Nucleic Acids Research, 2001, Vol. 29, No. 15 e74

The determination of complete human mitochondrial DNA sequences in single cells: implications for the study of somatic mitochondrial DNA point mutations

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Received March 20, 2001; Revised and Accepted June 13, 2001

ABSTRACT

Studies of single cells have previously shown intracellular clonal expansion of mitochondrial DNA (mtDNA) mutations to levels that can cause a focal cytochrome *c* oxidase (COX) defect. Whilst techniques are available to study mtDNA rearrangements at the level of the single cell, recent interest has focused on the possible role of somatic mtDNA point mutations in ageing, neurodegenerative disease and cancer. We have therefore developed a method that permits the reliable determination of the entire mtDNA sequence from single cells without amplifying contaminating, nuclear-embedded pseudogenes. Sequencing and PCR–RFLP analyses of individual COX-negative muscle fibres from a patient with a previously described heteroplasmic COX II (T7587C) mutation indicate that mutant loads as low as 30% can be reliably detected by sequencing. This technique will be particularly useful in identifying the mtDNA mutational spectra in age-related COX-negative cells and will increase our understanding of the pathogenetic mechanisms by which they occur.

INTRODUCTION

Human cells contain multiple copies of mitochondrial DNA (mtDNA) located in the mitochondrial matrix. The mitochondrial genome is highly compact and exhibits little redundancy throughout its sequence of 16 569 bp, which encodes 37 genes: two genes encode ribosomal RNAs, 22 encode transfer RNAs (tRNA) and 13 encode polypeptides, all of which are essential proteins of the mitochondrial respiratory chain (1). The only non-coding region is the 1.1 kb mtDNA control region, which contains elements crucial for mtDNA replication and transcription (2).

Mutations of the mitochondrial genome are widely recognised as important causes of disease (3). These cover a broad spectrum of clinical manifestations affecting many different tissues, although many patients typically present with a progressive neurological syndrome (4). The genetic lesion involves either single, large-scale rearrangements which tend to be sporadic or maternally inherited point mutations in protein encoding or RNA genes. Pathogenic mtDNA mutations frequently coexist with wild-type mtDNA, a phenomenon termed *heteroplasmy*, with higher levels of mutation accumulating in post-mitotic tissues such as skeletal muscle, heart and the central nervous system (5). Single cell and tissue culture studies of pathogenic tRNA mutations have shown that this ratio of mutant to wild-type mtDNA critically determines expression of the genetic defect, and hence the clinical phenotype, with mutant loads in excess of 90% required to cause respiratory chain dysfunction (6–8). Since this proportion varies between different tissues, the identification of causative mtDNA mutations may prove difficult.

Less clear is the role of somatic mtDNA mutations in human pathology. Lacking protective histones and many of the repair mechanisms associated with nuclear DNA, the mitochondrial genome is highly susceptible to oxidative damage (9,10), and hence mutation. The age-dependent accumulation of somatic mtDNA deletions to levels that affect mitochondrial function is well documented in neurological tissue (11,12), with clonal expansion of individual mutational events restricted to single cells. As the level of deletion exceeds a critical threshold, this causes a biochemical defect, as shown by a selective decrease in the histochemical activity of cytochrome *c* oxidase (COX), a component of the respiratory chain whose three major catalytic subunits are coded by mtDNA (13,14). Similar patterns of focal COX deficiency in individual neurones have been described in spinal cord from ALS patients (15) and choroid plexus and hippocampus in patients with Alzheimer's disease (16), although the precise molecular defect in these cells is as yet unknown. Whilst various PCR-based technologies exist for the study of mtDNA deletions in single cells (13,14,17,18), sensitive techniques that can identify mtDNA sequence variants in single cells are nevertheless required to investigate whether acquired mtDNA point mutations might be responsible for the biochemical defect in these COX-negative cells.

Our method for determining the complete mtDNA sequence of a sample uses a series of 28 overlapping PCR-amplified fragments that span the entire length of the mitochondrial genome (19). As each PCR reaction requires ~100 ng total DNA template to generate sufficient product to sequence, ~3 µg total DNA template is needed for a complete genome analysis. Absolute amounts of total mtDNA, estimated at ~0.1% of the

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Table 1. Oligonucleotide primer pairs used for the primary PCR amplification of mtDNA from single cell lysates

Forward primer	Nucleotide positions	Reverse primer	Nucleotide positions	Product size (bp)	Template for stage 2 PCR[a]
SC-A-for	503–520	SC-A-rev	2484–2463	1982	1–3
SC-B-for	2364–2386	SC-B-rev	4249–4228	1886	4–6
SC-C-for	4155–4175	SC-C-rev	6220–6199	2066	7–9
SC-D-for	6113–6133	SC-D-rev	8017–7996	1905	10–12
SC-E-for	7925–7944	SC-E-rev	9884–9863	1960	13–15
SC-F-for	9767–9784	SC-F-rev	11748–11727	1982	16–18
SC-G-for	11614–11635	SC-G-rev	13638–13617	2025	19–21
SC-H-for	13539–13559	SC-H-rev	15431–15409	1893	22–24
SC-I-for	15331–15350	SC-I-rev	836–815	2075	25, D1–D3

All nucleotide positions shown refer to the original Cambridge Reference Sequence report (1).
[a]See Table 2 for details of the primers used in the secondary PCR.

total genetic complement of a cell, have been calculated in the femtogram range in single muscle fibres (20). In order to generate sufficient template to sequence, we have therefore devised a two-stage PCR strategy that permits the accurate amplification and sequencing of the entire (16.6 kb) mitochondrial genome from single cells. Here we describe the identification of homoplasmic, neutral polymorphic variants to prove the fidelity of this protocol, and the sequencing of single muscle fibres from a patient with a heteroplasmic T7587C COX II mutation to demonstrate its capability of detecting levels of mtDNA heteroplasmy sufficient to cause a biochemical defect.

MATERIALS AND METHODS

Patients

Control muscle was obtained from a young individual (12 years old) who underwent muscle biopsy for the investigation of exercise-induced muscle pain. The biopsy findings were unremarkable, with no evidence of COX-negative fibres or signs consistent with mitochondrial myopathy. The patient with the heteroplasmic T7587C COX II mutation presented with an extensive history of fatigue and unsteadiness and has been reported previously (21). The mutation changes the initiating methionine to threonine, consequently impairing the initiation of protein translation. The biochemical threshold of the T7587C mutation has been shown to be low, with levels of 55–65% mutation required to cause a defect in COX activity (21).

Cell culture

Human osteosarcoma-derived cell lines either containing mtDNA (143B.TK$^-$, ρ^+) or lacking mtDNA (143B206 ρ^0) (kindly provided by Dr M. Davidson, Department of Neurology, Columbia University, New York, NY) were grown as previously described (22) and total DNA was extracted using standard protocols. The absence of mtDNA in the 143B206 ρ^0 cells had previously been confirmed by Southern blot analysis.

Histochemical analysis of muscle tissue

Transversely orientated blocks were frozen in isopentane and cooled to –190°C in liquid nitrogen. Cryostat sections (20 µm) of skeletal muscle were assayed for COX activities or, in some cases, both COX and succinate dehydrogenase activities (23), permitting the identification of COX-negative fibres.

Isolation of DNA from single cells

Individual muscle fibres were microdissected using fine glass capillaries and placed in a sterile microfuge tube containing 12 µl of lysis buffer (50 mM Tris–HCl, pH 8.5, 1 mM EDTA, 0.5% Tween-20, 200 µg ml^{-1} proteinase K) (24). Cells were incubated at 55°C for 2 h, followed by heat inactivation of the proteinase K at 95°C for 10 min.

Mitochondrial DNA sequencing

Primary PCR reactions. A series of overlapping primer pairs (SC-A–SC-I; Table 1) were designed to amplify the human mitochondrial genome in nine fragments of ~2 kb from the single cell lysate. All PCR amplifications were performed in a 50 µl volume containing 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% w/v gelatin), 0.2 mM dNTPs, 0.6 µM primers, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 1 µl single cell lysate. Reaction conditions were 94°C for 12 min and 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min. The final extension proceeded for 8 min. The capacity of these primer pairs to amplify nucleus-embedded mtDNA pseudogenes (25,26) was investigated by performing each of the nine primary PCR reactions using either 500 ng ρ^+ or ρ^0 cell DNA as template.

Secondary PCR reactions. During the course of extensive sequencing investigations of patients suspected of having pathogenic mtDNA mutations, we have designed 28 pairs of primers to generate overlapping fragments of between 600 and 700 bp that span the entire sequence of the human mitochondrial genome. To facilitate the direct sequencing of PCR-amplified products, all primers are tagged with M13 sequence. Forward primers are tagged with 18 nt of –21 M13 forward sequence (5′-TGTAAAACGACGGCCAGT-3′) and reverse primers

Table 2. M13-tagged oligonucleotide primer pairs used for the secondary PCR amplification of mtDNA templates

Forward primer	Nucleotide positions	Reverse primer	Nucleotide positions	Product size (bp)
1F	516–534	1R	1190–1172	675
2F	1138–1156	2R	1801–1782	664
3F	1756–1776	3R	2444–2426	689
4F	2395–2415	4R	3074–3054	680
5F	2995–3013	5R	3645–3627	651
6F	3536–3553	6R	4239–4219	704
7F	4184–4202	7R	4869–4852	686
8F	4832–4849	8R	5570–5551	739
9F	5526–5545	9R	6188–6171	663
10F	6115–6134	10R	6781–6761	667
11F	6730–6750	11R	7398–7379	669
12F	7349–7369	12R	8009–7990	661
13F	7960–7979	13R	8641–8621	682
14F	8563–8581	14R	9231–9212	669
15F	9181–9198	15R	9867–9848	687
16F	9821–9841	16R	10516–10497	696
17F	10394–10414	17R	11032–11013	639
18F	10985–11004	18R	11708–11689	724
19F	11633–11651	19R	12361–12341	729
20F	12284–12302	20R	13005–12987	722
21F	12951–12969	21R	13614–13595	664
22F	13568–13587	22R	14276–14258	709
23F	14227–14246	23R	14928–14911	702
24F	14732–14752	24R	15419–15400	688
25F	15372–15391	25R	16067–16048	696
D1F	15879–15897	D1R	16545–16526	667
D2F	16495–16514	D2R	389–370	446
D3F	315–332	D3R	803–786	489

tagged with 18 nt of the reverse M13 sequence (5′-CAGGAAACAGCTATGACC-3′). Each primer pair is designed to anneal optimally at 58°C, thereby permitting simultaneous amplification of the 28 PCR reactions required to amplify a complete genome. For amplification of templates generated in the primary PCR of single cell lysates, 2 µl of this initial PCR product was used as template in the secondary PCR amplification. Reaction conditions (30 cycles) were exactly as described above, apart from the inclusion of 10% DMSO in all reactions with the exception of 6F/6R, 8F/8R, 16F/16R, 17F/17R and 23F/23R (Table 2) and a shortening of the extension time to 1 min. Inclusion of DMSO in these amplification reactions has been noted to markedly decrease the efficiency of amplification (unpublished observations). PCR-amplified products were subsequently purified (QIAquick PCR purification kit; QIAGEN) to remove unincorporated primer and sequenced directly using BigDye terminator cycle sequencing chemistry (PE Biosystems) on an ABI 377 automated DNA sequencer. The sequences generated were compared to the revised Cambridge Reference Sequence (rCRS) (19) using Sequence Navigator and Factura software (PE Biosystems).

Quantification of T7587C mutation in single muscle fibres

The level of mutated mtDNA was determined by PCR–RFLP analysis essentially as described elsewhere (21). A 455-bp fragment encompassing the mutation site was amplified with the M13-tailed mismatch forward primer (M13 sequence shown in lower case, mismatch base in bold) L7565 (nucleotide positions 7565–7585, 5′-tgtaaaacgacggccagtGGCTAAATCCTATATAT**G**TTA-3′) and the reverse primer H8001 (nucleotide positions 8001–7984, 5′-TCAACGTCAAGGAGTCGC-3′). Following 30 cycles of amplification, an additional cycle was performed in the presence of 5 µCi [α-^{32}P]dCTP (3000 Ci/mmol). Labelled products were precipitated and equal amounts (1000–2000 c.p.m.) digested with 8 U *Hin*dII (Roche Molecular Biochemicals). Restriction fragments were separated through an 8% non-denaturing polyacrylamide gel, dried onto a support and analysed with ImageQuant software (Molecular Dynamics) following exposure to a PhosphorImager. A single *Hin*dII recognition site in the wild-type product generates fragments of 309 and 146 bp. The T7587C transition, in concert with the mismatch base in the forward primer, generates an additional recognition site cutting the 309-bp fragment into two smaller products of 272 and 37 bp.

RESULTS

Two-step PCR amplification of mtDNA

In order to generate sufficient template to sequence whole mtDNA genomes from individual cells, we designed nine pairs of PCR primers to amplify fragments of ~2 kb that encompassed the entire mitochondrial genome (Table 1). Each primer pair was tested using total homogenate DNA extracted from ρ^0 (mtDNA-less) and ρ^+ cells and shown to amplify true mtDNA sequences (Fig. 1).

Subsequently, these primers were used to amplify mtDNA using single cell lysate as template. Figure 2 shows the primary PCR reaction products for one pair of primers using DNA extracted from six individual muscle fibres as template. PCR products from single fibres are barely visible on the gel (Fig. 2A, fibre 5), whereas a clear, visible product is generated using a control homogenate (ρ^+ cell) DNA. Using 2 µl of the 50 µl first round PCR product as template for the subsequent PCR reactions with the sequencing primers (Table 2), products of the expected size were amplified from each of the single fibre lysates (Fig. 2B). Importantly, no visible PCR products are evident in either of the two negative control lanes, indicating that these PCR products must have been amplified from the single cell template DNA.

Whole genome sequencing from individual muscle fibres

We next investigated the quality of mtDNA sequence derived from single cell amplifications. Eighteen individual muscle fibres were picked from a 20 µm section of control muscle and the DNA isolated was subjected to two rounds of amplification using primer pair SC-I (forward and reverse) for the first round of amplification and primer pairs D1, D2 and D3 (forward and reverse) for the second round. Together, these primers amplify

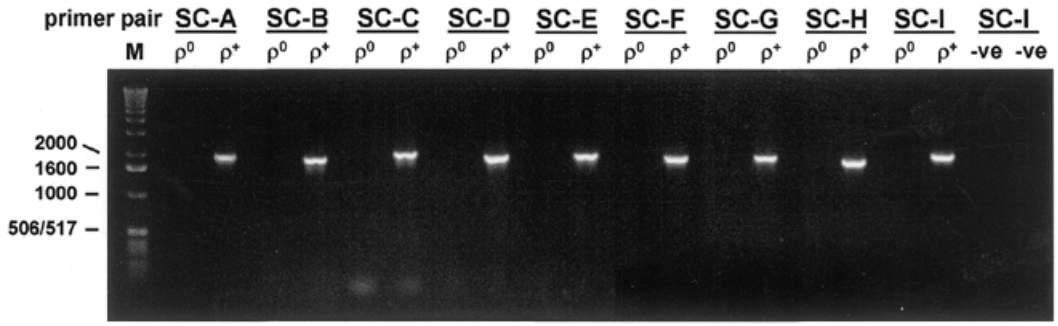

Figure 1. PCR amplification of ρ^+ and ρ^0 cell DNA using the nine primer pairs designed to amplify mtDNA from single cell lysates in the first round amplification. The predicted sizes of each of the products are given in Table 1. M, DNA size standard with sizes (bp) on the left; –ve, template omitted from PCR reaction.

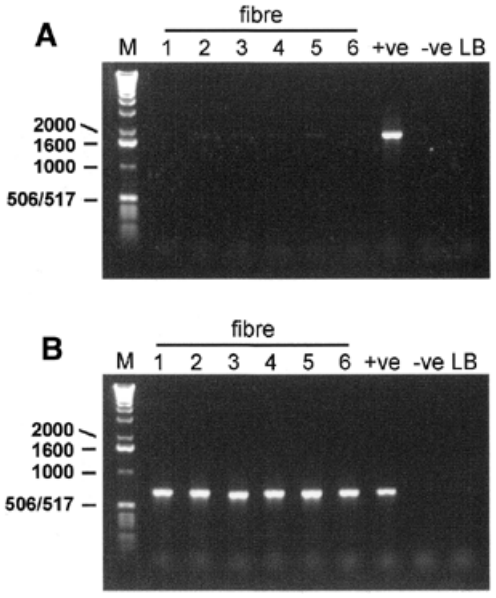

Figure 2. Two-step PCR amplification of mtDNA isolated from six individual muscle fibres. Representative gels of each round of amplification are depicted. (**A**) An aliquot of 1 µl total DNA template released by lysis of six individual fibres (1–6) was amplified (50 µl PCR reaction) by primer pair SC-D (Table 1). The expected size of this product was 1905 bp. (**B**) Amplified product (2 µl) from the first round of amplification was used as template in a second round of PCR with primer pair 12F/12R (Table 2). The expected size of this product was 661 bp. Appropriate controls are shown for each round of amplification. +ve, 500 ng ρ^+ DNA; –ve, template omitted from PCR reaction; LB, 2 µl aliquot of the single cell lysis buffer used in the DNA extraction; M, DNA size standard with sizes (bp) on the left.

across the polymorphic, non-coding control region or D-loop. All 18 fibres yielded excellent sequence, revealing 13 changes from the rCRS (Table 3) which were identical to the sequence derived from sequencing a DNA homogenate of this muscle (data not shown). No other sequence variations were observed that distinguished any fibre from the others and each change was represented in the MITOMAP database of sequence polymorphisms (27).

The DNA extracted from 9 of the 18 individual muscle fibres was subsequently subjected to further amplification with the remaining eight pairs of primary PCR primers and the entire coding region of mtDNA sequence deduced. The quality of the sequence information was excellent for every fibre, revealing a further 24 polymorphic variants from the rCRS (Table 4). These changes were present in all of the nine single fibres that were sequenced and were also present in the sequence from the muscle homogenate (data not shown). Most of these coding sequence changes had been previously described, whilst we have observed others (T1243C, A3505G, G5046A, C11674T, A11947G, T12414C and G15884C) in our own database of complete human mtDNA sequences (28). One silent change, G3531A in ND1, appeared to be novel.

Table 3. mtDNA D-loop sequence changes from the rCRS in individual muscle fibres (n = 18)

Nucleotide position	Nucleotide change	Comment
73	A→G	Common polymorphism[a]
143	G→A	Common polymorphism
189	A→G	Common polymorphism
192	T→C	Common polymorphism
194	C→T	Common polymorphism
195	T→C	Common polymorphism
196	T→C	Common polymorphism
204	T→C	Common polymorphism
207	G→A	Common polymorphism
263	A→G	Common polymorphism
311	C→CC	Common polymorphism
16223	C→T	Common polymorphism
16519	T→C	Common polymorphism

[a]Sequence variant documented in the MITOMAP database (27) as polymorphic.

Detection of mtDNA heteroplasmy in single cells

To establish the limits of detection of mtDNA heteroplasmy by single cell mtDNA sequencing, we performed experiments on individual muscle fibres from a patient with a well-characterised heteroplasmic T7587C mutation within the COX II gene (21).

Table 4. mtDNA coding sequence changes from the rCRS in individual muscle fibres (n = 9)

Nucleotide position	Gene	Nucleotide change	Amino acid change	Comment
709	12S rRNA	G→A	–	Common polymorphism
750	12S rRNA	A→G	–	Common polymorphism
1243	12S rRNA	T→C	–	Rare polymorphism[a]
1438	12S rRNA	A→G	–	Common polymorphism
2706	16S rRNA	A→G	–	Common polymorphism
3106	16S rRNA	C→CC	–	Common polymorphism
3505	ND1	A→G	T→A	Rare polymorphism
3531	ND1	G→A	Silent/P	Unreported polymorphism[b]
4769	ND2	A→G	Silent/M	Common polymorphism
5046	ND2	G→A	V→I	Rare polymorphism
5460	ND2	G→A	A→T	Common polymorphism
7028	COXI	C→T	Silent/A	Common polymorphism
8251	COXII	G→A	Silent/G	Common polymorphism
8860	ATPase 6	A→G	T→A	Common polymorphism
8994	ATPase 6	G→A	Silent/L	Common polymorphism
11674	ND4	C→T	Silent/T	Rare polymorphism
11719	ND4	G→A	Silent/G	Common polymorphism
11947	ND4	A→G	Silent/T	Rare polymorphism
12414	ND5	T→C	Silent/P	Rare polymorphism
12705	ND5	C→T	Silent/I	Common polymorphism
14766	Cyt b	C→T	I→T	Common polymorphism
15326	Cyt b	A→G	T→A	Common polymorphism
15884	Cyt b	G→C	A→P	Rare polymorphism
15924	$tRNA^{Thr}$	A→G	–	Common polymorphism

[a]Sequence variant documented in our own database of 66 complete mtDNA sequences (28).
[b]Sequence variant not previously reported.

Single fibre PCR–RFLP analysis confirmed that higher levels of the mutant 7587C allele were present in COX-negative fibres than COX-positive fibres (Fig. 3).

Next, by amplifying the DNA isolated from these individual fibres by the two-stage strategy, we were then able to correlate the quantifiable levels of the T7587C mutation with the sequence chromatogram. Using the primary PCR primers SC-D-for and SC-D-rev (Table 1) to amplify across the mutation site, the products obtained from both COX-positive and COX-negative fibres were used simultaneously as (i) template in the PCR–RFLP analysis of T7587C mutation load and (ii) template for PCR amplification with sequencing primer pair 12F/12R (Table 2). Representative data for this experiment are shown in Figure 4. Even in COX-positive fibres with loads of the 7587C allele in the region of 25–40%, there was an observable mutant C peak on the sequence chromatogram that was recognised by the software as different from the wild-type sequence. With higher levels of mutation, the relative proportion of C/T increased such that fibres with >65% mutant loads 'appeared' homoplasmic for the 7587C allele on the sequence chromatogram. A total of 26 individual muscle fibres (20 COX-negative, 6 COX-positive) were sequenced and every COX-negative fibre [mean level of mutant DNA 85.3 ± 11.8% (n = 20), range 54–98%] read a C on the sequence chromatogram. Together, these observations suggest that heteroplasmic mtDNA variants representing as low as 30% of the mtDNA genotype of a single cell can be detected by this sequencing method.

DISCUSSION

We have devised a two-stage PCR amplification strategy to determine the complete sequence of the human mitochondrial genome from single cells. The sequencing of 18 control regions and nine complete genomes equates to nearly 160 000 bases sequenced and every change from the rCRS observed was in agreement with the findings of other, independent experiments between different fibres. Furthermore, we were able to generate sufficient template to permit not only determination of the nucleotide sequence, but to subsequently investigate any potential pathogenic changes by other PCR-based technologies such as PCR–RFLP analysis (see Fig. 4).

The accumulation over time of somatic mtDNA mutations via clonal expansion of specific mutational events is a recognised phenomenon in non-dividing tissues. A broad spectrum of mtDNA deletions has been detected in various post-mitotic tissues of older individuals that are not observed in young control subjects (11,12). Despite these mutant mtDNAs accounting for <0.1% of total mtDNA in a tissue, individual cells within this tissue may contain very high levels of a single mutant mtDNA species (13). When the proportion of mutant mtDNA exceeds a critical threshold level, this results in an observable defect of respiratory chain function, which may eventually compromise organ function. In order to further our understanding of the role of somatic mtDNA mutations in ageing and other neurodegenerative disorders, it is essential to develop sensitive techniques that can detect and identify heteroplasmic mtDNA abnormalities in individual cells. Various PCR-based technologies exist for the study of mtDNA deletions (13,14,17,18), mtDNA copy number (20,29) and discrete mtDNA control region point mutations that have been implicated in the process of ageing (30,31). Nevertheless, the identification of novel point mutations is fraught with difficulties. Human mtDNA exhibits little redundancy of its coding sequence and, as such, a single base mutation in any region of the 16.6 kb genome could theoretically lead to a defect in mitochondrial protein synthesis and translation and hence oxidative phosphorylation. The ability to accurately determine the sequence of mitochondrial genomes from individual cells would therefore provide a powerful tool to investigate the pathophysiology of somatic mtDNA sequence variants.

Perhaps the biggest obstacle we faced in developing this technique was the generation of sufficient product to sequence the entire mitochondrial genome from a single cell containing femtogram amounts of template. Based on our findings, however, we are confident that even with such low amounts of starting template DNA from a single cell, the two-stage PCR faithfully amplifies only mtDNA sequence and does not result in the artefactual PCR amplification of mtDNA pseudogenes (25,26,32). By designing the nine primer pairs that generate template for the secondary, sequencing PCR reactions sufficiently far enough apart, co-amplification of mtDNA pseudogenes is

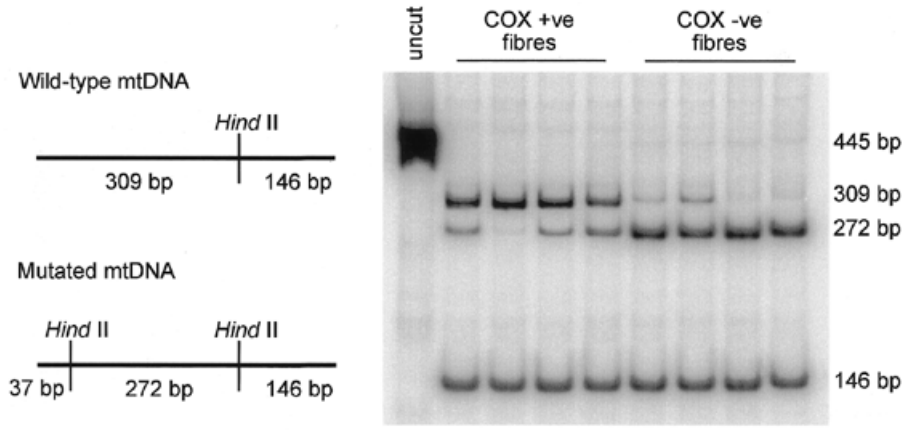

Figure 3. Quantification of the 7587C mutant allele in single muscle fibres expressing either a COX-positive or COX-negative phenotype. PCR and RFLP were performed as described in Materials and Methods and labelled products were separated through an 8% non-denaturing polyacrylamide gel. A schematic of the *Hin*dII digestion pattern expected in both wild-type and mutant mtDNA is shown on the left. Sizes of expected fragments (bp) are shown on the right of the gel.

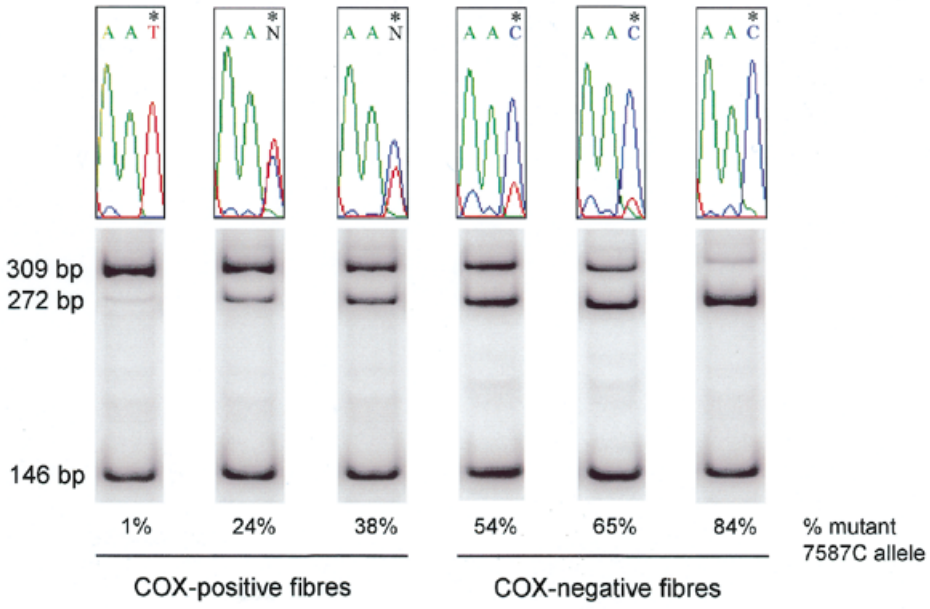

Figure 4. Detection of T7587C mtDNA heteroplasmy by sequencing individual muscle fibres. Products (2 µl) from the first round amplification of single cell DNA isolates by primer pair SC-D (Table 1) were used as template in both the PCR–RFLP analysis of T7587C mutation load and amplification with sequencing primer pair 12F/12R (Table 2). Comparative sequence chromatograms and RFLP gels are shown together. The mutated base is shown (*).

minimised. Since these primary PCR primers failed to generate a product when the mtDNA-deficient ρ^0 cell DNA was used as template, we conclude that only human mtDNA is amplified.

Somatic mtDNA mutations must accumulate to high levels before a biochemical defect is apparent and, as such, the identification of any unknown pathogenic mutation by sequencing is likely to be complicated by the coexistence of wild-type mtDNA in these cells. Many pathogenic mtDNA mutations are highly recessive, with expression of the biochemical abnormality exquisitely dependent on the ratios of the mutant and wild-type molecule. Experiments using primary cultures and transmitochondrial cybrids harbouring the A3243G $tRNA^{Leu(UUR)}$ and A8344G $tRNA^{Lys}$ point mutations have clearly demonstrated that mitochondrial function only becomes impaired with mutation loads in excess of 90% (6–8). The mutant load of these point mutations in COX-negative fibres from patients would be at a comparable level and would certainly be detected by sequencing through the relevant region of the genome. By studying a mutation (T7587C) with a low biochemical threshold for disease expression, an increasingly recognised phenomenon amongst mtDNA-encoded structural gene mutations (21,33–36), we confirmed that even the lowest level of mutant mtDNA observed in a biochemically affected cell (i.e. COX-negative) would be detected by this technique.

Recent studies have documented the accumulation of point mutations in the non-coding control region of mtDNA in fibroblast cell lines from old individuals, suggesting that they may play a role in ageing (30). One particular mutation (T414G), which resides adjacent to transcriptional start sites, was found in more than half of the cell lines investigated and reached levels of heteroplasmy nearing 50%. This mutation also accumulates in skeletal muscle (31), although it is absent in brain (37). The relevance of these findings to the role of mtDNA mutations in ageing remains contentious, but it highlights the need to search for these mutations both in coding and non-coding regions of the genome. We believe that the development of sensitive sequencing protocols as reported here that accurately and reliably determine the complete human mtDNA sequences in single cells will provide the methodological basis for investigating the role of somatic mtDNA mutations in ageing and neurodegenerative disease and begin to answer these questions. Furthermore, its application at the level of individual cells will help to further clarify the role of somatic mtDNA point mutations in cancer (38,39) and at what stage of the malignant process these mutations are fixed.

ACKNOWLEDGEMENTS

We would like to thank Dr Emma Blakely for her help in the development of these techniques. This work was supported by the Medical Research Council and the Wellcome Trust.

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