Nucleic Acids Research, 2006, Vol. 34, No. 13 ***e95***
doi:10.1093/nar/gkl516

Production of *trans*mitochondrial cybrids containing naturally occurring pathogenic mtDNA variants

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Received April 21, 2006; Revised July 5, 2006; Accepted July 6, 2006

ABSTRACT

The human mitochondrial genome (mtDNA) encodes polypeptides that are critical for coupling oxidative phosphorylation. Our detailed understanding of the molecular processes that mediate mitochondrial gene expression and the structure–function relationships of the OXPHOS components could be greatly improved if we were able to transfect mitochondria and manipulate mtDNA *in vivo*. Increasing our knowledge of this process is not merely of fundamental importance, as mutations of the mitochondrial genome are known to cause a spectrum of clinical disorders and have been implicated in more common neurodegenerative disease and the ageing process. *In organellar* or *in vitro* reconstitution studies have identified many factors central to the mechanisms of mitochondrial gene expression, but being able to investigate the molecular aetiology of a limited number of cell lines from patients harbouring mutated mtDNA has been enormously beneficial. In the absence of a mechanism for manipulating mtDNA, a much larger pool of pathogenic mtDNA mutations would increase our knowledge of mitochondrial gene expression. Colonic crypts from ageing individuals harbour mutated mtDNA. Here we show that by generating cytoplasts from colonocytes, standard fusion techniques can be used to transfer mtDNA into rapidly dividing immortalized cells and, thereby, respiratory-deficient *trans*mitochondrial cybrids can be isolated. A simple screen identified clones that carried putative pathogenic mutations in *MTRNR1*, *MTRNR2*, *MTCOI* and *MTND2*, *MTND4* and *MTND6*. This method can therefore be exploited to produce a library of cell lines carrying pathogenic human mtDNA for further study.

INTRODUCTION

The human mitochondrial genome (mtDNA) is a highly polymorphic molecule found in multiple copies in cells. The genome encodes 2 ribosomal RNAs and 22 tRNAs that are essential for translating the 13 mtDNA-encoded polypeptides in the mitochondrial matrix (1). Mutations of mtDNA can cause a wide spectrum of specialized disorders but have also been linked with common neurodegenerative disease and the ageing process itself (2–4). Our understanding of the exact molecular processes that mediate mitochondrial gene expression has been hampered by our inability to transfect mammalian mitochondria *in vivo*. Where possible, cell lines have been established from patients suffering with mtDNA disease and the cells have been investigated directly, or after transfer of the patient's mtDNA to a recipient cell line devoid of mtDNA (ρ^0) (5). Studies of such cell lines are manifold and have given an insight into critical residues of OXPHOS complexes both in their function and assembly (6–11), mt-tRNA residues essential for participation in intramitochondrial protein synthesis (12–14) and have led to the identification of a novel mechanism for translation-dependent decay (15). Given the polymorphic nature of the genome, however, it is often difficult to determine that a substitution is indeed pathogenic and is not a neutral polymorphism (16). This is particularly true for sequence variations in the two ribosomal RNA genes. In the absence of a transfection procedure for mitochondria, it is difficult to see how mt-rRNA nucleotides critical for mitoribosome assembly and

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function might be identified unless a large library of defined rRNA mutations was available.

In a recent study, we have shown that human colonic crypt stem cells accumulate a high level of mtDNA mutations as a consequence of ageing, resulting in respiratory chain deficiency (17). Many mutations were present in mt-tRNA, mt-rRNA and structural genes of cytochrome *c* oxidase (COX), leading to a mosaic pattern of COX-deficiency, which in some individuals accounted for up to 30% of the total number of crypts. Moreover, analysis of this tissue revealed a point mutation profile that is very different from that observed in patients with inherited mtDNA disease. Many missense and nonsense mutations in the *MTCO* genes were detected in COX-deficient crypts, whereas in the COX-normal cells there were frameshift and nonsense mutations in *MTND* and *MTCYB* genes that are highly likely to cause respiratory chain deficiency involving either complex I or complex III, respectively. As it appears that colonic stem cells can tolerate a high rate of clonal expansion, with little or no selection against pathogenic mtDNA mutations, our observations present an opportunity to study in detail the pathogenic nature of an almost limitless number of mtDNA mutations provided enough cells can be obtained to perform meaningful biochemical analysis. Colonocytes can be isolated from colonic sections and can be grown under strict conditions (18). This is a difficult procedure and the extensive doubling time of the cultured colonocyte precludes the routine expansion that would be necessary to produce sufficient material for standard analytical techniques. Further, successful growth and expansion of colonocytes defective in oxidative phosphorylation have never been demonstrated and derived primary cell lines would be prone to senescence. Therefore, the generation of a useful library of mtDNA mutations would only be possible if the mutated mtDNA could be transferred to immortalized ρ^0 cells.

In this paper we report the successful production of *trans*mitochondrial cybrids containing mtDNA from colonic crypts. These data infer that this method will be useful for the production of a large library of cell lines harbouring pathogenic mtDNA mutations.

METHODS

Preparation of human colonocytes

Normal human colonic tissue was obtained from two patients that had surgical specimens removed as treatment for adenocarcinoma. Human colonocytes were prepared according to the method of Whitehead *et al*. (18). Briefly, a 1 × 5 cm piece of tissue was trimmed of all muscle and the mucosal layer was washed in PBS. The tissue was incubated in 0.04% (v/v) sodium hypochlorite in PBS for 15 min to sterilize the surface, washed in PBS and then incubated in 3 mM EDTA plus 0.05 mM dithiothreitol (EDTA/DTT) in PBS for 90 min. After a PBS wash, the sample was shaken vigorously for 20 s to liberate the crypts from the submucosa. Crypts were transferred to a centrifuge tube and fresh PBS was added to the colonic tissue. The shaking step was repeated for three or four times until the crypt yield diminished. Crypt suspensions were centrifuged gently at 28 *g* for 5 min, resuspended in fresh PBS and pooled. After further centrifugation, the crypts were resuspended in 20 ml of 0.25% pancreatin in PBS and incubated for 90 min. The cell suspension was diluted with an equal volume of PBS and centrifuged at 170 *g* for 5 min. The cell pellet was resuspended in EDTA/DTT, pelleted and resuspended in PBS. The cell suspension was gently drawn through an 18-gauge needle, then through a 21-gauge needle to disrupt cell aggregates and, thus, a single cell suspension was obtained.

Preparation and fusion of cytoplasts

Cytoplasts were produced by centrifugation on a Percoll isopycnic gradient according to the method of Trounce *et al*. (19). Briefly, 2×10^7 colonocytes were resuspended in 20 ml of 1:1 mixture of Percoll/DMEM, enucleated by the addition of 20 μg/ml cytochalasin B and centrifuged at 44 000 *g* for 70 min at 20°C. Cytoplasts were recovered, diluted and pelleted at 600 *g* for 10 min. In order to check the efficiency of enucleation during development of the fusion method, cells were labelled with 1 μM Mitotracker (Molecular Probes) for 45 min before enucleation and 50 μg/ml DAPI after enucleation.

Cytoplasts were resuspended in DMEM, mixed with 143B ρ^0 cells in a 1:1 ratio and centrifuged at 1100 *g* for 5 min to form a pellet. DMEM was removed and the cells were resuspended in 0.8 ml of 50% (v/v) polyethylene glycol 1500 (BDH Limited). After 60 s, the cells were gently resuspended in DMEM and left to recover at 37°C for 45 min. Cells were then plated in DMEM supplemented with 4.5 mg/ml glucose, 10% (v/v) FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml uridine, 0.11 mg/ml sodium pyruvate and 2 mM L-glutamine at a variety of cell densities (5×10^3 to 2×10^4 ρ^0/ml). After 24 h, the medium was replaced with the supplemented DMEM lacking uridine to select against unfused ρ^0 cells. Medium was changed every 3 days and colonies were isolated using cloning rings before being transferred to 24-well plates for further expansion.

Cell fusions were also carried out with the A549.B2 ρ^0 lung carcinoma cell line and cytoplasts from the 143B *trans*mitochondrial cybrids 1.12 and 1.14 using the method outlined above. Twenty-four hours after fusion the medium was replaced with DMEM lacking uridine to select against unfused A549 ρ^0 cells. The medium was also supplemented with HAT media to select against non-enucleated 143B colonocyte cybrids. Clones were isolated as above.

Galactose growth assay for the selection of respiratory defective clones

All cybrid clones and corresponding parental and ρ^0 cell lines were plated in six-well plates at a density of 2.5×10^4 cells/well. Cells were grown in DMEM without glucose (Gibco, Invitrogen 11966-025) supplemented with 10% (v/v) FCS, 0.9 mg/ml galactose and 0.11 mg/ml sodium pyruvate. Cells were trypsinized from replicate plates after 3, 5, 7 and 10 days and total cell counts were taken and averaged.

Isolation of total cellular DNA

Cells were washed in PBS, trypsinized and digested overnight in 1% (w/v) SDS and 2 mg/ml proteinase K at 37°C. The DNA was purified by phenol/chloroform extraction followed by ethanol precipitation.

Genotyping of cybrid clones

PCR was performed with a GenePrint fluorescent STR Multiplex-GammaST kit (Promega, UK) according to the kit protocol recommendations. The GammaST kit contained four short-tandem repeat loci D16S539, D7S820, D13S317, D5S818 and one of each pair was labelled with fluorescein. PCR products were separated on a 5% Long Ranger polyacrylamide denaturing gel and detected by a laser-based detection system using the ABI 377 DNA sequencer (Perkin-Elmer, Warrington, UK). The gel image was analysed using Genescan 672 version 1.2 software (ABI-PerkinElmer). DNA fragments were sized using a 60–400 bp fluorescent ladder (Promega, UK). Data were imported into the Genotyper version 2.0 software (ABI-PerkinElmer, UK) to convert the DNA fragment size data into genotypes. Alleles in four loci were analysed for all samples.

Sequencing of mtDNA

The entire sequence of the mitochondrial genome was amplified by a two-stage strategy and a panel of M13-tagged primer pairs, essentially as described previously (20). PCR-amplified products were purified to remove unincorporated primer (Montage PCR filters, Millipore) and sequenced using BigDye (v3.1) terminator cycle sequencing chemistries on an ABI 3100 Genetic Analyser (Applied Biosystems). Sequence chromatograms were directly compared to the revised Cambridge reference sequence (rCRS) using SeqScape software (Applied Biosystems). All mtDNA sequence changes identified were first checked against the MITOMAP database of known, mtDNA polymorphic variants (http://www.mitomap.org) (21). If changes were not listed in MITOMAP, a further two databases (http://www.genpat.uu.se/mtDB/index.html) and that previously held at MitoKor (N. Howell, personal communication) were checked to verify whether reported sequence changes were polymorphic or likely to result in respiratory chain deficiency.

Enzymatic assays

Mitochondrial isolation and spectrophotometric assays of complexes I and IV and citrate synthase activities were carried out as described previously (22).

Gel electrophoresis and western blotting

Immunodetection analyses were performed on mitochondrial fractions isolated from the 143B cell lines and both cybrid clones. Samples (15 μg) were denatured and electrophoresed on a 15% SDS–polyacrylamide gel before transfer in CAPS buffer on to Immobilon-P transfer membrane (Millipore) at a constant current of 300 mA for 3 h. Monoclonal antibodies against the membrane proteins were purchased from Mito-Sciences (OR, USA) or Molecular Probes. Mitochondrially enriched fractions and Blue Native gels were essentially as described in (23). Briefly, control parental and fused cybrid cell lines (1×10^6 cells per sample) were harvested, washed in PBS and permeabilized with digitonin (4 mg/ml final) at 4°C for 10 min. Five volumes of ice-cold PBS were added followed by centrifugation at 10 000 *g* for 5 min at 4°C. Pellets were washed in PBS, resuspended in 1.5 M ε-amino caproic acid, 75 mM Bis–Tris, pH 7.0, solubilized with *n*-dodecyl β-D-maltoside and electrophoresed through a continuous 5–15% acrylamide gel with a 4% stacking gel. Transfer on to Immobilon membranes was performed in Towbin buffer for 1 h at 100 V.

Cytochrome spectral analyses

The cell pellets (containing $1.3–2.3 \times 10^7$ cells) were resuspended in 500 μl of 5% (w/v) ficoll and reduced by dithionite. Spectra were generated by scanning the cell suspensions with a single beam spectrophotometer built in-house and operating at room temperature.

Polarographic analyses

Oxygen consumption studies of intact cells were performed essentially as described previously (24), after resuspension in a Tris-based Mg^{2+}-, Ca^{2+}-deficient buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Tris–HCl, pH 7.4 at 25°C) and introduction into a high-resolution oxygraph chamber (Oroboros, Innsbruck). 2,4-Dinitrophenol (DNP) was titrated until maximal uncoupling was reached (30–60 μM), and respiration was inhibited by antimycin (20 nM). For substrate-specific respiration, a Tris-based permeabilization buffer was used [75 mM sucrose, 5 mM potassium phosphate, 40 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$, 0.35% (w/v) BSA and 30 mM Tris–HCl, pH 7.4 at 25°C] and digitonin was added at a concentration of 15 $\mu g/10^6$ cells. Traces were read after multiple substrate–inhibitor titration with pyruvate/malate (8/2 mM), glutamate (15 mM) and rotenone (100 nM), followed by succinate (10 mM) and antimycin (20 nM). Individual inhibitor-sensitive oxygen consumption rates were calculated as the time derivative of the oxygen concentration (DATLAB Analysis Software, Oroboros) and analysed using SPSS software. Data shown represent inhibitor-sensitive values as the means ± SD of 3–6 determinations and are expressed in fmol O_2/min/cell.

RESULTS

Generation and identification of respiratory-deficient *trans*mitochondrial cybrids

Human colonic crypts harbour substantial intercrypt heteroplasmy (mtDNA with sequence divergence). Further, individual crypts from regions of colon believed to be physiologically normally have been shown to carry mtDNA mutations that cause profound defects of cytochrome *c* oxidase activity. To determine whether it was possible to trap and identify these aberrant mtDNA molecules, cytoplasts were generated from a section of colon taken initially from donor 1 as detailed in Materials and Methods. Recipient osteosarcoma 143B ρ^0 cells were prepared and cytoplasts were fused. After fusion, cells were plated in a medium lacking uridine (to select against unfused ρ^0 recipients). After ~2 weeks, the majority of cells had died, with a small number of clones remaining, potentially carrying mtDNA from donor 1 cytoplasts. To assess whether these cells were *trans*-mitochondrial cybrids, 21 individual colonies were picked and expanded before DNA isolation as detailed. PCR amplification and DNA sequence analysis of the non-coding region confirmed that the cells contained mtDNA with a sequence identical to the donor 1 colonic crypts (data not shown).

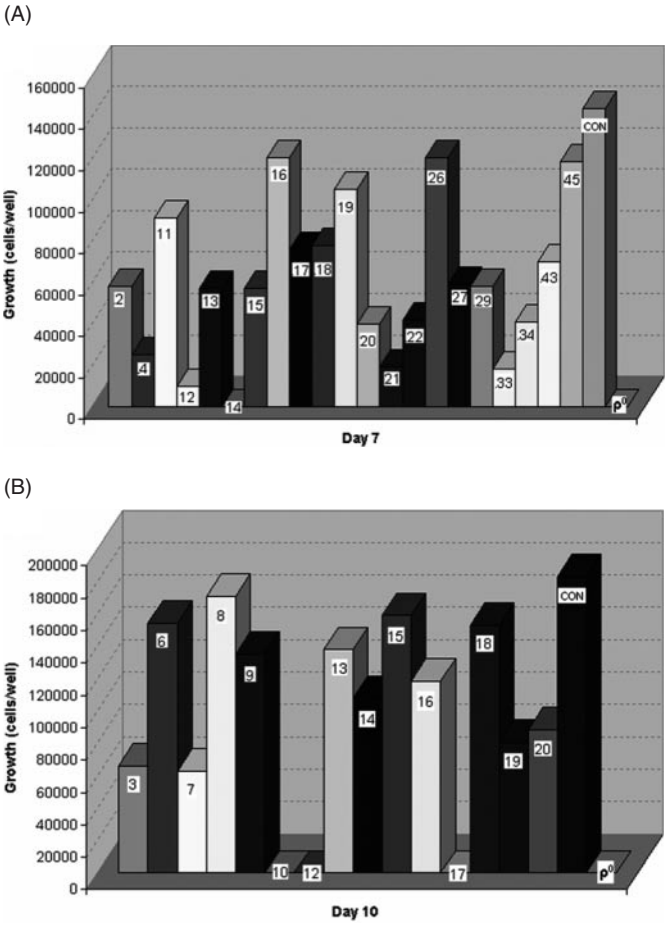

Figure 1. Growth of *trans*mitochondrial cybrid clones on galactose. Cybrids were generated as detailed in Materials and Methods (**A**). Twenty-one individual clones from donor 1 were assessed for growth in galactose. (**B**) Fifteen clones were also assessed from a second donor. Cell counts were performed and averaged from replicate plates after 3, 5 and 7 days (donor 1) and additionally after 10 days (donor 2). For brevity, cell counts are only shown for the final time point.

To determine whether the transferred mtDNA carried mutations that caused a respiratory deficiency, clones were screened for growth on a non-fermentable carbon source. All clones were expanded and grown as replicas with either glucose or galactose as a carbon source over a period of 7 days. As shown in Figure 1A, cell counts were variable between clones but similar trends were seen from two repeats. Two clones compromised for growth on galactose, 1.12 and 1.14, were taken for further study. First, to confirm that the nuclear background of the colonocyte cybrids was from the recipient osteosarcoma cell line alone, DNA was extracted from cultures of 143B ρ^0, clone 1.14 and from a homogenate sample of the colon from donor 1. Genomic alleles were compared at four different loci and analysed using Genotyper software. Figure 2 shows that clone 14 displayed an identical nuclear genotype to the 143B ρ^0 cell line and that the patient colon DNA contained different alleles at all four loci. As the 143B cells are notoriously aneuploid, the poor growth could also have been due to a defective gene in the host nucleus of the recipient clone or to aberrant gene dosage. Second, to confirm that the lack of growth

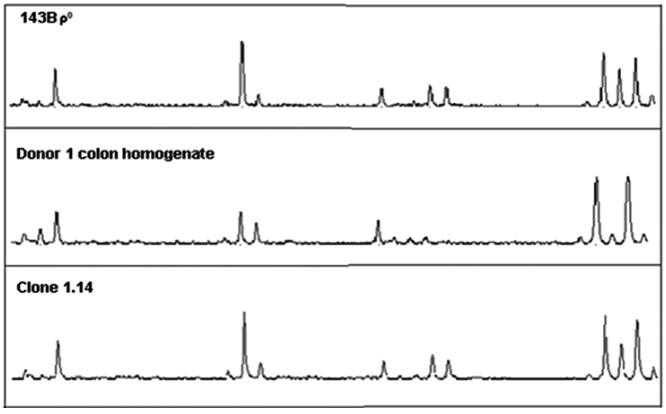

Figure 2. Genotype analysis of clone 1.14 DNA was extracted from cultures of the recipient 143B ρ^0, clone 1.14 and from a colon homogenate sample from donor 1. PCR was carried out as detailed in Materials and Methods using a fluorescent multiplex GammaSTR kit and PCR products were separated on a 5% polyacrylamide denaturing gel. The gel image generated was analysed with Genescan software to determine the sizes of PCR fragments and Genotyper software was then utilized in order to convert the fragment size into genotypes. Analysis of the four loci showed that the genotype of the 143B ρ^0 cell line is identical to that of clone 1.14 yet different to the donor colon, confirming the nuclear background of the cybrid.

was due to the transferred mtDNA, cytoplasts were prepared from the two clones and fused to a second ρ^0 cell line, the lung carcinoma A549.B2. Both resultant *trans*mitochondrial cybrids were severely compromised for growth on media containing galactose as a sole carbon source, confirming that the mutations were indeed mtDNA borne (data not shown).

Identification of pathogenic mtDNA mutations captured from colonic crypts

During the production of the A549 cybrids, a colonic sample was retrieved from a second donor and cybrids were prepared as detailed above. Similar to the cybrids produced from donor 1, 15 resultant clones showed variable growth on galactose (Figure 1B). To identify the mutations underlying the respiratory defect, total mitochondrial genomic sequencing was employed on DNA extracted from clones 1.12 and 1.14 plus six putative respiratory-compromised cybrid clones from the second donor and compared against the sequence of mtDNA extracted from the donor tissue. Table 1 summarize the base substitutions that were unique to the clones and Supplementary Tables 1 and 2 list all the sequence variations from the revised Cambridge Reference Sequence. Clone 1.14 carried a single 5958T>C base transition from the donor homogenate mtDNA, in *MTCOI*. RFLP analysis was unable to detect any remaining wild-type DNA consistent with this being a homoplasmic mutation (Supplementary Figure 1). Clone 1.12, in distinction, carried three transitions in rRNA, two in the gene encoding the 16S mt-rRNA (*MTRNR2,* 1758T>C;3039T>C) and one encoding the 12S mt-rRNA (*MTRNR1*, 1411G>A). In each case, no heteroplasmy was apparent by sequence analysis. Although we did not formally assess levels of heteroplasmy for these three sites, in our experience we have never seen >10% mutation load when a site appears homoplasmic by sequencing and is then

subjected to RFLP analysis (Taylor *et al*., unpublished data). From the second fusion, three of the clones contained heteroplasmic mtDNA mutations, as judged by repeat DNA sequence analysis. These mutations were present in different *MTND* genes and predicted amino acid replacements in highly conserved positions. We are unable to conclude that all six clones derived from donor 2 harboured pathogenic mtDNA mutations. For confirmation of their pathogenicity, it would be necessary to re-fuse these cytoplasts into naïve ρ^0 cells, as had been performed with the two clones from the first fusion. In this respect, it is interesting to note that mtDNA from clone 2.10 was unable to grow on galactose yet full genome sequencing did not reveal any variation from the donor's DNA, consistent with the compromised growth being due to a nuclear-borne defect.

Table 1. Unique base substitutions identified in *trans*mitochondrial cybrids

Clone	Base substitution*	Affected gene	Predicted change
1.12	1411 G>A	*MTRNR1*	—
	1758 T>C	*MTRNR2*	—
	3039 T>C	*MTRNR2*	—
1.14	5958 T>C	*MTCOI*	Y19H
2.3	11055 T>A*	*MTND4*	L99Q
2.7	2943 G>A	*MTRNR2*	—
2.10	—	—	—
2.12	14361 A>G*	*MTND6*	W105R
2.17	5458 T>C*	*MTND2*	I330T
2.19	4647 T>C	*MTND2*	F60L

The entire mitochondrial genome was sequenced from two and six respiratory-deficient independent cybrids generated from donors 1 and 2, respectively. Listed are all the substitutions unique to the cybrids and not present in mtDNA amplified from donor homogenate. All base substitution differing from the revised Cambridge Reference Sequence (34) are reported in Supplementary Tables 1 and 2. Asterisks denote that the substitution is present in heteroplasmic form as confirmed by repeat DNA sequencing. No substitutions were apparent in mtDNA from 2.10, suggesting that this clone carries an altered nuclear genotype.

Analysis of *trans*mitochondrial cybrids

Following confirmation that their mtDNA carried pathogenic mutations, the two clones 1.12 and 1.14 were expanded and the mitochondria was isolated for further analysis. For 1.12, western blots showed a considerable decrease in the steady-state levels of proteins in complexes I and IV, consistent with a global mitochondrial translation defect (Figure 3A). This was supported by the low level of the assembled complexes and in-gel activities as determined by one-dimensional BN-PAGE (Figure 3B and Supplementary Figure 2). In contrast, 1.14 which harboured a single mutation in *MTCOI* retained normal steady-state levels and activity of complex I, but BN-PAGE assays showed a profound decrease

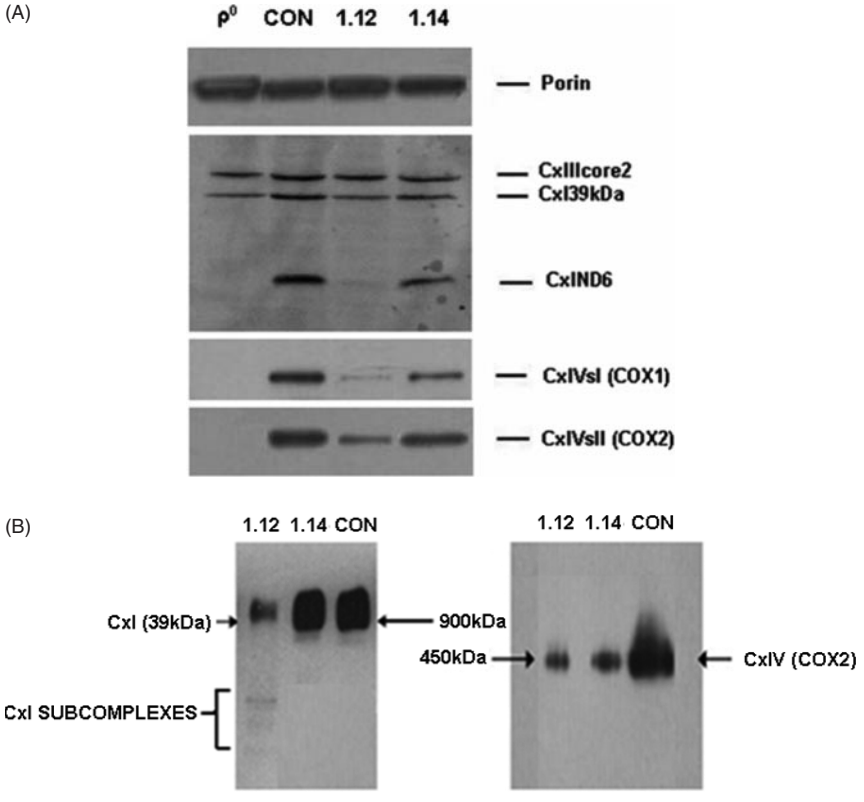

Figure 3. Western blot analysis of respiratory-deficient *trans*mitochondrial cybrids. Mitochondrial protein was isolated from 143B.ρ^0 (ρ^0), parental (Con) and the two *trans*mitochondrial cybrids 1.12 and 1.14. (**A**) Fifteen micrograms were subjected to western blot analysis using antibodies against complex I (nuclear-encoded 39 kDa or mtDNA-encoded ND6), complex III (nuclear-encoded core 2) and complex IV (mtDNA-encoded COX1 and COX2). All mtDNA-encoded polypeptides are substantially depleted in clone 1.12, consistent with a general translation defect. Clone 1.14 harboured lower steady-state levels of COX proteins but were still substantially higher than either the 143B cell line lacking mtDNA (ρ^0) or 1.12. Equal loading of mitochondrial protein was confirmed with an antibody specific to the outer membrane protein, porin. (**B**) A total of 100 μg of protein was subjected to a non-denaturing gel before visualization of intact complex I and complex IV with anti-39 kDa and COX2 antibodies, respectively. Clone 1.12 is depleted for both holoenzymes, whereas 1.14 has a specific decrease in complex IV. Minor subcomplexes of complex I can be seen in mitochondria isolated from 1.12.

in COX assembly and activity (Figure 3 and Supplementary Figure 2). This decrease was supported by biochemical analysis, where complex IV activity when normalized to citrate synthase was 0.29 compared to 2.13 for the 143B parentals. Complex I activities were similar (complex I/CS 0.29 cf. 0.32 for parentals). Interestingly, western analysis of 1.14 consistently showed a lower but significant level of the mtDNA-encoded COX subunits I and II. Spectroscopic analysis of mitochondria isolated from the two clones and controls are shown in Figure 4A. These data are broadly in agreement with the other assays, showing a profound decrease of cytochrome aa_3 in both clones, with an apparent sparing of cytochromes bc_1 in 1.14 consistent with an assembled complex III that is low or absent in 1.12. Polarographic studies underline the *in vivo* consequences on oxidative phosphorylation in these cell lines (Figure 4B). Inhibitor-sensitive oxygen consumption was abolished in the 143B ρ^0 cell line (data not shown), and intact cell respiration (ENDO) was decreased to ~14 and 60% in 1.12 and 1.14 cell lines, respectively, when compared to parental controls. When substrate-specific respiration was measured in permeabilized cells, 1.14 showed only an approximate 25% reduction in comparison to the profound complex IV defect measured by spectrophotometric analysis. These data suggest that complex IV exerts a lower control strength on respiration in 143B cells than was reported previously (25). This difference may be explained by the aneuploid nature of this cell line, as a weaker COX control strength was more recently detailed (26). In the case of the 1.12 clones respiration studies reiterated the finding of a global mitochondrial translation defect.

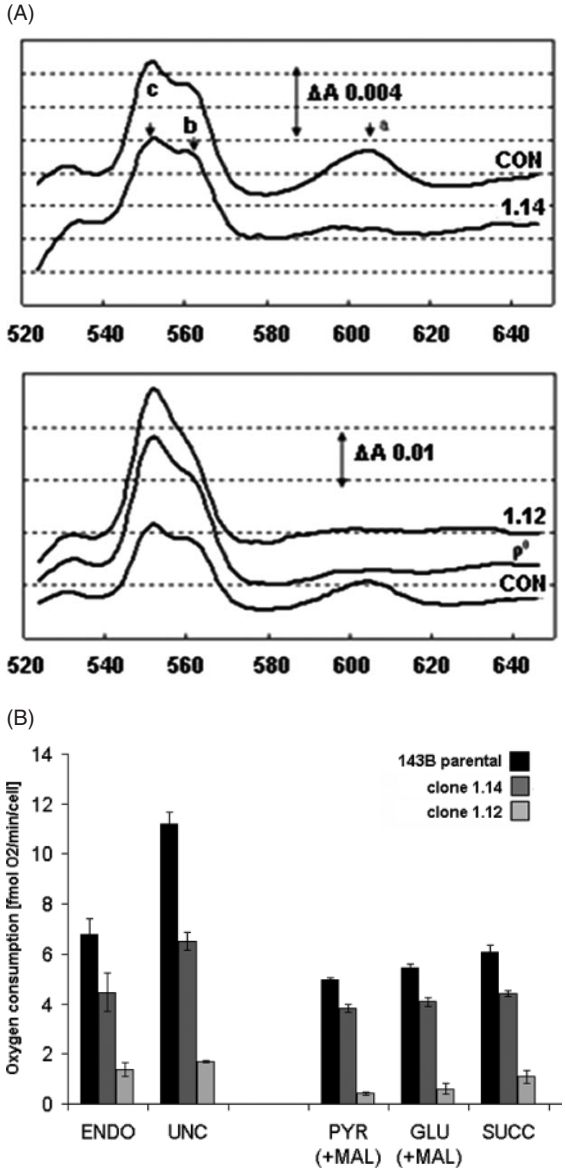

Figure 4. Spectrophotometric and polarographic analyses of respiratory-deficient *trans*mitochondrial cybrids. (**A**) Whole cell suspensions of *trans*mitochondrial cybrids 1.12 and 1.14 along with 143B. ρ^0 and parental cells were dithionite reduced and scanned at room temperature. Distinct peaks can be seen corresponding to cytochrome $c + c_1$ (c), b (b) and aa_3 (a) for the 143B parental line. There is little evidence of cytochrome aa_3 in either of the clones or the ρ^0 line a decreased level of complex IV. Cytochrome b depletion is also noted in 1.12, consistent with defective mitochondrial translation. (**B**) Cells (143B parental, clone 1.14, clone 1.12) were suspended in incubation media and assessed for respiration as detailed in Materials and Methods. Clone 1.12 exhibits a major decrease in endogenous respiration rate when compared to the control 143B parental cells. All lines showed a stimulation of activity on uncoupling of the respiratory chain by dinitrophenol (UNC). Again, the respiration rate of 1.12 with all respiratory substrates was substantially reduced, consistent with a defect in mitochondrial translation. Clone 1.14 showed a decreased rate of respiration when compared to 143B parentals, but this depletion was not marked, suggesting that complex IV does not have a major control strength for respiration in these cells.

DISCUSSION

Mitochondrial gene expression is essential for life in mammals yet our knowledge of this process is far from complete. This is in part due to our inability to transfect the mitochondrial genome, precluding the testing of many hypotheses. The molecular analysis of cell lines established from patients has led to an increased understanding in this area and researchers have benefited by being able to identify mtDNA mutations that are undoubtedly pathogenic. However, the number of cell lines carrying confirmed pathogenic mutations is still limited. The discovery that we all harbour pathogenic mtDNA mutations in our colonocytes, often at very high level is surprising, although variants have previously been identified in other tissues and successfully transferred to ρ^0 cells. Trounce *et al*. (27) reported the transfer of murine mtDNA from neuronal synaptosomes into ρ^0 cells. Although a low level of *trans*mitochondrial cybrids contained mtDNA variants, no pathogenic mutations were identified. Hayashi and co-workers (28) also found that synaptosomes in mice are a useful source of variant mtDNA molecules. In this case pathogenic mutations were found and have been extremely useful for the production of heteroplasmic mice, but the mutations identified were of large-scale mtDNA deletions. These are unlikely to he particularly informative about the function of specific nucleotides, as the mammalian mitochondrial genome is highly compact and large-scale deletions would invariably remove several vital genes. Crucially, human colonocytes harbour point mutations of the mitochondrial genome. Furthermore, sequence analysis has shown that the spectrum of these mutations is very different from those currently associated with inherited mtDNA disease (17). Therefore, in the absence of being

able to manipulate the genome at will, if we are to increase our understanding of mitochondrial gene expression it could be of great importance to try and trap such mutations. In this report, we have focused on two *trans*mitochondrial cybrids that are unable to respire efficiently. One cybrid, 1.14, carried a single mutation in the *MTCOI* gene predicting a Y19H mutation. Position Y19 is highly conserved between species and it is clear that Y19H causes a major defect in assembly of cytochrome *c* oxidase. Intriguingly, the original paper reporting the crystal structure of bovine heart cytochrome *c* oxidase considered the equivalent residue to be a potential member of a proton channel (29). Blue native gel and biochemical assays, however, suggest that the mutation does not impair activity of the assembled complex in the cybrids, although more detailed study would be needed to completely preclude such a role for this residue.

A library of mutations in mtDNA-encoded polypeptides would promote our understanding of structure–function relationships, but the identification of functionally important residues in the mitochondrial rRNAs is also of great importance. The second cybrid, 1.12, contained three substitutions in the *MTRNR* genes. The mitochondrial genome is extremely polymorphic, a phenomenon that has been of great use to evolutionary biologists. This variation, however, has complicated the association of disease with mtDNA mutation. For example, from 13 nt substitutions or rearrangements in *MTRNR1* that have been associated with maternally inherited hearing loss, only two have been confirmed to cause disease (21). These mutations have a very subtle effect on respiratory function and the defect only seems to be apparent on treatment with aminoglycoside antibiotics. The most common A1555G substitution occurs close to the 3′ end of the 12S mt-rRNA and is believed to introduce a new base pair at the base of a stem–loop structure close to the decoding site, facilitating access of the antibiotic to the ribosomal subunit leading to impaired or inaccurate translation (30,31).

MTRNR2 encodes the 1558 nt 16S mt-rRNA. This is the larger mt-rRNA species and 75 polymorphisms are reported in mitomap alone (21). Only three substitutions, however, have been associated with disease and none has been confirmed. Therefore, if we are to learn more about critical regions and nucleotides of mt-rRNAs, it is unlikely that current patient cell lines will prove to be a valuable source. Cryo-EM studies have helped to resolve the human mitoribosome to 13.5 Å, but although these studies will be useful in predicting crucial rRNA structures it will be necessary to support these predictions with direct mutational analyses (32,33). How, therefore, can we identify critical nucleotides in mt-rRNAs? The identification of the pathogenic mutation in the second clone 1.12 (and the putative pathogenic mutation in 2.7) illustrates the power of generating a library of *trans*-mitochondrial cybrids from colonocytes, although it is complicated by the concurrence of three individual mutations all appearing to be present at high levels, consistent with the mutations being present on the same molecule. A folding prediction for the human 12S mt-rRNA places the 1411G>A substitution in the middle of the stem of hairpin 42 in domain III. This is likely to cause a substantial weakening in the stem, possibly resulting in a translation defect. Folding predictions for human 16S mt-rRNA are incomplete, particularly towards the 5′ terminal where 1758T>C is located. This region, which replaces domain I of the bacterial 23S homologue, appears to be unstructured and its functional importance is unclear. The second 16S mutation, 3039T>C close to the 3′ end falls in the loop region of helix 92 in domain V. This is unlikely to cause a major structural perturbation but it is in an area of strong conservation between species and, hence, its importance cannot be ruled out. To be certain of the functional role of these individual nucleotides it will be necessary to select clones with mtDNA that carries only one of these mutations. In the absence of substantial recombination, it is unlikely that this will be possible for these particular mutations, but the power of the approach is clear. Identifying crucial residues in screens of large libraries of colonocyte-derived cybrids will complement the ever-increasing structural studies of the mammalian mitoribosome and with the bioinformatics tools available, a functional profile of both rRNA gene products will soon be generated.

In summary, we have been able to confirm that the human colon contains variant mtDNA and a subset of those variants are pathogenic. These mutations can be trapped as *trans*mitochondrial cybrids and as well as generating mutations that may prove to be informative in structure–function studies of these OXPHOS components, we believe this approach will be very useful for mapping critical functional residues in mitochondrial rRNA genes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

ACKNOWLEDGEMENTS

We wish to thank Richard Whitehead, Ian Trounce and Sheila Patel for their kind help and suggestions. Z.M.A.C.-L., R.W.T., D.M.T. and R.N.L. would also like to thank the Wellcome Trust for financial support (Award 074454). This work was partially supported by the European Community's sixth Framework Programme for Research, Priority 1 'Life sciences, genomics and biotechnology for health', contract number LSHM-CT-2004-503116. Funding to pay the Open Access publication charges for this article was provided by the Wellcome Trust.

Conflict of interest statement. None declared.

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