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Nucleus-driven Multiple Large-Scale Deletions of the Human Mitochondrial Genome: A New Autosomal Dominant Disease

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Summary

We studied several affected and one nonaffected individuals belonging to three unrelated pedigrees. The pathological trait was an autosomal dominant mitochondrial myopathy due to large-scale multiple deletions of the mitochondrial genome. Clinically, symptomatic patients had progressive external ophthalmoplegia, muscle weakness and wasting, sensorineural hypoacusia, and, in some cases, vestibular areflexia and tremor. The muscle biopsies of all patients examined showed ragged-red fibers, neurogenic changes, and a partially decreased histochemical reaction to cytochrome c oxidase. Multiple mtDNA heteroplasmy was detected in the patients by both Southern blot analysis and PCR amplification, whereas the unaffected individual had the normal homoplasmic hybridization pattern. These findings confirm and add further details to the existence of a new human disease—defined clinically as a mitochondrial myopathy, genetically as a Mendelian autosomal dominant trait, and molecularly by the accumulation of multiple, large-scale deletions of the mitochondrial genome—that is due to impaired nuclear control during mtDNA replication.

Introduction

Mitochondrial myopathies are clinically heterogeneous disorders associated with morphologically abnormal mitochondria and variably impaired oxidative metabolism in the muscle. Cerebral, renal, and cardiac symptoms may also be present, implying a multisystemic defect (Lombes et al. 1989; Zeviani et al. 1989*a*).

Mitochondria depend on the complementation of two separate genomes, the nuclear genome and the mitochondrial genome (mtDNA) (Wallace 1987). While nuclear genes are transmitted as allelic Mendelian traits, mtDNA genes have their own, peculiar mode of transmission, i.e., maternal inheritance (Hutchinson et al. 1974; Wallace 1987).

From a genetic standpoint a mitochondrial disease could be due to (1) defective transcription or translation of mitochondrial proteins coded by nuclear genes, (2) mutations of mtDNA genes, or (3) mutations of nuclear genes which control mtDNA gene expression.

Most of the human diseases associated with defects of mtDNA genes are either sporadic or maternally transmitted (Wallace 1987, 1989). In particular, mtDNA heteroplasmy, due to large-scale deletions of mtDNA (Holt et al. 1988), was often found in clinical phenotypes characterized by progressive external ophthalmoplegia (PEO), with or without the other features of the Kearns-Sayre syndrome (Zeviani et al. 1988; Holt et al. 1989; Moraes et al. 1989). Most were sporadic cases showing the same deletion in various tissues, the disease being likely the result of a single, clonally amplified somatic mutation (Johns et al. 1989*a*; Shanske et al. 1990; Zeviani et al. 1990).

Alternatively, we have recently described a family with autosomal dominant PEO and multiple mtDNA deletions, in which the Mendelian inheritance of the trait points to a mutation of a nucleus-encoded factor, resulting in the damage of mtDNA (Zeviani et al. 1989*b*).

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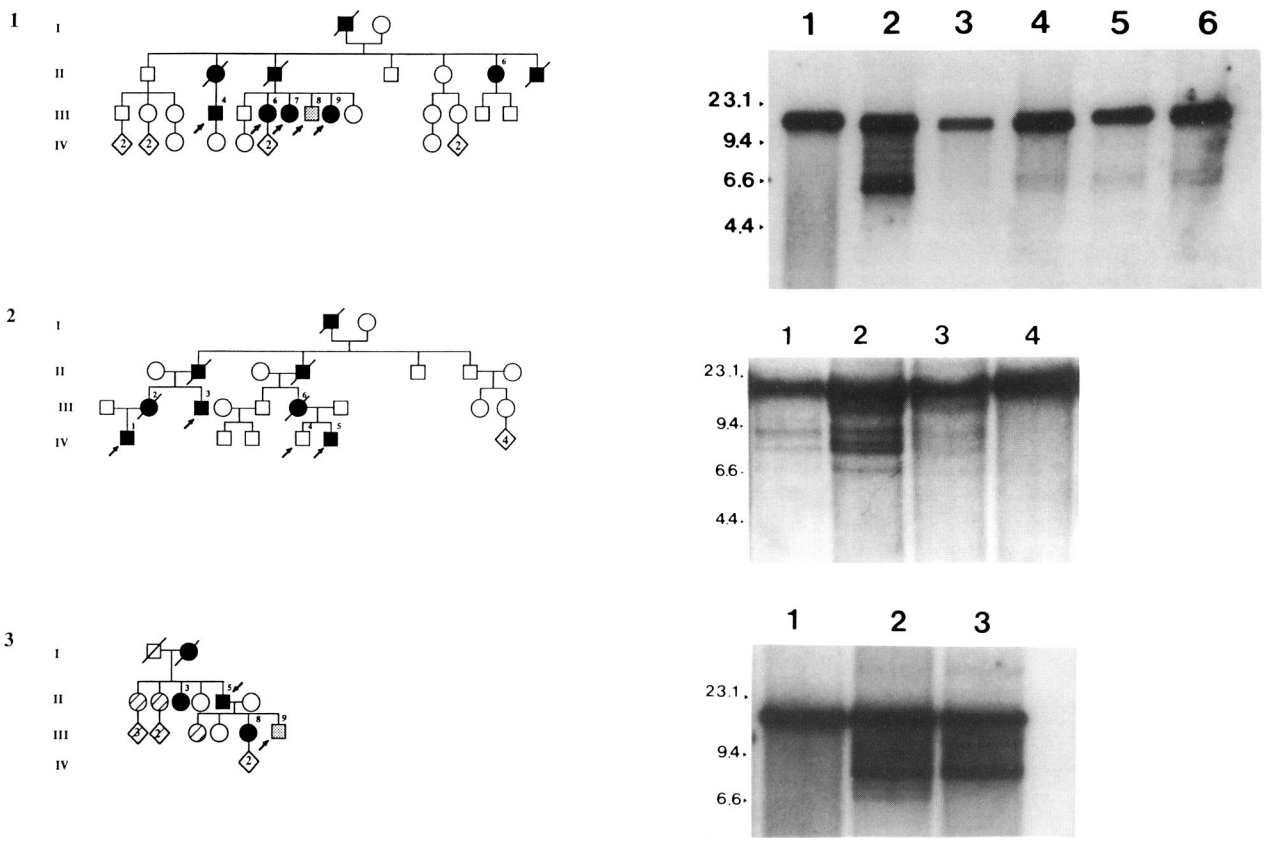

Figure 1 Family pedigrees and Southern blot analysis of mtDNA. *Left,* Pedigrees of families 1–3. Solid symbols indicate clinically symptomatic individuals. Gray-shaded symbols indicate presymptomatic individuals. Striped symbols of pedigree 3 indicate individuals whose clinical attribution is uncertain because of lack of available information. Barred symbols indicate deceased individuals. Arrows indicate the propositi. *Right,* Southern blot analysis on total muscle DNA hybridized with radiolabeled purified human mtDNA. *Top, Pvu*II-digested muscle DNA of propositi of pedigree 1. Lane 1, normal individual. Lane 2, Patient III-4. Lane 3, Patient III-6. Lane 4, patient III-7. Lane 5, Patient III-8. Lane 6, Patient III-9. *Middle,* Muscle DNA, digested with either *Pvu*II or *Bam*HI, of propositi of pedigree 2. Lane 1, Patient III-3 (*Bam*HI). Lane 2, Patient IV-1 (*Bam*HI). Lane 3, Patient IV-5 (*Pvu*II). Lane 4, Normal subject IV-4 (*Pvu*II). *Bottom, Pvu*II-digested muscle DNA of propositi of pedigree 3. Lane 1, Normal individual. Lane 2, Patient II-5, Lane 3, Patient III-9. Marker size is in kb.

We have now extended our investigation to two other members of this family and to several members of two additional families with similar clinical features. The study of these pedigrees confirms the existence and extends the molecular characterization of a new inherited disease in humans that is due to the abnormal interaction of a nuclear gene product with the mitochondrial genome.

Patients and Pedigrees

The three pedigrees are shown in figure 1 (*left*). Affected members were considered as either symptomatic or presymptomatic. Diagnosis was based on the following:

1. Clinical examination: symptomatic patients had adult-onset PEO, proximal muscle weakness and wasting, sensorineural hypoacusia, and, in several cases, vestibular areflexia. Retinal degeneration was absent in all the examined patients, but nearsightedness due to bilateral cataract was present in the elder patients (II-6 and III-4, pedigree 1; III-3, pedigree 2; and II-5, pedigree 3). Tremor, ataxia, and chronic sensorimotor peripheral neuropathy were present in all patients of pedigree 2. Moderately elevated levels of serum lactate at rest were noticed in most of the cases. Symptoms steadily progressed with the age of the patients. Presymptomatic patients appeared normal at the clinical examination.

2. Light-microscopy examination of muscle biopsies: both symptomatic and presymptomatic patients had ragged-red fibers, neurogenic changes, and a partially decreased histochemical reaction to cytochrome c oxidase.

3. Biochemical studies: several affected members of the three pedigrees had variably reduced activities of cytochrome c oxidase in their muscle homogenates.

4. Electromyographic studies: all patients had myopathic changes at the electromyographic examination; moreover, all patients belonging to pedigree 2 had electrophysiological signs of peripheral nerve involvement. In particular, motor- and sensory-nerve conductions were profoundly reduced in amplitude, while velocities of conduction were within the range of controls, indicating that the nerve fiber degeneration was predominantly axonal.

Several members (II-6, age 53 years; III-4, age 38 years; III-6, age 33 years; and III-7, age 28 years) of pedigree 1 were described in an earlier study as having multiple mtDNA deletions (Zeviani et al. 1989*b*). In the present study, we extended the clinical and molecular-genetic analysis to two patients (III-8, age 27 years; and III-9, age 25 years). Patient III-8, who appeared normal at the physical examination, had mild electrophysiological and morphological signs of muscle involvement. He was considered presymptomatic. Patient III-9 had bilateral ptosis and weakness. She was considered symptomatic.

Four individuals of pedigree 2 were analyzed: IV-1, a 42-year-old affected man, son of an affected woman (III-2) deceased at age 62 years; III-3, his 61-year-old maternal uncle; and his two second-degree cousins, IV-5, a 30-year-old man, and IV-4, the latter's healthy 31-year-old brother.

In pedigree 3, three subsequent generations were affected: the trait was transmitted from a woman (I-2) to her daughter (II-3, now 53 years old) and son (II-5, 48 years old) and from the latter to his daughter (III-8, now 20 years old) and son (III-9, 18 years old). Patient III-9 was clinically normal, but he had "myopathic" signs at the EMG examination, and his muscle biopsy showed numerous fibers which were ragged-red and/or negative to COX reaction; he was considered presymptomatic. Some members of this pedigree were clinically undefined because information was not available (see fig. 1, *left*).

Material and Methods

Preparation of Total DNA

Total DNA was isolated from 50–150 mg of frozen muscle, by using the method of Davis et al. (1986), modified by Zeviani et al. (1988). Muscle open biopsies were performed with local anesthesia after informed consent was obtained. A portion of each sample was stored in melting isopentane for morphological studies; the remaining portion was immediately frozen in liquid nitrogen for biochemical and molecular-genetic studies.

Preparation of mtDNA Probes

Mitochondria were isolated from normal human muscle according to a method described elsewhere (Husain and Steenkamp 1983). mtDNA, isolated and purified by the method of Palva and Palva (1985), was "linearized" by digestion with the restriction enzyme *Pvu*II, and 20 ng were labeled in the presence of [α^{32}P-dCTP] (Amersham) by the random-primer method (Feinberg and Vogelstein 1983).

Southern (1975) Blot Analysis

Five to 10 μg of total DNA were digested with either *Pvu*II or *Bam*HI, two restriction enzymes which "linearize" the mtDNA by digesting it at unique sites (nucleotides [nt] 2500 and 14258, respectively) (Cambridge sequence of human mtDNA; Anderson et al. 1981). Southern blot analysis was then performed essentially as described by Zeviani et al. (1988).

DNA Amplification by the Polymerase Chain Reaction (PCR) (Saiki et al. 1988)

Selection of fragments amplified from mutant mtDNAs was obtained by using pairs of oligonucleotides which primed on widely separated regions of mtDNA. Since the efficiency of the amplification is inversely proportional to the length of the template, deletion-containing fragments are selectively amplified because of their smaller size.

To obtain a PCR-based gene mapping of the deleted areas, the entire mtDNA molecule was "scanned" thoroughly by using the following pairs of oligonucleotides (Cambridge sequence), synthesized in a Gene-Assembler Plus apparatus (Pharmacia-LKB): (1) "sense" primers—*mt-100,* from nt 81 to nt 110; *EcoRI 4121,* from nt 4110 to nt 4139; *PstI 6914,* from nt 6900 to nt 6924; *XbaI 7440,* from nt 7430 to nt 7454; *XbaI 8286,* from nt

8270 to nt 8294; *PstI 9024,* from nt 9010 to nt 9034; *HindIII 11680,* from nt 11670 to nt 11694; *KpnI 16133,* from nt 16115 to 16144; and *ApaI 16459,* from nt 16445 to nt 16474—and (2) "antisense" primers—*RC KpnI 16133,* reverse complementary of *KpnI 16133; RC Apa I 16459;* reverse complementary of *Apa*I 16459; *RC KpnI 16052,* reverse complementary of the mtDNA sequence from nt 16035 to nt 16064; *RC An. 530,* reverse complementary of the mtDNA sequence from nt 515 to nt 544; *RC mt 100,* reverse complementary of mt 100; *RC BamHI 14258,* reverse complementary of the mtDNA sequence from nt 14240 to nt 14269; *RC EcoRI 5274,* reverse complementary of the mtDNA sequence from nt 5260 to nt 5289; *RC XhoI 14955,* reverse complementary of the mtDNA sequence from nt 14940 to nt 14969; and *RC RsaI 15813,* reverse complementary of the mtDNA sequence from nt 15800 to nt 15824.

The Gene-Amp kit (Perkin Elmer–Cetus) was used in all the experiments. Amplification was performed on 1–5 ng of total DNA in a DNA thermal-cycler apparatus (Perkin Elmer–Cetus) for 25 cycles. One cycle was as follows: heat denaturation for 1.5 min at 94°C, primer annealing for 2 min at 55°C–60°C, and primer extension for 2.5 min at 72°C.

In these conditions, PCR almost invariably failed to amplify normal muscle DNA, which was included in each experiment as a control to check against the possibility of aspecific priming. Rarely, however, a high-molecular-weight band, corresponding in size to the wild-type fragment, was amplified from both the controls' and the patients' templates.

Direct Sequencing of Double-stranded DNA (Winship 1989)

PCR-amplified DNA corresponding to approximately 0.5 pmol was extracted and purified from 1% agarose gel by using the glass-beads method (Geneclean™ kit; Genenco). DNA was resuspended in 10 μl of 10% dimethylsulfoxide (DMSO), 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl (1 × Sequenase™ buffer; USB, Cleveland), and 100 pmol of the suitable primer, heat denatured for 3 min at 98°C and immediately frozen in liquid nitrogen. The Sequenase kit and protocol for the dideoxy chain-termination sequencing method (Sanger et al. 1977) using [α-^{35}S-dATP] (Amersham) were used in each experiment, except that the first reaction was carried out for 2 min on ice in the presence of a 10% final concentration of DMSO. Samples were loaded on a 5.5% polyacrylamide denaturing gel and electrophoresed in 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA). The gel was soaked for 20 min in 10% methanol and 10% acetic acid and was vacuum-dried and autoradiographed at room temperature for 12–24 h by using Kodak XAR-5 films with an intensifying screen.

Results

Southern Blot Analysis

Southern blot analysis of *Pvu*II- or *Bam*HI-digested muscle DNA from symptomatic and presymptomatic patients, hybridized with radiolabeled purified human mtDNA, showed the presence of mtDNA heteroplasmy, because, in addition to a major 16.5-kb band corresponding to the linearized, wild-type mtDNA (Cambridge sequence), several smaller bands, corresponding to deleted mtDNAs, were also detected (fig. 1, *right*). By contrast, normal individuals, including subject IV-4 (pedigree 2), had the wild-type homoplasmic hybridization pattern.

Identification of Two PvuII Polymorphisms in Pedigree 2

Restriction analysis of *Pvu*II-digested muscle DNA from patients III-3 (pedigree 2) (fig. 2, *bottom*), and IV-1 (pedigree 2) and from available lymphocyte DNA from patient III-2 (pedigree 2) (not shown) showed the presence of two additional *Pvu*II sites, 2,393 bp upstream and 2,314 bp downstream (Cambridge sequence sense) from the known *Pvu*II site at nt 2652. We amplified the corresponding regions, from nt 530 to nt 100 and from nt 4700 to nt 5055, and directly sequenced the DNA fragments. The polymorphic *Pvu*II sites were identified at nt 259 and nt 4964, respectively. The first *Pvu*II polymorphism was caused by a CA→TG dinucleotide transition at nt 261–262 (CAGCCA→CAGCTG), while the second was produced by a T→C transition at nt 4965 (CAGTTG→CAGCTG). Neither patient IV-5 nor his unaffected brother IV-4, both of whom belonged to a different branch of the same pedigree 2, showed the *Pvu*II polymorphisms (fig. 1, *right*).

Southern Blot–Based Deletion Mapping

A set of probes corresponding to different regions of mtDNA was used to map the deleted areas in different individuals. Total DNA was cut with restriction endonucleases recognizing two or three sites in wild-type mtDNA, to improve the electrophoretic separation—

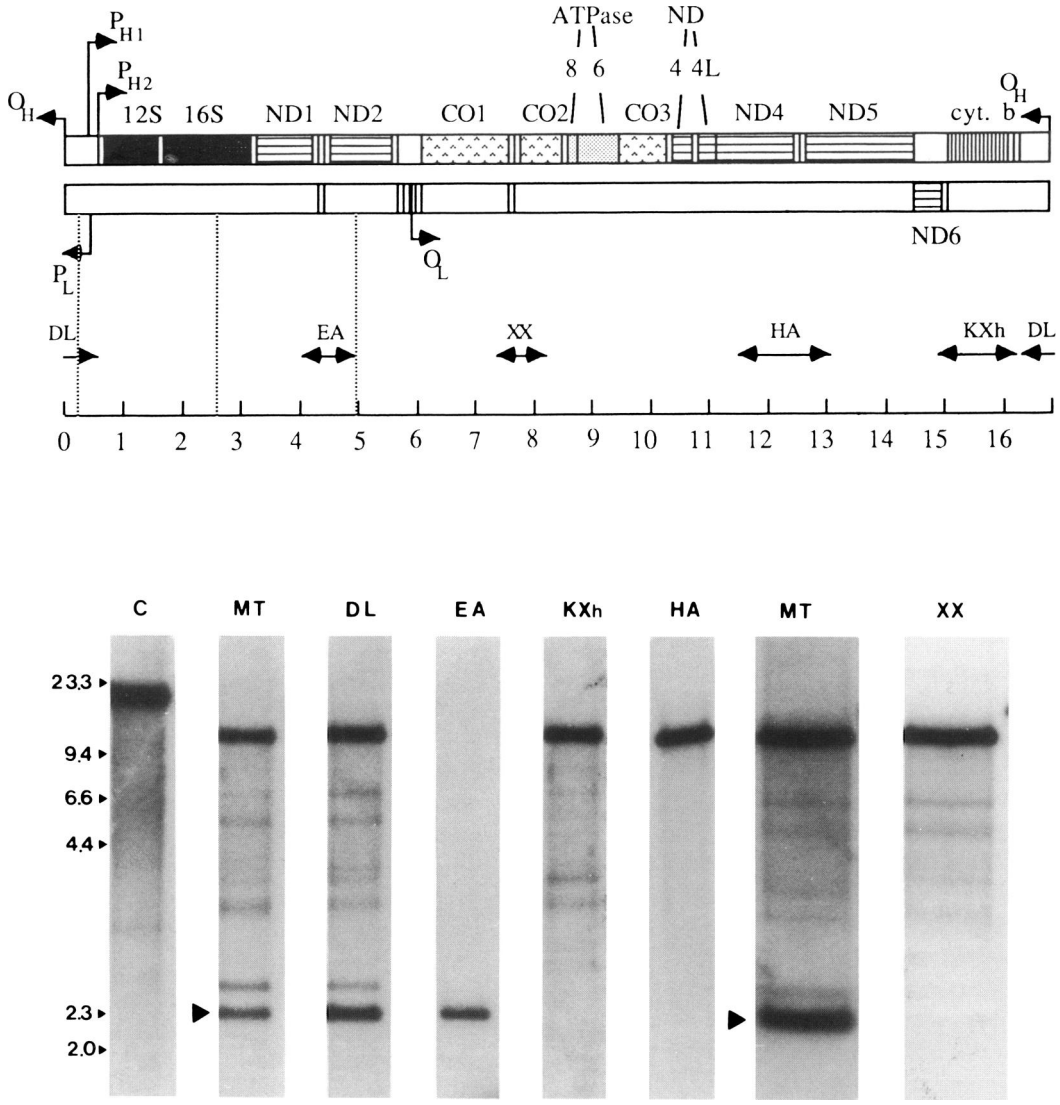

Figure 2 Southern blot–based mapping of the mtDNA deletions. *Top*, Scheme of mtDNA and probes used for gene mapping of deleted mtDNAs. The maps at the top represent the heavy (upper map) and light (lower map) strands of linearized mtDNA, showing the genes for the subunits of NADH-CoQ reductase (ND1–ND6), cytochrome c oxidase (CO1–CO3), cytochrome b (cyt. b), and ATP-synthase (ATPase 6 and ATPase 8) and for the 12S and 16S ribosomal RNAs. The transfer RNAs are indicated by small open boxes. The origins and the direction of heavy-strand (O_H) and light-strand (O_L) replication and the promoters for the initiation of heavy-strand (P_{H1} and P_{H2}) and light-strand (P_L) transcription are indicated by arrows. Double arrows indicate the extension and localization of hybridization probes DL, EA, XX, HA, and KXh (see Results for details). Dotted vertical lines indicate the three *Pvu*II sites found in wild-type muscle mtDNA of patients III-3 and IV-1 (pedigree 2) (see Results). The bar at the bottom represents the length of the map (in kb); progression from 0 to 16 follows the direction of the Cambridge sequence (Anderson et al. 1981). *Bottom*, Southern blot analysis of *Pvu*II-digested muscle DNA from patient III-3 (pedigree 2). Each lane shows the results of hybridization with probe MT, corresponding to total mtDNA, and probes DL, EA, XX, HA, and KXh. The arrowheads in MT lanes indicate the bands corresponding to the *Pvu*II polymorphisms shown in fig. 2, *top*. Since two of the three fragments generated by digestion with *Pvu*II had a nearly identical size, they appear as a single, ~2.3-kb band. Marker size is in kb.

and therefore the identification—of individual bands corresponding to deletion-containing areas. For instance, figure 2 (*bottom*) shows the results obtained in patient III-3 (pedigree 2) carrying the two *Pvu*II polymorphic sites discussed above. Total muscle DNA was cut with *Pvu*II and probed with (fig. 2, *top*): (1) the entire mtDNA molecule (MT), (2) DL, a 969-bp PCR fragment from nt 16133 to nt 530 (Cambridge sequence

sense), encompassing the D-loop region, (3) EA, an 804-bp PCR fragment from nt 4123 to nt 4927, entirely contained within the 2,314-bp-long *Pvu*II fragment, in close proximity with the origin of light-strand replication (O_L), (4) XX, an 848-bp PCR fragment from nt 7440 to nt 8288, (5) HA, an 890-bp PCR fragment from nt 11680 to nt 12570, and (6) KXh, a 1,095-bp PCR fragment from nt 14955 to nt 16050. The same pattern was obtained by using either the entire mtDNA or DL as probes, indicating that the D-loop region was conserved in all the deleted mtDNAs. Only the ~2.3-kb band corresponding to the 2,314-bp *Pvu*II polymorphic restriction fragment was hybridized by probe EA, again suggesting that this region was not affected by recombinations and was therefore presumably conserved in both the wild type and the mutants. By contrast, disappearance of bands was observed with probes XX, KXh, and HA. As expected, these probes did not recognize the ~2.3-kb band corresponding to the polymorphic *Pvu*II fragments, but they also failed to hybridize a number of bands belonging to deleted genomes. This result indicates that the mtDNA regions corresponding to probes HA, KXh, and XX were preferentially contained within deleted areas, as compared with regions corresponding to probes DL and EA, which appeared conserved in most mtDNA species. It is also obvious that the region most commonly involved in deletions contained HA. Similar results were obtained by adapting the same experimental design to DNA samples from other patients (not shown).

PCR-based Deletion Mapping

Figure 3 (*top*) shows some of the results of PCR amplifications of deletion-containing regions of mtDNA. Multiple fragments were obtained from all patients by using pairs of primers which "explored" a wide portion of mtDNA, spanning from the origin of replication of the heavy strand (O_H), including the D-loop region, to O_L (Cambridge sequence), (heavy-strand [H-strand] replication sense; see legend to fig. 2, *top*). As in our previous report (Zeviani et al. 1989*b*), PCR-amplified fragments were consistently obtained with pairs of oligonucleotides including, on one side, the *RC KpnI 16133* or the *RC An. 530* primers, which hybridize sequences at the termini of the mtDNA D loop. However, PCR fragments were also obtained by amplifying mtDNA regions which exclude the D-loop, such as those encompassed by primers *RC RsaI 15813* and *XbaI 8286*, *RC XhoI 14955* and *XbaI 7440*, or *RC BamHI 14258* and *PstI 9024* (H-strand replication sense). By contrast, no fragments were obtained by amplifying the portion of mtDNA spanning from O_L to O_H (H-strand replication sense), suggesting that this region was conserved in most of the mutant mtDNA species.

It is interesting that in several cases PCR fragments produced in different, unrelated patients by the same pairs of primers were apparently the same. An example is given in figure 3 (*bottom*). This result suggests that deletions are due to a molecular mechanism common to the three pedigrees, possibly acting on "hot-spot" sequences scattered throughout a wide area of mtDNA.

Finally, the possibility that the fragments were produced by multiple aspecific priming during PCR amplification was ruled out in two ways. First, no fragments were produced from normal mtDNA, with the occasional exception of a large fragment, most likely corresponding to the wild-type template. Second, in several cases we directly sequenced the entire collection of fragments from each PCR amplification in both directions, using, for each reaction, either the sense or the antisense oligonucleotide as sequencing primers. Although the sequences became soon more and more ambiguous—and eventually unreadable—because of multiple frameshifts due to deletion breakpoints, the first 100–150 bases on each side were unambiguous and invariably corresponded to the appropriate priming region. Two of these sequences, obtained by using *XbaI 7440* and *RC RsaI 15813* as primers, are shown in figure 4.

Conservation of the D Loop

To verify whether the D loop, a crucial region containing both the O_H and the heavy-strand transcriptional promoters was conserved in the mutant species, we examined PCR fragments which, because they were obtained by using either *RC An. 530* and *PstI 9024* (fig. 5*A*) or *RC An. 530* and *XbaI 7440* (not shown), could include recombinations within the D loop. The fragments were then digested with *Kpn*I, because a *Kpn*I restriction site is present at the very end of the D loop (nt 16130). As shown in figures 5*A* and 5*B*, *Kpn*I digestion of muscle DNA from the patients produced a major band of ~1 kb, as would be expected if the corresponding *Kpn*I site was consistently conserved in all fragments. The DNA of the gel shown in figure 5*B* was then blotted onto a nitrocellulose filter and hybridized with the ~1-kb PCR fragment obtained with the two oligonucleotides *RC An. 530* and *KpnI 16133*, a fragment which encompasses the entire D-loop region. Results confirmed that all recombinations occurred downstream from the *Kpn*I 16130 site, because the probe, which hybridized with the whole set of un-

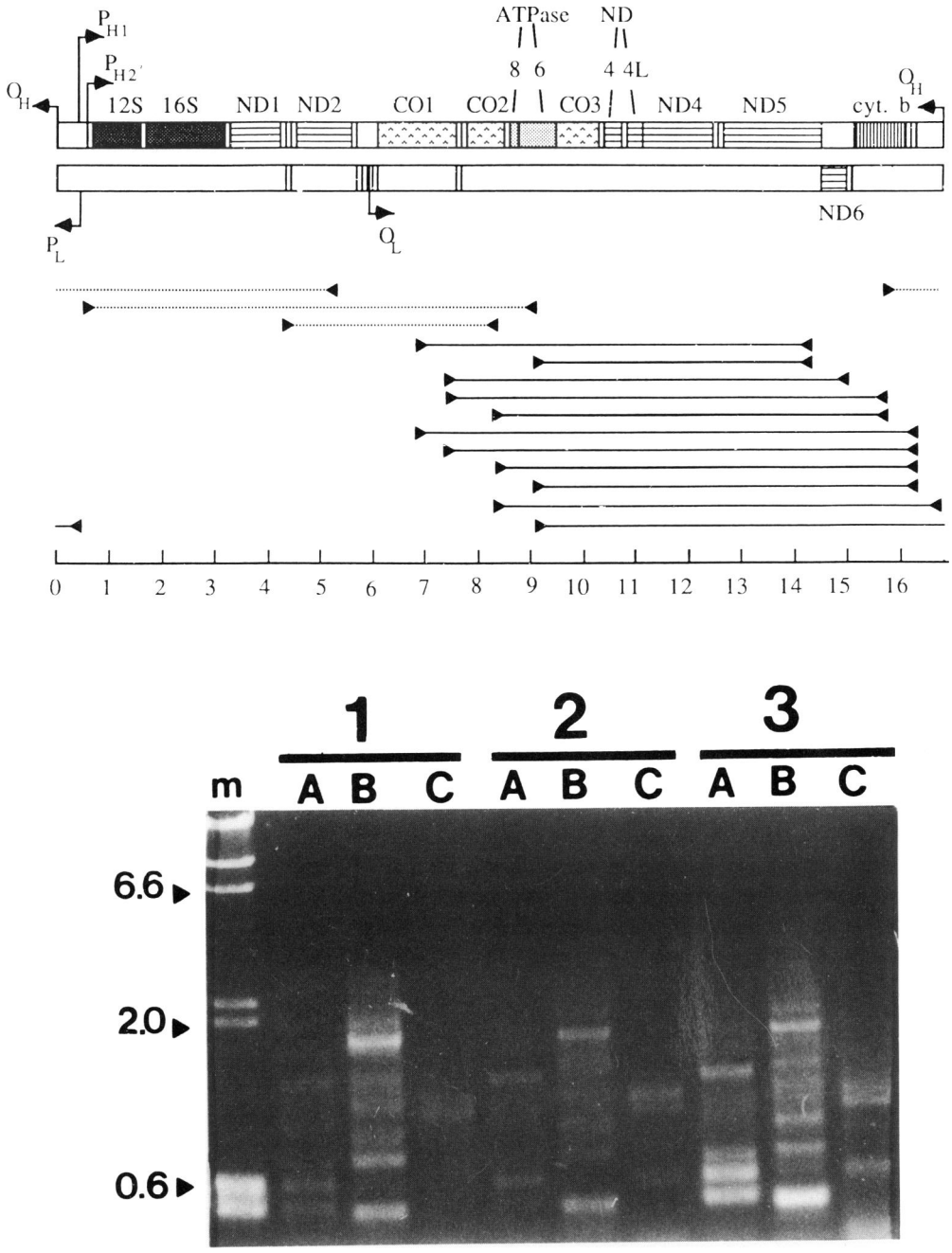

Figure 3 PCR-based mapping of mtDNA deletions. *Top*, Maps at top represent linearized human mtDNA as in fig. 2, *top*. Solid double arrows indicate the positions and distances of primers used for PCR amplification of breakpoint-containing DNA fragments. Dotted double arrows indicate the positions and distances of primers used in PCR experiments that failed to produce DNA amplification fragments. The bar at the bottom represents the length of the map (in kb); progression from 0 to 16 follows the direction of the Cambridge sequence. *Bottom*, PCR amplification fragments. Lanes A, Amplification using *RC RsaI 15813* and *XbaI 8286* as primers. Lanes B, Amplification using *RC KpnI 16133* and *XbaI 7440*. Lanes C, Amplification using *RC BamHI 14258* and *PstI 9024*. Group 1: PCRs on muscle DNA from patient III-9, pedigree 1. Group 2: PCRs on muscle DNA from patient IV-1, pedigree 2. Group 3: PCRs on muscle DNA from patient II-5, pedigree 3. Marker size is in kb.

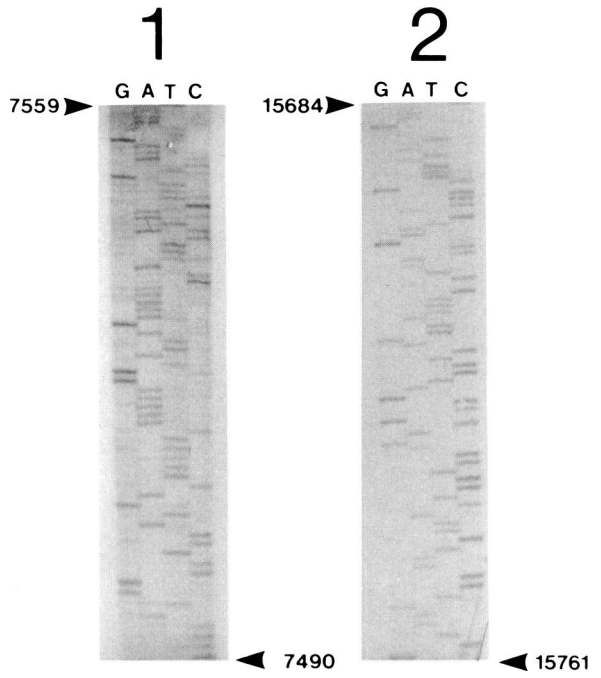

Figure 4 Direct sequencing of a whole PCR amplification set. Fragments were obtained from muscle mtDNA of patient III-3 (pedigree 2) by amplifying with *XbaI 7440* and *RC RsaI 15813*. *1*, Nucleotide sequence from nt 7490 to nt 7559 (Cambridge sequence), performed by using the *XbaI 7440* primer. *2*, Nucleotide sequence from nt 15761 to nt 15684, performed by using the *RC RsaI 15813* primer.

digested fragments, recognized only the 1-kb band into which they had "collapsed" after digestion with *KpnI* (not shown).

Direct Sequencing of PCR Fragments

The conservation of the D-loop region was further confirmed by the direct sequencing of the whole collection of PCR fragments from two samples, belonging to patients III-9 (pedigree 1) and IV-4 (pedigree 2). As in other cases, the sequences obtained by using either *RC An. 530* on one side and either *PstI 9024* or *XbaI 7440* on the other side as primers corresponded to the expected regions of mtDNA, indicating that the amplification was specific. However, the reading frames of the sequences obtained by using *PstI 9024* or *XbaI 7440* contained an increasing number of ambiguities, occurring after the initial 100–200 bases from the primer. By contrast, the sequences obtained by using *RC An. 530* had no ambiguities, being readable up to nt ~130. Sequence analysis was then continued on overlapping regions throughout the entire D loop, by using

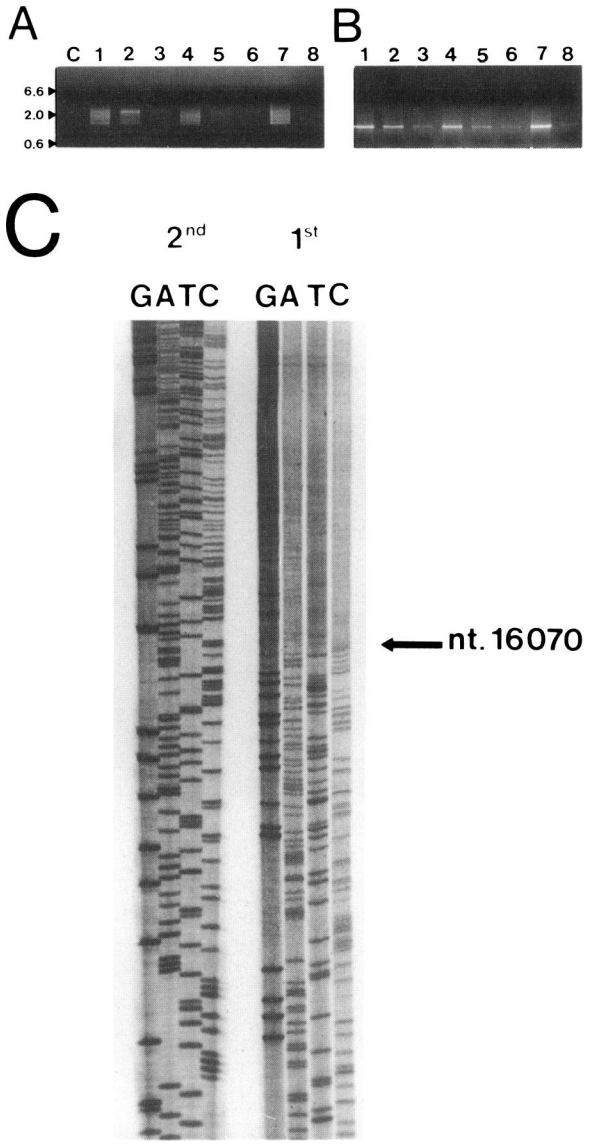

Figure 5 Conservation of D loop. *A*, PCR fragments obtained using *RC An. 530* and *PstI 9024* as primers. Lane C, Muscle DNA from control subject. Lane 1, Muscle DNA from Patient III-4, pedigree 1. Lane 2, Muscle DNA from Patient III-8, pedigree 1. Lane 3, Muscle DNA from Patient III-9, pedigree 1. Lane 4, Muscle DNA from Patient III-3, pedigree 2. Lane 5, Muscle DNA from Patient IV-1, pedigree 2. Lane 6, Muscle DNA from Patient IV-5, pedigree 2. Lane 7, Muscle DNA from Patient II-5, pedigree 3. Lane 8, Muscle DNA from Patient III-9, pedigree 3. Marker size is in kb. *B*, Same fragments after digestion with *KpnI*. *C*, Nucleotide sequence of PCR fragments obtained from muscle DNA of patient IV-5, pedigree 2, by using *RC An. 530* and *PstI 9024* as primers. Fragments were directly sequenced using *RC ApaI 16430* as the sequencing primer. The first and second loadings, as well as the position of nt 16070 (Cambridge sequence), are indicated.

RC mt 100 and *RC ApaI 16430* as "internal" primers. The DNA fragments were thoroughly readable from nt ~100 to nt ~16400 and from nt ~16430 to nt ~16070, where multiple ambiguities ensued (fig. 5*C*). To demonstrate that the latter were not due to secondary structures of the template, we also sequenced a wild-type PCR fragment encompassing the portion of the D loop from nt 16133 to nt 15925 and found the sequence readable throughout (not shown). Our interpretation was that no recombinations, which would have produced multiple reading ambiguities, occurred either (1) from position 530, at one end of the PCR fragments, to position 16070, at the end of the D-loop region or (2), as expected, beyond the *KpnI* site at nt 16130.

We also directly sequenced single PCR fragments extracted from agarose gel and reamplified by PCR. In particular, sequence analysis was carried out on three sets of fragments: the first set was obtained by using the oligonucleotide *RC KpnI 16133* on one side and oligonucleotide *XbaI 7440* on the other side; the second set was obtained by using *RC RsaI 15813* and *PstI 9024;* and the third set was obtained by using *RC RsaI 15813* and *XbaI 7440*. In the first set of fragments, the breakpoints of the deletions were localized within a short region spanning from nt 16070 to nt 16080, a result similar to those of previous observations (Zeviani et al. 1989*b*). In the second and third sets of fragments, which could not contain the 16070–16080 region, we found new breakpoints scattered throughout the mtDNA. In all cases, deletions appeared to occur across flanking direct repeats of variable length. An example of these results is given in figure 6.

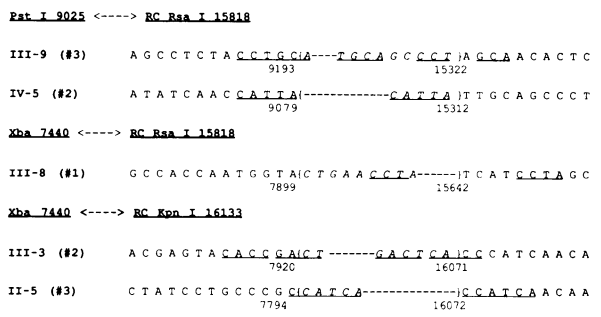

Figure 6 Nucleotide sequences of breakpoint regions of single PCR fragments. Numbers refer to nucleotide positions in the Cambridge sequence of human mtDNA L strand. Breakpoints are indicated by parentheses. Italic letters indicate nucleotides which are included within deletions. Regions of homology, at each side of the deletions, are underlined. The primers used for each amplification and the patients to whom each sequence refers are also indicated.

Discussion

We found multiple mtDNA deletions in several individuals belonging to three independent pedigrees. They had clinical and/or laboratory findings of a disease characterized by adult-onset PEO, as well as other symptoms of mitochondrial encephalomyopathy.

We believe that the mtDNA heteroplasmy was the cause of the clinical phenotype, since (1) all the affected and none of the unaffected tested individuals, including one healthy relative in pedigree 2, had deletions, and (2) the PEO phenotype is commonly associated with mtDNA deletions (Zeviani et al. 1988; Holt et al. 1989; Moraes et al. 1989).

In these three pedigrees the trait was autosomal dominant, because transmission was through both paternal and maternal lineages, and both sexes were affected in subsequent generations. Furthermore, two *Pvu*II restriction polymorphisms were present in the mtDNA of maternal members of one of the two lineages studied in pedigree 2. The first polymorphism, at nt 260–261, was located within the D loop. It is known that since this region contains no genes it is subjected to less stringent selective pressure, compared with the rest of the mitochondrial genome. As a consequence, the D-loop region retains the fastest evolutionary rate and the highest intraspecific variability (Anderson et al. 1981; Clayton 1982; Wallace 1987). In addition, the *Pvu*II polymorphism is not comprised within functionally important regions (such as the so-called consensus-sequence blocks [CSBs]), which are also the most conserved portions of the D loop (Clayton 1982). The second polymorphism, which occurred in the ND2 gene, does not modify the protein reading frame of the corresponding subunit, because the new triplet, AGC, codes for the same amino acid (serine) as does the "wild type" AGT. Therefore, it is unlikely that either mutation can play a role in the pathogenesis of the disease. However, the finding that the polymorphisms were present only in one of the two lineages, which are linked to each other by affected ancestral males, indicates that the mtDNA lesions, which were found in individuals belonging to both lineages, were produced on two independently inherited mitochondrial genotypes.

The molecular details of mutant mtDNAs were studied by a combination of Southern blot, PCR-based gene mapping, and sequence analysis. By the latter method, we confirmed our previous observation (Zeviani et al. 1989*b*) that a hot spot for mutations was localized at the end of the D-loop region (H-strand replication sense)

between nt 16070 and nt 16080. However, in the present study we found that this was not the exclusive site, because breakpoint-containing PCR fragments were also produced by amplifying regions, comprised between the two origins of replication (H-strand replication sense), well downstream from the end of the D loop. This region includes genes for cytochrome b, the three subunits of cytochrome c oxidase, the two subunits of ATPase, and five subunits of NADH dehydrogenase, as well as for numerous tRNAs. These data were confirmed by Southern blot analysis of mtDNA by using differential hybridizations with probes scattered throughout the mitochondrial genome. We had identical results also by reexamining the original patients first described by us (Zeviani et al. 1989*b*). Although we cannot exclude that deletions could also affect the portion of the molecule from O_L to O_H (H-strand replication sense), the latter seemed to be relatively conserved. In particular, data obtained by PCR-based gene mapping, differential Southern blot hybridization analysis, and direct sequencing of multiple PCR fragments all suggested that both O_L and O_H were spared from deletions, supporting the idea that molecular rearrangements which loop out these regions cannot be perpetuated. Similar results were recently reported by Yuzaki et al. (1989), in a study showing multiple mtDNA deletions apparently transmitted as an autosomal recessive trait.

On the basis of the sequences already analyzed in several members of pedigrees #1 (Zeviani et al. 1989*b;* and present paper), #2 and #3 (present paper), and in sporadic patients (Schon et al. 1989; Shoffner et al. 1989; Johns et al. 1989*b;* Mita et al. 1990; Zeviani et al. 1990), most deletions occur across flanking direct repeats. Commonly invoked mechanisms for the generation of length variation in the presence of repeated sequences are unequal crossing-over during recombination and slipped mispairing during replication (Efstratiadis et al. 1980). The data that are presently available suggest that recombination may not occur in the mitochondrial system of multicellular animals (Clayton 1982; Wallace 1987). Therefore, although recombination cannot be formally ruled out, a more likely mechanism could be the slippage and mismatching of single strands during replication. Slipped mismatching has been proposed to explain the mtDNA size variation observed in a number of lower animal species (Densmore et al. 1985; Rand and Harrison 1989), as well as the occurrence of single, sporadic mtDNA deletions in man (Shoffner et al. 1989). In our patients, this hypothesis is strongly supported by the observation that the portion of the mtDNA molecule containing most of the deletions is exposed as single strands for considerable periods during replication, so that slip-mismatches across repeats may well occur in this area, as compared with the rest of the molecule, in which much smaller tracts are exposed as single strands for a shorter time (Clayton 1982).

Naturally occurring mtDNA size variation and heteroplasmy is common in lower—but not in higher—animals. Homeothermic animals generally have the smallest and least variable mitochondrial genomes, while poikilotherms have slightly larger mtDNAs which are considerably more variable in size, suggesting stronger selection for smaller, highly conserved mtDNAs in animals with higher metabolic rates (Wallace 1982; Moritz et al. 1987). Heteroplasmy in lower animals was observed only in isofemale lineages, suggesting maternal inheritance (Densmore et al. 1985; Rand and Harrison 1989). Heteroplasmic mtDNA samples, however, were much more frequent in triploid than in diploid lizards (*Cnemidophorus tesselatus*) (Densmore et al. 1985), suggesting that altered nuclear background may be of importance in promoting mtDNA length variation.

Genetic analysis of our pedigrees indicates that the mitochondrial genomes were damaged by a dominantly inherited mutation of a nuclear gene. By the above discussion, it is likely that this gene encodes for a protein involved in the nucleus-controlled mechanism of mtDNA replication. For instance, strand slippage and mispairing could occur between homologous regions, because of a defect in one of the mtDNA binding proteins which are believed to prevent DNA branch migration during the synthesis of the nascent H-strand (Van Tuyle and Pavco 1981; Mignotte et al. 1985). Both the search for genes coding for known candidate proteins and linkage analysis with genetic markers are currently underway to identify the gene responsible for this new human disease.

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