manner proposed for the MERRF 8344G:C mutation.

The observation of complex I deficiency in 206.3243 cybrids provides a parallel with the biochemical phenotype in a subset of patients with partial mtDNA deletions. Polargraphy on isolated muscle mitochondria of patients with partial deletions identified three sub-groups (31–32); patients with respiratory rates in the normal range, patients with multi-enzyme deficiencies, and patients with a specific deficiency of complex I. It was noted that the deleted protein encoding genes, in patients with a specific complex I deficiency, all specified subunits of complex I (31–32). The observation of a specific deficiency of a complex I subunit in 206.3243 cybrids goes some way to explaining the similarities in histopathology and clinical phenotype between the MELAS mitochondrial tRNA gene mutation and partial mtDNA deletions (15,32).

MATERIALS AND METHODS

Cell culture

Patient myoblasts and control fibroblasts were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), including pyruvate and 10% fetal bovine serum (FBS), prior to fusion with 143B.206.TK\textsuperscript{−} cells. Fusions were performed as described (10). The resultant cybrid cell lines and parental cells (143B.TK\textsuperscript{+}) were maintained in DMEM with 10% FBS with 5-bromodeoxyuridine (100 µg/ml). The p\textsuperscript{+} cells were additionally dependent on 50 µg/ml uridine (6). Tissue culture reagents were purchased from Life Technologies (Bethesda, MD). Cybrid clonal cell lines from the fusion of p\textsuperscript{−} cells with MELAS myoblasts were termed 206.3243, whereas cybrids repopulated with mitochondria from cells of a healthy adult male were called 206.con.

Biochemical and DNA analyses

The rate of oxygen consumption of digitonin-permeabilised cells was measured by a modification of the method of Granger and Lehninger (19). Cells were harvested following trypsinization, resuspended at 1 × 10\textsuperscript{5} cells/ml in buffer A [20 mM HEPES, pH 7.1, 10 mM MgCl\textsubscript{2}, 250 mM sucrose and 0.01% (w/v) digitonin] and incubated on ice for 1 min. In our hands, incubation times of <1 min led to greater variation in the rate of oxygen consumption, particularly for complex I substrates. Results from 3 min incubations were not discernibly different from 1 min incubations. Following incubation on ice, nine volumes of buffer A without digitonin were added, with mixing, and the cells were then centrifuged at 400 × g for 60 s. The cell pellet was resuspended in 1/10 volume of buffer A, and placed on ice. Within 5 min, the cells were diluted twenty fold, to 5 × 10\textsuperscript{6} per ml with buffer B (20 mM HEPES, pH 7.1, 250 mM sucrose, 10 mM MgCl\textsubscript{2}, 2 mM potassium phosphate, 1.0 mM ADP) in a Clark type oxygen electrode (Hansatech) at 37°C with stirring. An aliquot from each experiment was retained for protein estimation (20). Substrates and inhibitors were prepared immediately prior to use and stored on ice. Final concentrations were 5 mM for sodium pyruvate, sodium succinate and ascorbic acid. For other reagents, the final concentrations were 200 µM TMPD, 100 mM rotenone, 4 mM antimycin A and 1 mM potassium cyanide. Intact cell oxygen consumption rates were also determined in a Clark type oxygen electrode with 500–1000 µl of 5×10\textsuperscript{6} cells/ml in RPMI 1640 medium without glucose (Life Technologies) (6). All oxygen consumption rates were expressed as fmol O\textsubscript{2}/min/cell.

The proportion of 3243G:C and 3243A:T mtDNA was estimated by Southern blotting Apol digests of amplified mtDNA (3122–3558). The blots were analysed with a GS 250 molecular imager (Bio-Rad) following hybridization to radiolabelled mtDNA. A control amplified fragment of human mtDNA (8278–9956) was co-digested with the 3122–3558 PCR product and was required to undergo complete digestion before proceeding with Southern blotting. Changes in the levels of 3243G:C and 3243A:T mtDNA have been observed over time (9,10), however, the percentage 3243G:C mtDNA was stable in all the cybrids analysed here (data not shown). The relative amounts of mitochondrial and nuclear DNA were determined by probing Southern blots simultaneously with labelled placental mtDNA and 18S ribosomal RNA. Standard enzymatic assays for cytochrome c oxidase (COX) (21), succinate cytochrome c reductase (SCCR) (22) and citrate synthase (CS) (23), were used. The methods of lactate and pyruvate measurement were adapted from Noll (24). Briefly, pyruvate production was determined by measuring the oxidation of NADH, in the presence of LDH, when combined with RPMI 1640 medium after 24 or 48 h incubation with cultured cells. Lactate was measured similarly except that the reduction of NAD was determined in the presence of hydradine hydrate 0.1% (v/v).

Mitochondrial translation products were labelled specifically by incubating approximately 5 × 10\textsuperscript{5} cells with 100 µCi/ml 35S-methionine (NEN) for 30 min in the presence of 10 µg/ml emetine. Following washing and harvesting, the cells were lysed in 500 µl of 1% SDS and vortexed for at least 30 s. Equal amounts of protein, as determined by the Bradford method (25), were loaded on to 15–20% gradient gels and electrophoresed at a constant 30 mA for 6–10 h. Gels were fixed overnight in 30% methanol, 10% acetic acid and treated with ‘Amplify’ (Amer-sham) prior to drying and autoradiography for 1–7 days at ~70°C. Translation products were assigned according to Chomyn et al. (11), with additional reference to pre-stained molecular weight standards (Bio-rad).

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