Genes in the 16.6 kb human mitochondrial DNA (mtDNA) are concerned exclusively with bioenergy production. Mutations in mtDNA can, therefore, lead to bioenergy decline and so contribute to various age-related degenerative diseases and even to 'natural' ageing (1–3). Large deletions in mtDNA occur in tissues of patients with mitochondrial myopathies and also occur in normal ageing, particularly in postmitotic tissues characterised by high energy demands and low rates of cell division, notably skeletal muscle, cardiac muscle and brain (4–11). The frequency in ageing of deletion-bearing mtDNA is much lower than that in overt mitochondrial diseases, but does regularly increase with advancing age. The age-associated deletions affecting mtDNA frequently include a 4977 bp deletion that is known as the 'common deletion' and is generated between a pair of 13 bp direct repeats, located at nucleotide (nt) positions 8470–8482 and nt 13447–13459, respectively (Table 1, Deletion 1). This deletion was detected by the polymerase chain reaction (PCR) in every postmitotic tissue examined, including skeletal muscle, heart and brain, from adult humans 40 years and over (4,7,13). In addition, a 7436 bp deletion, occurring between a pair of 12 bp direct repeats at nt 8637–8648 and nt 16073–16084 (Table 1, Deletion 2), has been observed in many adult postmitotic tissues, along with other deletions (2,6,9,14).

In our studies on mtDNA deletions, skeletal muscle tissue from a 54-year-old Chinese male (AW) with the chronic fatigue syndrome (CFS) was found to exhibit an unusual pattern of mtDNA deletions. PCR analysis was carried out to detect the common 4977 bp mtDNA deletion in total cellular DNA extracted from a biopsy of left biceps of AW. As a reference, we used DNA from skeletal muscle of KK, a deceased 69-year-old female subject in whom mtDNA deletions have been previously characterised (6). When the conventional oligonucleotide primers L7901 (nt 7901–7920) and H13650 (nt 13650–13631) were used, the product from AW of 0.87 kb (Fig. 1A, Lane 1) was 0.1 kb larger than the product of 0.77 kb representing the common 4977 bp deletion in KK (Fig. 1A, Lane 2). We determined whether the PCR product from the mtDNA of AW was in fact derived from subgenomic mtDNA carrying a deletion by the primer shift method (15), using primers L7901 and H13928 (nt 13928–13905) which should amplify a segment of mtDNA 278 bp larger than that from L7901 and H13650. The products from AW and KK increased 0.28 kb to 1.15 kb (Fig. 1A, Lane 3) and 1.05 kb (Fig. 1A, Lane 4), respectively. A further pair of primers, L8282 (nt 8282–8305) and H13851 (nt 13851–13832), also previously used to detect the 4977 bp deletion (14), yielded the expected 0.59 kb product carrying the breakpoint of the 4977 bp deletion from KK (Fig. 1A, Lane 6), but a 0.69 kb product from AW (Fig. 1A, Lane 5). Hence, it is clear that the PCR products were amplified from subgenomic mtDNA molecules, and that the product of AW was consistently 0.1 kb larger than that of KK.

These results could have either of two explanations. First, there is in both subjects a deletion at the same excision sites, but with AW there is an insertion into mtDNA of a sequence of 0.1 kb which lies between the excision sites and the 3' ends of the two nearest primers used for PCR (L8282 and H13650). Second, the muscle of AW contains another deletion of about 4.88 kb but lacks a detectable level of the common 4977 bp deletion. To test the first explanation, segments of mtDNA in non-deleted molecules, including the region between the excision sites of the common 4977 bp deletion and the nearest primers (L8282 and H13650), were amplified by PCR. Thus PCR products were amplified using primers L8282 and H8540 (nt 8540–8521) that yielded a 0.26 kb product (Fig. 1A, Lanes 7 and 8), or primers L13145 (nt 13145–13168) and H16075 that yielded a 0.51 kb product (Fig. 1A, Lanes 9 and 10). The products in each case were of the same size for AW and KK, indicating that there was no insertion in the regions of mtDNA tested. Thus muscle tissue of AW indeed had a mtDNA deletion that differed from the age-related common 4977 bp deletion.

The novel deletion in AW was characterised by identifying the excision sites by DNA sequencing. Each of the 0.87 kb, 1.15 kb and 0.69 kb primer shift PCR products from mtDNA of AW (Fig. 1A, Lanes 1, 3 and 5, respectively), which contained the putative deletion breakpoint, was cloned into pUC18. The inserted DNA in each plasmid was sequenced using primers L8282 and H13650 to identify the breakpoint. This defined a novel mtDNA deletion, 4881 bp in length (Table 1, Deletion 3), that was identical in each cloned mtDNA segment tested. This deletion occurred between a pair of 7 bp direct repeats (CTTCAAA) situated at nt 8625–8631 and 13506–13512; precisely one copy of the repeated sequences was retained in subgenomic molecules.

The common 4977 bp deletion was tested for in 60 muscle samples obtained at autopsy from 20 adults aged 40–70 years, courtesy of the Victorian Institute of Forensic Pathology; samples were taken from diaphragm, deltoid and quadriceps muscles. The DNA was amplified by PCR using primers L8282 and H13851 and the expected 0.59 kb product bearing the 4977 bp deletion was demonstrable in every sample. Thus, the collective results from the literature and from the present...
Table 1. Nucleotide sequences of the deletion breakpoint regions

<table>
<thead>
<tr>
<th>Deletion number</th>
<th>Deletion size (bp)</th>
<th>Sequence</th>
<th>Direct repeat</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4977</td>
<td>8470</td>
<td>ACTACCACCTACCTCCCTACCA... (AAGCC... TATCA... TCCTCCCTCACC) TTGGCAGCCTT</td>
<td>13 bp</td>
</tr>
<tr>
<td>2</td>
<td>7436</td>
<td>8637</td>
<td>CCAAATATCTCATCAACAACCG (ACTAA... TCACCCATCAACAACCG) CTATGTATTT</td>
<td>12 bp</td>
</tr>
<tr>
<td>3</td>
<td>4881</td>
<td>8625</td>
<td>TGATCCCAACTCTCCAAA (TATCT... TTCTACATCCAAA) GACCACATCA</td>
<td>7 bp</td>
</tr>
<tr>
<td>4</td>
<td>7683</td>
<td>8387</td>
<td>GCCCCAACCTAAATACTACCG (TATTGACCAC... TATTGACTCA) CCCATCAACAACCGCTATGT</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>7459</td>
<td>8612</td>
<td>ATCATTTATTTCCCTCTCCT (ATTGATCCCC... ATTGACTCAG) CCATCAACAACCGCTATGT</td>
<td>None</td>
</tr>
</tbody>
</table>

The numbering of nucleotides is according to Anderson et al. (12) and the number indicates the position of the nucleotide below the last digit. Direct repeat sequences are underlined. The breakpoints are indicated by parentheses and the nucleotides shown inside the parentheses (indicated by italics) were deleted.

Figure 1. PCR analysis that distinguishes mtDNA deletion patterns in two subjects. (A) Evidence for a novel mtDNA deletion in AW in place of the common 4977 bp deletion. DNA from the skeletal muscle of AW and the reference subject KK was amplified using the primers indicated below each pair of lanes. Lane M contains λ DNA digested with EcoRI and HindIII as DNA size markers. The sizes of some marker fragments are shown at the left. The positions and sizes of some bands amplified by PCR are indicated at the right; asterisks indicate PCR products from AW that contain a novel 4881 bp deletion (Table 1, Deletion 3). (B) Different sets of multiple mtDNA deletions in the skeletal muscle of AW and KK. DNA from the skeletal muscle of the two subjects was amplified using the primers L7901 and H13650. The positions and sizes of prominent bands amplified by PCR are shown at the right; asterisks indicate PCR products from AW that contain novel deletions (0.96 kb, Deletion 4 and 1.18 kb, Deletion 5, in Table 1). Lane M is the same as in Panel A.

study suggest that the lack of a detectable level of the common 4977 bp mtDNA deletion in muscle from AW was indeed exceptional.

Since sequence variations in one copy or both copies of the 13 bp direct repeats could have prevented the generation of the common 4977 bp deletion in AW, we obtained mtDNA sequence information from two particular PCR products shown in Fig. 1A (Lanes 7 and 9), which each contained one of the putative 13 bp direct repeat sequences in mtDNA of AW. Both copies of the 13 bp direct repeat sequences were found to be identical to those published by Anderson et al. (12). In addition, the DNA sequence in the vicinity of the direct repeats was compared with the published sequence (12). Among 256 nucleotides sequenced, between nt 8328 and 8583, only a C to T transition was found in the mtDNA of AW at nt 8426, 44 nucleotides from the 13 bp repeat. No variation was detected among 352 nucleotides from nt 13261 to 13612. Hence, the undetectable quantities of the common 4977 bp deletion in
mtDNA of AW could not be attributed to any sequence variation within either of the two 13 bp direct repeats, or to significant variations around the repeats.

Primers L7901 and H16540 have been successfully used for the detection of multiple mtDNA deletions in tissues of adults, represented as a series of PCR products of distinct size in a single amplification reaction (2,6,16). Accordingly, this pair of primers was used for PCR analysis of total cellular DNA from muscle of AW and KK (Fig. 1B). Several PCR products were amplified from both samples, indicating multiple deletions. In the PCR products from mtDNA of AW, there were two prominent bands ~1.18 kb and 0.96 kb in size (Fig. 1B, Lane 1). DNA from the more prominent product (0.96 kb) was cloned into pUC18 and was found by sequencing to carry a 7683 bp deletion, representing a segment from nt 8388 to 16070, which did not have direct repeat sequences near the breakpoint (Table 1, Deletion 4). Similarly, DNA from the 1.18 kb product was cloned into pUC18 and a 7459 bp deletion was identified by sequencing between nt 8613 and nt 16071; as for the 7683 bp deletion, no direct repeat sequences were found to flank this deletion, or to lie in its immediate vicinity (Table 1, Deletion 5).

On the other hand, the PCR products from KK (Fig. 1B, Lane 2), described by Zhang et al. (6), differed from that of AW. These included a prominent product of 1.2 kb representing a 7436 bp deletion, excised at a pair of 12 bp direct repeats (Table 1, Deletion 2), that is frequently encountered, whereas an equivalent band for AW could not be detected (Fig. 1B, Lane 1). The other minor bands in PCR products from muscle mtDNA of AW were not characterised but were mostly different from those seen in the PCR products from muscle mtDNA of KK.

The 4977 bp common deletion has been shown by Baumer et al. (16) to be homogeneous (class A), such that precisely one copy of the 13 bp direct repeat is retained in every subgenomic molecule. By contrast, an 8.04 kb deletion was found to be heterogeneous (class B), such that the amplified PCR product carrying the deletion represented up to nine or more closely related deletions involving the same pair of 5 bp direct repeats (16). The repeats in that case were a sequence CCCAT located both at nt 8030-8034 and nt 16071-16075; the breakpoints differed at the base pair level in each member of the heterogeneous family of 8.04 kb deletions.

One end of the breakpoints of the novel 7683 bp and 7459 bp deletions of AW was located at nt 16071 and 16072, respectively, in precisely the same region from which one end of the 8.04 kb heterogeneous deletion family is generated. We therefore asked whether the deletions characterised in this study were homogeneous (class A) or heterogeneous (class B) by applying a procedure similar to that introduced by Baumer et al. (16). We found that both of these newly identified deletions in the muscle of AW (Table 1, Deletions 4 and 5) were homogeneous (class A deletions), although one end of their breakpoints lies in the ‘hot spot’ region (nt 16070–16075) from which the family of 8.04 kb class B deletions originates (16).

Whether there is a relationship in AW between the unusual mtDNA deletions and the chronic fatigue syndrome is uncertain. Perhaps some aspect of his mtDNA metabolism differs from that of normal individuals, indicated by a predisposition to unusual types of mtDNA deletions. Thus direct repeats may not be primarily involved in the generation of deletions in AW. It is our experience that mtDNA deletions without direct repeats are comparatively rare in normally ageing individuals, since hitherto only three such deletions have been reported (5).

If indeed AW has some aberrations in mtDNA metabolism, the possibility is that there are accompanying disturbances in mitochondrial bioenergy production, presuming that replication and expression of mtDNA are also affected. Hence, among the acknowledged multiple causes of the chronic fatigue syndrome (17,18), there could be cases explicable by dysfunctional mitochondrial energy production. Behan et al. (19) reported the occurrence of mitochondria with ultra-structural abnormalities in muscle biopsies of patients with postviral fatigue syndrome, but analysis of mtDNA in those patients was not reported. It will be important for future work to establish whether involvement of mitochondrial dysfunction occurs regularly in the chronic fatigue syndrome, and also whether there is an identifiable subset of patients in whom analysis of mtDNA would be informative. This is clinically important since this possible subset of patients could derive benefit from intensive therapy with suitable redox compounds (1,20).

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REFERENCES