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Detection and characterization of mitochondrial DNA rearrangements in Pearson and Kearns-Sayre syndromes by long PCR

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Abstract We used a strategy based on long PCR (polymerase chain reaction) for detection and characterization of mitochondrial DNA (mtDNA) rearrangements in two patients with clinical signs suggesting Pearson syndrome and Kearns-Sayre syndrome (KSS), respectively, and one patient with myopathic symptoms of unidentified origin. Mitochondrial DNA rearrangements were detected by amplification of the complete mitochondrial genome (16.6 kb) using long PCR with primers located in essential regions of the mitochondrial genome and quantified by three-primer PCR. Long PCR with deletion-specific primers was used for identification and quantitative estimation of the different forms of rearranged molecules, such as deletions and duplications. We detected significant amounts of a common 7.4-kb deletion flanked by a 12-bp direct repeat in all tissues tested from the patient with Pearson syndrome. In skeletal muscle from the patient with clinical signs of KSS we found significant amounts of a novel 3.7-kb rearrangement flanked by a 4-bp inverted repeat that was present in the form of deletions as well as duplications. In the patient suffering from myopathic symptoms of unidentified origin we did not detect rearranged mtDNA in blood but found low levels of two rearranged mtDNA populations in skeletal muscle, a previously described 7-kb deletion flanked by a 7-bp direct repeat and a novel 6.6-kb deletion with no repeat. These two populations, however, were unlikely to be the cause of the myopathic symptoms as they were present at low levels (10–40 ppm). Using a strategy based on screening with long PCR we were able to detect and characterize high as well as low levels of mtDNA rearrangements in three patients.

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Introduction

Large-scale structural rearrangements of the 16 569-bp human mitochondrial genome, such as single deletions, multiple deletions, or duplications, have been associated with mitochondrial respiratory chain disorders such as the Kearns-Sayre syndrome (KSS), progressive external ophthalmoplegia, adult-onset diabetes mellitus with deafness, Pearson syndrome (Ballinger et al. 1992; Wallace 1992; Superti-Furga et al. 1993; Rötig et al. 1995), and Mendelian-inherited myopathies (Kaukonen et al. 1996). Patients are heteroplasmic, harbouring a mixture of rearranged and intact mitochondrial DNA (mtDNA), and the severity of the disease is correlated with the amount of rearranged mtDNA. Moreover, mtDNA rearrangements also occur in non-mitochondrial diseases such as Parkinson's disease (Mann et al. 1992), Alzheimer's disease (Corral-Debrinski et al. 1994), and ischaemic heart disease (Corral-Debrinski et al. 1991). In addition, post-mitotic tissues and oocytes of elderly subjects have been found to harbour low levels of mtDNA rearrangements that accumulate during ageing (Cortopassi and Arnheim 1990; Chen et al. 1995), supporting the hypothesis that a heterogeneous array of mtDNA mutations increases with age in parallel with a decrease in the capacity of oxidative phosphorylation in mitochondria and metabolic decline (Melov et al. 1995).

Mitochondrial DNA rearrangements flanked by direct or no repeats have been described and suggested to occur via different pathways (Mita et al. 1990). However, mechanisms of slipped-replication or legitimate recombination via direct repeats were suggested as the major cause of human mtDNA deletions (Mita et al. 1990; Rötig et al. 1991).

So far Southern blot analysis, requiring hybridizations using several restriction enzymes and mtDNA probes, has been the basic approach for detection of mtDNA rearrangements. However, Southern hybridizations have a low sensitivity and failed to detect low levels of rearranged

mtDNA in patients and "aged" controls (Holt et al. 1988; Cortopassi and Arnheim 1990; Ozawa et al. 1990; Kawashima et al. 1994). A screening strategy via multiprimer polymerase chain reaction (PCR) has been presented by Ernst et al. (1994). We used a strategy based on long PCR for analysis of mtDNA from three patients and detected and characterized variable amounts of rearranged mtDNA in different tissues.

Subjects and methods

Human subjects

Studies were carried out on two patients with clinical signs suggesting a mitochondrial disorder, on one patient suffering from myopathy of unclear origin and on control persons of different ages (5, 12, 30, 56, 63, and 76 years). The control persons were normal individuals with no signs of mitochondrial dysfunction, and blood samples and skeletal muscle biopsies were taken with informed consent during operations due to other indications.

Patient 1 (male) suffered from severe lactic acidois from the second day after birth. Therapy-resistant anaemia, cholestasis, and nuclear vacuolization in precursor cells of the bone marrow were noted, but no clinical symptoms of pancreatic insufficiency. He developed coagulopathy and a liver biopsy showing signs of cholestasis and microvesicular fat accumulation was made at the age of 2 months. He died from liver dysfunction at the age of 3 months and analysis of autopsy material revealed severe lipid storage, haemosiderosis, and cholestatic cirrhosis. A tentative diagnosis of Pearson syndrome (OMIM # 557000) was made. Skeletal muscle, liver, pancreas, brain, and heart muscle samples were available at autopsy. Biochemical analysis of the mitochondrial respiratory complexes of autopsy material revealed normal activities in skeletal muscle, but reduced activity of complexes III and IV in liver tissue. A blood sample from his clinically normal mother and blood from umbilical cord from a healthy younger brother were available.

Patient 2 (female) presented with symptoms suggesting KSS (OMIM # 530000), such as short stature, ataxia, tremor, retinitis pigmentosa, basal ganglia calcification, hypothyreosis and, at the age of 13 years, the onset of external ophthalmoplegia. A skeletal muscle biopsy was taken at the age of 13 years and histochemical analysis revealed red-ragged fibres, subsarcolemmal accumulation of mitochondria, and an increase in neutral lipid and glycogen in muscle fibres. Biochemical analysis showed reduced activity of complexes I and III in skeletal muscle. Her mother and two sisters did not show any clinical symptoms.

Patient 3 (female) presented with myopathic symptoms of unidentified origin, including muscle pain and weakness since early childhood. The patient's sister and one brother showed the same symptoms. One brother was apparently healthy. Blood and a skeletal muscle biopsy were obtained at the age of 43 years. Skeletal muscle was normal on histochemical and electron microscopic analysis. Biopsy material was limited and no further analysis was made.

DNA purification and PCR analysis

Total DNA was extracted from peripheral blood lymphocytes and tissue samples according to standard protocols, and quantified by spectrophotometry. Amplification of mtDNA fragments larger than 2 kb was carried out using the Expand Long Template PCR System (Boehringer Mannheim, Switzerland) and of fragments up to 2 kb using *Taq* DNA polymerase (Boehringer Mannheim) (conventional PCR). Amplifications were performed in a 50-µl reaction mix containing 16 pmol of each primer and 50–200 ng of total DNA (tissue). The PCR primers covered the following regions of the Cambridge sequence (Anderson et al. 1981) (Fig. 1): D1A [nucleotides (nt) 336–363], D1B (nt 282–255), OLA (nt 5756–5781), OLB (nt 5745–5721), mt2 (nt 261–242), mt4 (nt 683–664), COI/IIB (nt 7840–7821), mt7 (nt 8167–8186), mtK1 (nt 8278–8297), ATP8A (nt 8354–8373), ATP6A (nt 8531–8550), ATP6B (nt 8676–8654), mt9 (nt 8859–8878), mt11 (nt 9121–9139), COIIIB (nt 9840–9821), ND4Aint (nt 11080–11100), mt5 (nt 11728–11747), ND4B (11910–11891), HB (12412–12394), RH1065 (nt 15149–15174), mt3 (nt 15787–15806), mt10 (nt 15933–15915), and D2B (nt 16148–16127). All primers were synthesized by Microsynth, Balgach, Switzerland. Primer pairs OLA/OLB and D1A/D1B were designed for amplification of the complete intact mitochondrial genome (16.6 kb) but were also used in combination with other primers for conventional PCR. Primers BP1, BP2, BP3/6, and BP3/7 were designed for the breakpoint junctions detected in the three patients. For junctions without direct repeats, 17–19 nt (5′–3′) of the primers corresponded to one side of the breakpoint junction and 7 nt to the other side: BP2 (nt 12104–12122:15844–15850), BP3/6 (nt 9474–9490:16072–16078)). For junctions carrying direct repeats, primers were selected that contained 8–13 nt (5′-3′) annealing to one side of the boundary, including the direct repeat, and only 3–4 nt binding to the other side of the repeat: BP1(nt 8629–8648:16085–16087), and BP3/7 (nt 8604–8623:15663–15666). Breakpoint primers were used in combination with ATP6B and COI/IIB, with HB and ND4B, and with COIIIB, ATP6B, and COI/IIB. Amplification conditions for conventional PCR were, after an initial 2-min denaturation step at 95°C, 15 s denaturation at 95°C, 15 s annealing at 58°C, and 1 min elongation at 72°C for 28 cycles. In the last cycle elongation was prolonged for 5 min.

For long PCR the amplification conditions were, after an initial 2-min denaturation step at 94°C, 10 s denaturation at 94°C, 30 s annealing at 65°C for D1A/B, OLA/B (62°C for BP1, BP3/7 and 56°C for BP2, BP3/6), and, depending on the fragment length, 8–12 min elongation at 68°C for 10 cycles. Amplification was continued for another 20 cycles with an extension of the elongation step of 20 s for each cycle. In the last cycle, elongation was prolonged for 7 min. Amplification was performed using a GeneAmpPCR System 2400 (Perkin Elmer) thermal cycler. A volume of one-tenth of the final amplification product was loaded on a 1% agarose gel either directly, or after digestion with restriction enzymes as indicated according to the manufacturer's specifications (Boehringer Mannheim). Ethidium bromide-stained agarose gels were UV illuminated and recorded with a video camera.

Southern blot analysis

Total DNA extracted from tissue (6 µg) and from peripheral blood lymphocytes (10 µg) was digested with *Bam*HI, electrophoresed on 0.8% (w/v) agarose gels, and transferred to nylon membranes (Hybond+, Amersham). The membranes were hybridized with probes labelled with [^{32}P]dCTP by random priming.

Quantification of rearranged mtDNA

Quantitative evaluation of rearranged versus intact mtDNA was performed as described by Cortopassi and Arnheim (1990). Mitochondrial DNA was linearized with *Pvu*II before amplification. Primers D2B, mt3, ATP8A, mtK1, ATP6A, mt4, and COIIIB were used for patient 1, mt5, HB, and D2B for patient 2, and mt2, mt3, mt11, mt10, RH1065, and mt7 for the two rearranged mtDNA populations of patient 3. We tested different population-specific primers to rule out selection by the different primers, and all gave the same quantitative results. For estimation of duplicated and deleted mtDNA in patient 2 we used primers BP2, HB, and ND4B. Mitochondrial DNA analysed by Southern blotting was quantified using a BioRad video densitometer.

DNA sequencing and computer analysis

The amplification products were purified via a spin column (PCR purification Kit, Qiagen, Switzerland). One-fifth volume of the amplification product was cycle sequenced using the ABI PRISM

Dye Terminator Cycle Sequencing Kit FS (Perkin Elmer) using a GeneAmpPCR System 9600 (Perkin Elmer) thermal cycler. Sequencing primers were those used in the previous PCR amplification. The reaction products were resolved on an Applied Biosystems 373A DNA Sequencer (ABI, Perkin Elmer) and analysed with Collection, Analysis, SeqEd (ABI), and GCG (Genetics Computer Group of the University of Wisconsin, USA) computer programs. Analysis of the Cambridge sequence (Anderson et al. 1981) for repeats was performed using PatScan (Mathematics and Computer Science Division, Argonne National Laboratory, USA) and data filters under UNIX. "Repeat" denoted a repeated sequence of nucleotides. We counted all direct and inverted repeats that could possibly flank a deletion. Repeats that were a symmetrical subset of longer repeats were not counted. The mtDNA sequences of patients were analysed for repeats by scanning the region 15 bp 5′ and 3′, respectively, of each breakpoint site.

Enzymatic assays

Activities of the enzyme complexes of the electron transport chain in muscle and liver tissue were determined as described previously (Krähenbühl et al. 1994, 1996).

Results

Detection of mtDNA rearrangements

In order to screen for mutations in the mitochondrial genome, primers were selected from regions that are essential for mtDNA replication (Hixson et al. 1986) and are not usually removed by deletions. They contain the origin of L-strand replication (O_L, 5721–5781) and the origin of H-strand replication (O_H) as well as the L-strand promoter (P_L) (191–445).

For detection of mtDNA rearrangements we amplified mtDNA from total DNA with primer pairs D1A, D1B and OLA, OLB (Fig. 1). Amplification of intact mtDNA led to a 16.6-kb fragment corresponding to the complete mt genome (16516 bp for primer pair D1A, D1B and 16559 bp for OLA, OLB, respectively) (Fig. 2, lane 1).

We analysed tissue samples from different patients (1–3) and controls of different ages. In Fig. 2, amplifications of mtDNA from skeletal muscle using primers OLA/B are shown. The 30-year-old control presented with the full-length mtDNA of 16.6 kb (Fig. 2, lane 1) and cleavage with *Sac*I, cutting at nt 40 and 9647 of the Cambridge sequence, resulted in fragments of 6962, 5705, and 3892 bp (Fig. 2, lane 2). Patient 1, however, presented with a fragment of about 9.1 kb in length (lane 3) and digestion with *Sac*I confirmed that he carried rearranged mtDNA lacking about 7.4 kb (lane 4). Amplification (lane 5) and digestion (lane 6) of mtDNA from patient 2 revealed that she harboured rearranged mtDNA lacking about 3.7 kb. In contrast to patients 1 and 2, patient 3 demonstrated the full-length mtDNA in addition to two populations of rearranged mtDNA (lane 7). Digestion with *Sac*I resulted in fragments originating from the intact mtDNA portion and an additional 4.3-kb band (lane 8). Better resolution revealed the 3.9-kb band to be a double band, suggesting that patient 3 carried two rearranged mtDNA populations lacking about 7 and 6.5 kb, respectively. In the 76-year-old control the full-length mtDNA as well as a fragment ladder were detected (lane 9).

Simultaneous amplification of fragments of variable size depends on size differences and the proportion of intact versus rearranged mtDNA. Simultaneous amplification of rearranged and intact mtDNA as observed in patient 3 and the 76-year-old control indicated low levels of rearranged mtDNA. We amplified complete mtDNA from

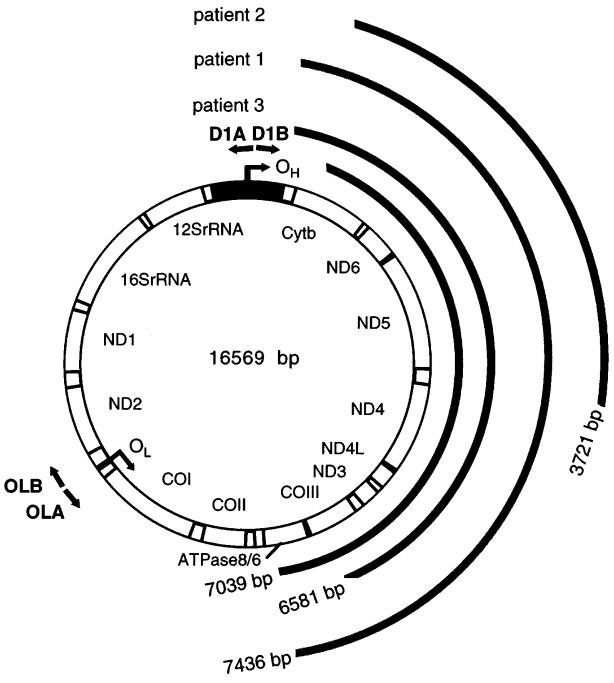

Fig. 1 Map of the human mitochondrial genome showing the binding sites of the primers D1A, D1B, OLA, and OLB. The deleted mitochondrial DNA (mtDNA) segments found in patients 1, 2, and 3 are marked. (O_H, O_L origins of replication of H and L strands, *ND1–6*, *ND4L* subunits 1–6 and 4L of NADH dehydrogenase, *COI–COIII* subunits 1–3 of cytochrome *c* oxidase, *ATPase 8/6* subunits 8 and 6 of ATP synthase, *Cytb* cytochrome *b*. Genes for tRNAs are not indicated)

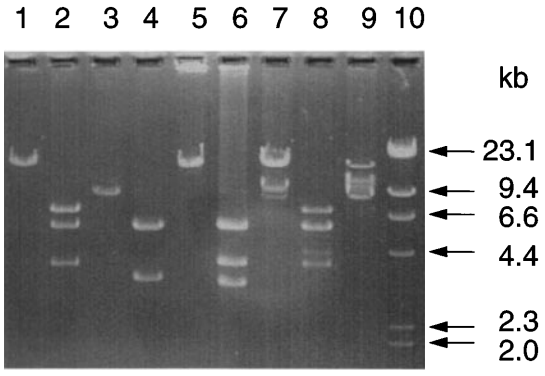

Fig. 2 Agarose gel demonstrating complete mitochondrial genome amplified from skeletal muscle DNA using primer pairs OLA, OLB (*lanes 1, 3, 5, 7, 9*) and after digestion with *Sac*I (*lanes 2, 4, 6, 8*). *Lanes 1, 2* 30-year-old control, *lanes 3, 4* patient 1, *lanes 5, 6* patient 2, *lanes 7, 8* patient 3, *lane 9* 76-year-old control, *lane 10* molecular weight markers, as indicated

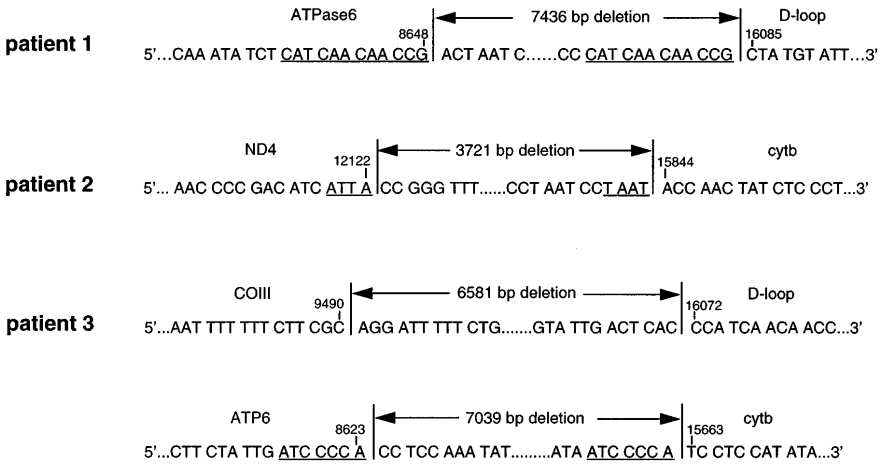

Fig. 3 Nucleotide sequence flanking the breakpoint junctions in patients 1, 2, and 3. Direct and inverted repeat sequences are *underlined*

skeletal muscle and blood from control subjects aged 5, 12, 30, 56, 63, and 76 years and detected mtDNA rearrangements only in the muscle biopsy from the 76-year-old control. The pattern of rearranged mtDNA found in the 76-year-old control also varied within the biopsy (data not shown). Patient 1 exhibited the mtDNA rearrangement in all tissues tested (skeletal muscle, heart muscle, brain, pancreas, and fibroblasts; data not shown), whereas peripheral blood lymphocytes from his mother and from the umbilical cord of a recently born brother did not show any rearranged mtDNA (data not shown), suggesting a spontaneous mutational event either in the maternal germline or early in his development. In the blood sample from patient 3 we did not detect rearranged mtDNA.

Long PCR using primer pair D1A, D1B gave identical results to primer pair OLA, OLB (data not shown). Specificity of the amplifications was also confirmed by sequencing of the PCR products.

Localization of the mtDNA rearrangements

Results from restriction mapping of the amplified complete mtDNA were used for PCR-based mapping performed by conventional PCR across the deleted mtDNA region using different primers. Breakpoint junctions were identified by direct sequencing of the amplification products (Fig. 3).

Direct sequencing revealed in patient 1 a common mtDNA deletion of 7436 bp flanked by a 12-bp direct repeat (Bader-Meunier et al. 1994; Melov et al. 1995) (Fig. 3). The deletion removed most of the ATPase6 gene and all genes from COIII to $tRNA^P$ (Fig. 1). In addition we detected a novel mtDNA polymorphism in the breakpoint region present in both mtDNA populations: a neutral, apparently homoplasmic 8610T→C transition in the ATPase6 gene.

Patient 2 harboured a novel 3721-bp deletion flanked by a 4-bp inverted repeat in skeletal muscle. The deletion removed all genes from $tRNA^H$ to $tRNA^E$ and almost the complete cytochrome *b* gene. Sequencing of the breakpoint junction revealed a new mtDNA polymorphism present in each of the different mtDNA populations: a neutral, apparently homoplasmic 11776T→C transition in the ND4 gene.

In patient 3 we detected two deletions in skeletal muscle, a novel 6581-bp deletion with no repeat removing two-thirds of the COIII gene and all genes from $tRNA^G$ to $tRNA^P$ and a previously known 7039-bp deletion flanked by a 7-bp direct repeat (Tanaka et al. 1989) that removed most of the ATPase6 gene, all genes from COIII to $tRNA^E$, and most of the cytochrome *b* gene. Near the breakpoint junctions in the mtDNA populations we found differences from the Cambridge sequence: polymorphism 9477G→A, leading to a V→I exchange in the COIII gene (Marzuki et al. 1991), and a new 8609C→T transition, resulting in substitution of L for P in the ATPase6 gene. As the 8609 transition was apparently homoplasmic and affected a non-conserved amino acid, it is thought to be a polymorphism.

Southern blot analysis

For detection of all mtDNA rearrangements by Southern blotting, restriction enzymes linearizing all mtDNA species and probes binding to the non-deleted mtDNA regions must be selected. Patients 1 and 2 were also analysed by Southern blotting, confirming results by long PCR. Additional hybridizations using restriction enzymes that cut in the deleted mtDNA regions and probes that bind to the deleted and non-deleted mtDNA regions enable distinctions to be made between the different species of rearranged mtDNA molecules, such as deletions and duplications; results from patient 1 are shown in Fig. 4. Distinction between nicked deletion monomers, dimers and multimers requires two-dimensional electrophoresis (Poulton et al. 1993, 1994). For full molecular characterization by Southern blotting several hybridizations and localization of the mtDNA deletions are necessary. Figure 4a demonstrates linearized full-length mtDNA from the parents and patient 1 (lanes 1–7). Patient 1, in addition, presented in all tissues tested two faster-migrating bands corresponding to a higher amount of linearized (due to shearing) and a lower amount of supercoiled deletion monomers (lanes

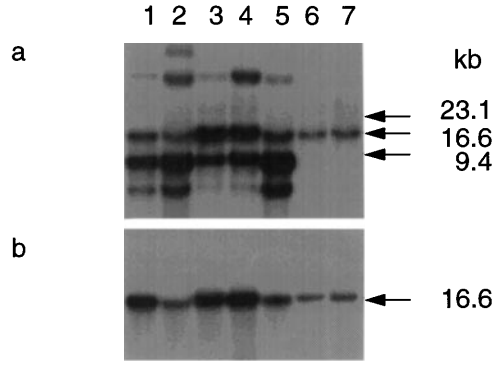

Fig. 4a, b Southern blot analysis of DNA from CNS, pancreas, heart muscle, skeletal muscle, and liver of patient 1 (*lanes 1–5*), and from blood from the patient's father (*lane 6*) and mother (*lane 7*). Total DNA was digested with *Bam*HI, which cuts within the deleted region, and hybridized with probe A [nucleotides (nt) 4621–5380], binding to the non-deleted mtDNA region (**a**), and with probe B (nt 14812–15933), binding to the deleted mtDNA region (**b**)

1–5). The slower-migrating bands are presumably nicked deletion monomers (lanes 1–5) and deletion dimers or multimers (lane 2). In Fig. 4b, only full-length mtDNA is seen and no slower-migrating duplicated mtDNA molecules were detected in tissues from patient 1. Southern blot analysis of skeletal muscle DNA from patient 2 indicated the existence of duplicated mtDNA (data not shown). Insufficient material was available from patient 3 to allow Southern blots to be performed. The detection limit of Southern blot analysis was about 3% and low levels of mtDNA rearrangements, e.g. due to ageing, were not detected by Southern blotting (data not shown; Melov et al. 1995).

Detection of duplications by long PCR

In order to identify duplicated mtDNA in the different patients we designed primers (BP1, BP2, BP3/6, and BP3/7, respectively) binding to the breakpoint junctions of the three patients. Specific amplification of duplicated mtDNA from skeletal muscle from patient 2 is shown in Fig. 5a, b. Long PCR using the breakpoint primer (Fig. 5a) in combination with a backward primer (HB for patient 2) located in the non-duplicated region specifically amplified duplicated mtDNA from patient 2 (Fig. 5b, lane 2). Using a backward primer (ND4B for patient 2) located in the duplicated region, duplicated and deleted mtDNA was simultaneously amplified, resulting in a higher yield (lane 6). Specific amplification was confirmed by negative control samples (lanes 5 and 9), restriction analysis (lanes 3, 4, 7, and 8), and direct sequencing. Long PCR using breakpoint primers BP1, BP3/6, and BP3/7 in combination with backward primers binding to the duplicated mtDNA regions of the different mtDNA populations (COI/IIB, ATP6B, and COI/IIB, respectively) specifically amplified rearranged mtDNA from tissues of patients 1 and 3. However, no duplicated mtDNA was detectable in tissues

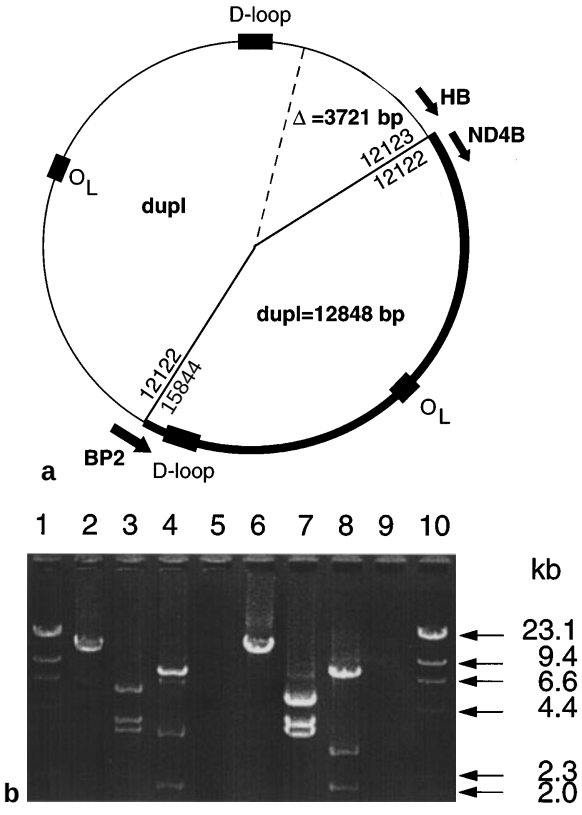

Fig. 5 **a** Map of the duplicated mtDNA molecule found in patient 2. The *marked segment* indicates the mtDNA region (12848 bp) inserted into full-length mtDNA (16569 bp), resulting in a duplicated molecule of 29417 bp. (*dupl* duplicated region, Δ non-duplicated region) **b** Amplification of duplicated mtDNA from skeletal muscle of patient 2 using breakpoint primer BP2. *Lanes 2–5* amplification using primers BP2, HB: amplification of duplicated mtDNA from patient 2 (*lane 2*) and after digestion with *Eco*RV (*lane 3*) and *Pst*I (*lane 4*); *lane 5* 30-year-old control. *Lanes 6–9* amplification using primers BP2, ND4B: coamplification of duplicated plus deleted mtDNA from patient 2 (*lane 6*) and after digestion with *Eco*RV (*lane 7*) and *Pst*I (*lane 8*); *lane 9* 30-year-old control. *Lanes 1, 10* size markers

from patients 1 and 3 using backward primers annealing to the non-duplicated mtDNA regions (ATP6B, COIIIB, and ATP6B, respectively; data not shown). Amplification of duplicated human mtDNA by long PCR has also been reported by Fromenty et al. (1996). In contrast to these authors, we specifically amplified duplicated mtDNA using breakpoint primers containing direct repeats.

Quantification of rearranged mtDNA

The amount of rearranged mtDNA in the different patients was evaluated by a three-primer method as described by Cortopassi and Arnheim (1990). The method is based on selective amplification of deleted and intact mtDNA fragments of similar size. One forward primer binding to both populations is used in combination with two different backward primers. One anneals to the deleted mtDNA region, amplifying only intact mtDNA, and the other one binds

Table 1 Proportions (%) of the different rearranged mtDNA populations found in the three patients by three-primer polymerase chain reaction. – not detected, *n.a.* not analysed

Patient	Rearrangements of mtDNA		Rearranged mtDNA (%) in					
			Blood	Skeletal muscle	Liver	Pancreas	CNS	Heart muscle
1		Rearranged	[a]	60	90	> 95	80	20
		Duplicated	[a]	–	–	–	–	–
2		Rearranged	n.a.	70				
		Duplicated	n.a.	12				
		Deleted	n.a.	58				
3	6.6-kb	Rearranged	–	0.004				
		Duplicated	–	–				
	7 kb	Rearranged	–	0.001				
		Duplicated	–	–				

[a] No DNA could be extracted from peripheral blood lymphocytes of patient 1

on the other side of the deletion breakpoint, amplifying exclusively rearranged mtDNA. Quantification of rearranged mtDNA from Southern blotting gave corresponding results. We used UV illumination for visualization and intend to use fluorescence-labelled primers that will provide more exact and rapid quantification. The ratio of deleted to duplicated mtDNA was estimated by three-primer PCR using the breakpoint-specific primers. Results are presented in Table 1. Patients 1 and 2 harboured large amounts of rearranged mtDNA in the tissues analysed. In contrast, in skeletal muscle from patient 3 rearranged mtDNA populations were observed at about 40 and 10 ppm (parts per million), respectively. As shown above, they could still be detected using the breakpoint-specific long PCR, indicating that the detection limit for rearranged mtDNA missing about 7 kb is at least 10 ppm using long PCR.

Discussion

For detection of mtDNA rearrangements we designed two primer pairs, located at the O_H (D1A, D1B) and O_L (OLA, OLB) sites, respectively, that are essential for replication (Hixson et al. 1986). We amplified the complete mitochondrial genome using both primer pairs (D1A/D1B = 16 516 bp and OLA/OLB = 16 559 bp) in order to encompass all mtDNA rearrangements, including deletions that might remove either the O_L (Ballinger et al. 1992, 1994) or the O_H site.

We analysed one patient with Pearson syndrome (patient 1), one patient with KSS (patient 2), and one patient suffering from myopathic symptoms of unidentified origin (patient 3), and control subjects of different ages. Rearranged mtDNA was detected in all tissues tested from patient 1 and in skeletal muscle from patient 2, which was the only tissue available. No rearranged mtDNA was found in blood from patient 3, while two rearranged populations were detectable in skeletal muscle. Simultaneous amplification of short deleted mtDNA and full-length mtDNA had already indicated low levels of mtDNA rearrangements. Quantitative analysis revealed high amounts (20–95%) of rearranged mtDNA in tissues from patients 1 and 2 and low levels (10–40 ppm) in skeletal muscle from patient 3. Low levels of rearranged mtDNA have been suggested to accumulate in post-mitotic tissues with ageing (Cortopassi and Arnheim 1990; Melov et al. 1995). However, when blood and skeletal muscle from control subjects aged 5, 12, 30, 56, 63, and 76 years was tested by long PCR, rearranged mtDNA was detected only in skeletal muscle from the 76-year-old control. So far no representative population studies have been performed on the frequency and amount of mtDNA rearrangements in ageing skeletal muscle.

Mitochondrial DNA rearrangements were identified by restriction analysis of the complete amplified mt genomes, followed by PCR-based mapping and direct sequencing of the amplification products. A frequently observed 7.4-kb deletion flanked by a 12-bp direct repeat (Bader-Meunier et al. 1994; Melov et al. 1995) was found in patient 1 and a novel 3.7-kb deletion that was flanked by a 4-bp inverted repeat was detected in patient 2. Patient 3 carried a previously described 6.6-kb deletion flanked by a 7-bp direct repeat (Tanaka et al. 1989) and a novel 7-kb deletion with no repeat.

The use of long PCR with deletion-specific primers revealed that rearranged mtDNA from patient 2 with KSS was present in the form of deletions as well as duplications, while in patient 1 with Pearson syndrome only deleted mtDNA was detectable. Low levels of deleted but no duplicated mtDNA were identified in patient 3. Thus, for the first time, the form of low levels of rearranged mtDNA has been analysed.

Patients 1 and 2 were analysed by long PCR and Southern blotting, but, unfortunately, insufficient material was available from patient 3 to perform Southern blot analysis. Results determined by long PCR were in accordance with those obtained from Southern blot analysis.

Genetic analysis on mtDNA from patients 1 and 2 confirmed clinical and biochemical findings suggesting a mitochondrial respiratory chain disorder. The low levels of rearranged mtDNA detected in skeletal muscle from patient 3 were not likely to be the cause of the myopathic symptoms and might be a phenomenon of ageing or a consequence of another unrecognized metabolic disease. In addition mtDNA from patient 3 was analysed for the

most common mtDNA point mutations associated with mitochondrial myopathies but no critical point mutation was discovered. In agreement with findings from histochemical and electron microscopic analysis of skeletal muscle we suggest that the myopathic symptoms of patient 3 were not the result of a mtDNA mutation.

Our findings that rearranged mtDNA was present in the form of deletions as well as duplications in patient 2, suffering from KSS, while no duplicated mtDNA was detectable in patient 1, with Pearson syndrome, confirm the studies of Poulton et al. (1994), who suggested that duplications are a characteristic of KSS, while no duplications were detectable by Southern blot analysis in patients suffering from Pearson syndrome alone.

We identified two novel mtDNA rearrangements flanked by no repeats and a short symmetrical inverted repeat, respectively. The most frequently observed mtDNA rearrangements are the "common" 4977-bp deletion and the 7436-bp deletion, the latter being present in patient 1, which are precisely flanked by long (13- and 12-bp, respectively) perfect direct repeats. In addition, deletions flanked by shorter direct repeats as well as deletions with no repeats adjacent to the breakpoint have been described and recombination has been suggested to occur mainly via direct repeats (Mita et al. 1990; Rötig et al. 1991). In contrast, deletions exclusively flanked by inverted repeats have not been observed. Only one deletion with short partly imperfect inverted repeats in the breakpoint region, which were suggested to form a stem-loop structure, has been described by Fischel-Ghodsian et al. (1992). Apparently there is no mechanism that mediates recombination of mtDNA via inverted repeats. We analysed the Cambridge sequence (Anderson et al. 1981) for the presence of repeats and found a high abundance of 4-bp inverted repeats (259 201 of 4-bp inverted repeats in total and 67 595 of 4-bp inverted repeats that are separated by 1.5–8 kb and localized in the deletion-prone region between the O_L site and nt 16200). The obvious absence of any deletions flanked by long inverted repeats and the abundance of 4-bp inverted repeats in the Cambridge sequence suggest that the 4-bp inverted repeat found in patient 2 is not of biological significance and the recombination event may have been mediated by the same mechanism as for deletions with no flanking repeat.

The efficiency and high sensitivity of long PCR have directed several research groups to use long PCR for detection of mtDNA rearrangements in patients with mitochondrial diseases as well as with ageing and other deleterious effects on mtDNA (Cheng et al. 1994; Li et al. 1995; Melov et al. 1995; Reynier and Malthiery 1995). It has to be considered that long PCR might miss low levels of rearranged mtDNA with small deletions in the presence of abundant wild-type mtDNA. However, we were able to detect a mtDNA fragment of about 13 kb in the presence of abundant wild-type mtDNA in skeletal muscle from the 76-year-old control (Fig. 2, lane 9). We describe a strategy for detection and characterization of human mtDNA rearrangements. As long PCR enables analysis of large sample numbers, it may contribute more data to the understanding of mtDNA rearrangements. In addition the sensitive and specific identification of duplications by long PCR may help elucidate their supposed role as intermediates in the formation of deletions (Poulton et al. 1993) and in the development and severity of mitochondrial diseases.

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