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A Direct Repeat Is a Hotspot for Large-Scale Deletion of Human Mitochondrial DNA

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Kearns-Sayre syndrome (KSS) and progressive external ophthalmoplegia (PEO) are related neuromuscular disorders characterized by ocular myopathy and ophthalmoplegia. Almost all patients with KSS and about half with PEO harbor large deletions in their mitochondrial genomes. The deletions differ in both size and location, except for one, 5 kilobases long, that is found in more than one-third of all patients examined. This common deletion was found to be flanked by a perfect 13–base pair direct repeat in the normal mitochondrial genome. This result suggests that homologous recombination deleting large regions of intervening mitochondrial DNA, which previously had been observed only in lower eukaryotes and plants, operates in mammalian mitochondrial genomes as well, and is at least one cause of the deletions found in these two related mitochondrial myopathies.

KEARNS-SAYRE SYNDROME (KSS) IS a multisystem mitochondrial disorder defined by the presence of ophthalmoplegia and pigmentary retinopathy with onset before age 20 and at least one of the following: high cerebrospinal fluid (CSF) protein content, blockage in heart conduction, or ataxia (*1*). Morphologically, KSS patients display "ragged red fibers" (RRF) in muscle sections; RRF are a morphologic hallmark of proliferating mitochondria in muscle and are seen in muscle sections stained with modified Gomori trichrome as red patches (*2*). KSS is ultimately fatal. Progressive external ophthalmoplegia (PEO) and, frequently, RRF are also seen in ocular myopathy, but there is no systemic involvement and the disease is rarely fatal. Biochemically, both KSS and PEO often show reduced respiratory chain enzyme activity, particularly that of cytochrome c oxidase (CO) (*3*, *4*).

We found that 13 of 15 patients with KSS and about half of all patients with PEO had large-scale deletions of mitochondrial DNA (mtDNA), ranging in size from 1.3 to 7.6 kb (*4*). Similar results have also been obtained by others (*5*). The size and location of the deletions, and the number of deleted mtDNA relative to the number of normal mitochondrial genomes, differed among patients and did not appear to be correlated to the presentation or the severity of the disease phenotype.

The 29 deletions we studied were mapped by analyzing each deleted genome for the absence of known restriction sites on the mtDNA map (*6*). Using this method, however, we were unable to specify the precise breakpoint of any deletion. Nevertheless, we showed that all the deletions were in regions of the mitochondrial genome containing structural components of the respiratory chain; no deletions were found in the ribosomal RNA genes, or in the region of either the origins of heavy- or light-strand replication, or of heavy- and light-strand transcription (*7*). Of the 29 deletions, 11 (3 in patients with KSS and 8 in patients with PEO) mapped to an identical location in the mtDNA, with deletion breakpoints about 5 kb apart, extending from the ATPase8 gene (*8*) of complex V on the left, to the gene encoding a subunit of NADH dehydrogenase (ND5) of complex I on the right. We have now found a 12th patient (with KSS) who also harbors this common deletion.

Using the polymerase chain reaction (PCR) (*9*), we amplified selectively the region of the deleted genome spanning the deleted mtDNA in a number of patients with this common deletion and determined the exact site of the deletion breakpoint by DNA sequencing (*10*, *11*).

We sequenced mtDNA from two patients with KSS and three patients with PEO who

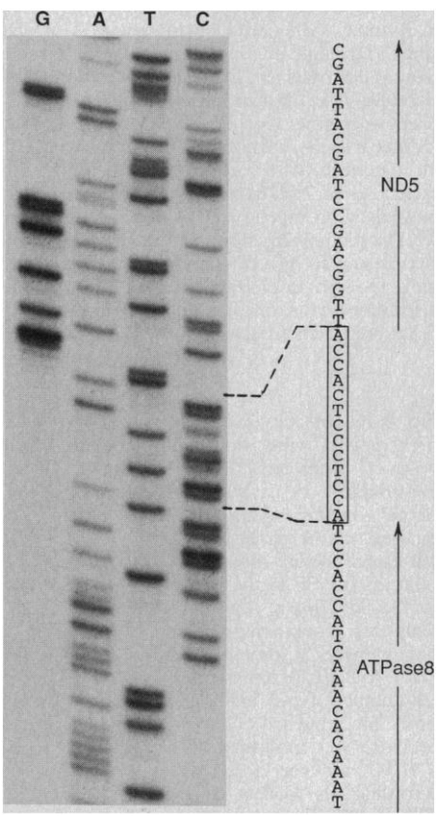

Fig. 1. Autoradiogram of a DNA sequencing gel in the region of the deletion breakpoint. The sequence (dideoxy reactions G, A, T, and C) is of the L-strand, reading 5′ to 3′ from bottom to top (arrows). The single 13-bp direct repeat (boxed) in the deleted mtDNA is downstream of the 5′ portion of the ATPase8 gene and upstream of the 3′ portion of the ND5 gene; the intervening 4977 bp are deleted (Fig. 2).

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had the identical deletion breakpoint (Fig. 1). The deletion was 4,977 bp long, with the breakpoint on the left side at nucleotide position 8,483 [numbering of (*6*)] within the ATPase8 gene, and on the right side at position 13,460 within the ND5 gene. The deletion was flanked by a perfect 13-bp repeat (Fig. 2); one repeat was found immediately prior to the left deletion breakpoint, whereas the other 13-bp repeat was found (in normal, undeleted mtDNA) at the extreme 3′ end of the deleted region, just prior to the right deletion breakpoint. The fusion gene thus created encodes an mRNA that is out-of-frame in the ND5 portion of the predicted transcript, resulting in a premature termination codon 12 nucleotides beyond the deletion breakpoint. Rather than encoding an ATPase8 protein of 68 amino acids [deduced molecular weight of 7.9 kD (*6*)], this mRNA, if translated, would encode a truncated protein 42 amino acids long (that is, about 5 kD).

Although we did not sequence the deletion in the mtDNA of the other 7 patients harboring the common deletion, PCR analysis showed that all 12 contained a deleted mtDNA genome. When the mtDNA was amplified with the same oligonucleotide primers as used for the five sequenced deletions, it displayed an identically sized DNA fragment on agarose gels (Fig. 3), indicating that all 12 patients most likely carry the identical deletion.

The presence of 12 patients with KSS or PEO who have the identical deletion implies that the 13-bp repeat found at mtDNA positions 8,470 to 8,483 and 13,447 to 13,460 is a preferred target, or hotspot, for deletion. A computer search (*12*) of the mitochondrial genome for the presence of other long, perfect direct repeats showed the presence of four 13-bp repeats and one 15-bp repeat. (There are also 253 different 10-bp repeats, 58 11-bp repeats, and 16 12-bp repeats, which are surprisingly high numbers for a 16.5-kb genome.) Of the largest repeats, the three 13-bp repeats not associated with the common deletion (at mtDNA map positions 10/2275, 535/4430, and 2210/10614) and the 15-bp repeat (at positions 3674/11748) have left ends in the area between the D-loop region and the origin of light-strand replication, and would thus be predicted to be nonviable for the propagation of deletions. Only the 13-bp repeat found in the common deletion is in a region representing a viable target for deletion.

The presence of a large-scale deletion flanked by direct repeats in the mtDNA implies that this deletion was caused by a homologous recombination event. Moreover, the fact that 12 of 30 examined deletions in mtDNA of patients with KSS or PEO are flanked by this particular direct repeat implies that homologous recombination is a significant source of deletion in these diseases.

The involvement of direct repeats in the creation of microdeletions, on the other hand, has been observed. Wrischnik *et al.* (*13*) found a deletion of one of two tandem 9-bp repeats in the 3′ untranslated region of the human CO II gene. This particular polymorphism was found in 12 individuals; since it was in the 3′ untranslated region of CO II, it did not result in any overt pathology. The D-loop region has also been found to be a region containing small heterogeneities among human and bovine mtDNA; in this case the area of individual variation involved only a few nucleotides within a polypyrimidine stretch on the L strand located within a conserved sequence region (*14*).

Although large-scale deletions of mtDNA had not been observed previously in mammals, they have been well documented in other eukaryotes. Mitochondrial recombination has been observed in yeast rho$^-$ petite mutants, often, but not always, involving repeated elements (*15*). Similar types of deletions involving repeated elements in mtDNA have also been observed in stopper (*stp*) mutants of *Neurospora crassa* (*16*) and in DNA associated with the senescence phenotype of *Podospora anserina* (*17*). Large-scale deletions flanked by repeats have also been found in the mtDNA of plants, usually involving intragenomic homologous recombination (*18*). Although no clearly identified recombination machinery has been identified in mammalian mitochondria (*19*), recombination of mtDNA has been inferred from the analysis of interspecific somatic cell hybrids (*20*), and an endonuclease associated with recombination has been found in mouse cell mitochondria (*21*). Our results imply that there is a mechanism for rearranging human mtDNA, apparently via homologous recombination.

We can only speculate as to how the 5-kb deletion arose. Even though dimeric mtDNA circles have been identified in mammals (*7*), it is unlikely that this deletion arose as a result of intergenomic recombination by unequal crossing-over. An unequal crossing-over event between two mtDNAs at one of the 13-bp repeats, followed by resolution of the double-length dimer mtDNA, would result in one genome with three repeats (21.5 kb rather than 16.5 kb) and one with only one repeat (11.5 kb). Since the three-repeat mtDNA contains all the origins of replication and transcription, it ought to be viable and segregate into adult tissues, much in the same way the one-repeat (deleted) mtDNA has been amplified in the muscle tissues of the patients in our study. When digested with Pvu II (which cuts only once in human mtDNA) one would expect to see three mtDNA fragments in DNA blots, 21.5, 16.5, and 11.5 kb in size. However, in more than 30 KSS and PEO patients with deleted mtDNAs, including the 12 described here, we have never observed any mtDNA larger than 16.5 kb after Pvu II digestion.

A more likely possibility is that the deletion arose by means of an intragenomic recombination event. In this case, homologous recombination at the 13-bp repeats would generate the 11.5-kb deleted mtDNA containing one repeat plus a small (presumably circular) 5-kb subgenomic fragment, the deleted region, containing the other 13-bp repeat. Since the 5-kb deleted region

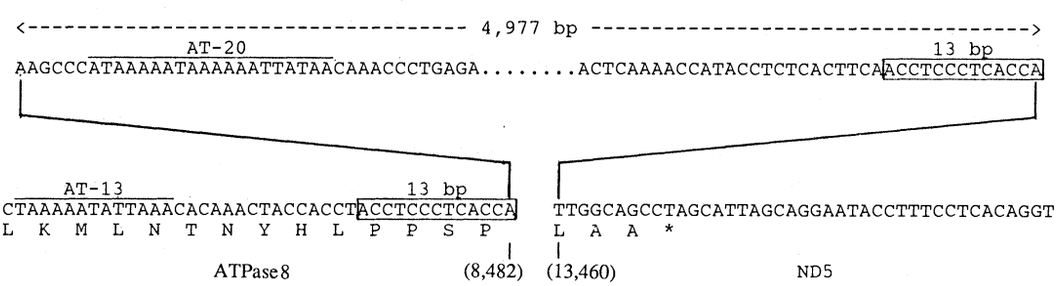

Fig. 2. DNA sequences (reading 5′ to 3′ on the L-strand) in the region of the common deletion. The DNA sequence found in the deleted genome is on the lower line, with the intervening deleted DNA shown on the upper line (bracket). The sequences flanking the two 13-bp repeats (boxed) are shown, including the AT-rich regions flanking the left-side repeat (overlined). The amino acid sequence (one-letter code) deduced from the ATPase8/ND5 fusion gene is shown below the DNA sequence. Abbreviations for the amino acid residues are as follows: A, Ala; H, His; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; S, Ser; T, Thr; and Y, Tyr.

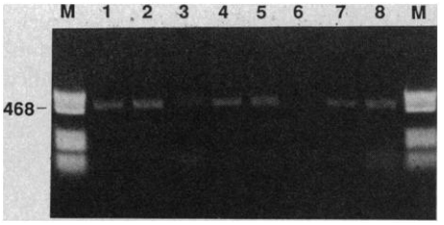

Fig. 3. Ethidium bromide staining of a 1% agarose gel containing PCR-amplified mtDNA from one patient with the common deletion analyzed by DNA sequence analysis as in Figs. 1 and 2 (lane 1), compared to that for seven other patients whose amplified DNA was not sequenced (lanes 2 to 8). About 10% of the total PCR reaction was loaded in each lane. The size of the amplified fragment, in bp, predicted from the sequence analysis, is at the left. M, markers of pBR322 digested with Hae III.

contains only structural genes and no origins of replication, it almost certainly was lost soon after the initial rearrangement occurred. An intragenomic event deleting this region via nonhomologous recombination would also produce nonviable subgenomic fragments. That we found no remnants (that is, a 5-kb circular or linear fragment) of such a putative initial recombination event in the adult skeletal muscle mtDNA of our patients is consistent with this interpretation.

Intragenomic rearrangement via slipped mispairing (*22*) was suggested as a likely mechanism for the generation of the small deletions in the D-loop and CO II 3′ untranslated regions (*13, 14*). Since slipped mispairing requires regions of single-stranded DNA on both the donor and target strands, it is a plausible model for removing a tandem repeat or for eliminating a few adjacent repeated bases. In our situation, however, slipped mispairing would have to operate over a span of 5 kb. Since there are two displaced origins of replication in mammalian mtDNA, daughter-strand synthesis is continuous on both strands (*7*); thus, neither repeat on the displaced (single-stranded) parental H-strand originating from the origin of replication on the H-strand has a partner on the complementary parental L-strand with which to hybridize, because daughter H-strand DNA has already been laid down on the parental L-strand. On the other hand, even the generation of a small region of single-stranded DNA adjacent to one of the repeats in the replicated duplex might be sufficient to allow for slipped mispairing to occur. It is significant that the human mitochondrial genome contains long regions of homopurine/homopyrimidine stretches that may exhibit an altered DNA conformation (*23*) and which may be susceptible to DNA bending that allows for just such a "bubble" to open (*24*). DNA sequences in the polypyrimidine block in the D-loop–conserved sequence region that display length heterogeneity (5′-CCCCCCCTCCCCCGCTTCT-3′ on the L-strand), the deleted 9-bp repeat in the CO II gene (5′-CCCCCTCTA-3′), and the core of the KSS/PEO 13-bp repeat itself (5′-CCTCCCTC-3′) all have the potential to form bent DNA. Furthermore, the left side 13-bp repeat at position 8470 to 8482 in the ATPase8 gene is flanked by two AT-rich regions, 13 and 20 nucleotides long (Fig. 2), which also have the potential to form bent DNA (*25*). Such altered DNA structures may not be confined to the polypurine/polypyrimidine tract, but may extend on the 5′ side of the polypurine-containing strand for at least 200 bp (*25*). Since putative bent-DNA regions may form a triple helix with a displaced single-stranded loop, called H-DNA (*26*), homologous recombination in the region of the 13-bp repeat, perhaps via slipped mispairing, now becomes a distinct possibility. Thus, both the repeat sequence itself and the sequence context in which it lies may render this region particularly susceptible to the formation of single-stranded DNA on supercoiling, either by bending at the polypyrimidine stretch or by bending or melting out at the flanking AT-rich regions. Since supercoils are introduced into mammalian mtDNA only after replication has ended (*7*), any putative recombination event that occurs via a mechanism invoking the extrusion of single-stranded DNA under torsion might occur in two phases: slipped mispairing before replication, and resolution of mispaired intermediates during replication.

A detailed analysis of the other deletions in KSS and PEO may enable us to clarify the exact mechanism of deletion of large regions of human mitochondrial DNA.

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10. A segment of the patient mtDNA encompassing the deletion was amplified by the PCR (*9*), with a pair of synthesized single-stranded oligonucleotide primers (Genetic Designs) corresponding to mtDNA sequences located upstream and downstream of the breakpoint. The upstream oligonucleotide, called XBA-8289F, had the sequence 5′-CCCTCTACC-CCCTCTAGAGCCCACTGTAAAGC-3′ (corresponding to nucleotides 8274 to 8305) and the downstream oligonucleotide, called STU-13705B, had the sequence 5′-GGCTTCCGGCTGCCA-GGCCTTTAATGGGG-3′ (corresponding to nucleotides 13692 to 13720). Twenty-five cycles of amplification were completed with 1 μg of patient DNA, 100 pmol of each primer, 20 nmol of each deoxynucleoside triphosphate (dNTP), and 2.5 units of Taq Polymerase (Perkin-Elmer Cetus) in 50 m*M* KCl, 10 m*M* tris, *p*H 8.3, 1.5 m*M* $MgCl_2$, and 0.01% gelatin in a total volume of 100 μl; annealing was at 55°C for 2.5 min, extension was at 72°C for 4 min, and denaturation was at 94°C for 1.5 min. In the last cycle, the extension proceeded for 10 min without any denaturation step. Because the distance between the two primers is less than 500 bp in the deleted genomes, but nearly 5 kb in the normal mtDNA, only the deleted genomes were amplified. The amplified mtDNA of the patient shown in lane 1 of Fig. 3 was digested with Xba I and Stu I, which cleave in the XBA-8289F and STU-13705B primer regions, respectively. After electrophoresis through 1% low melting temperature agarose, the two orientations of the cleaved DNA were subcloned into M13mp18 and M13mp19 digested with Xba I + Hinc II (designated clones mKSS3a and mKKS3b, respectively); the single-stranded M13 templates were sequenced with the Sequenase kit (USB) and separated by electrophoresis through thin 5% denaturing polyacrylamide gels, as described (*11*). To highlight the region flanking the deletion in this patient (Fig. 1), the PCR-amplified DNA fragment was digested with Hae III, subcloned into M13mp19 digested with Hinc II, and the L-strand was sequenced from the Hae III site at position 8391, with Taq polymerase (Perkin-Elmer Cetus) according to the manufacturer's directions. For the remaining four samples, the amplified DNA fragment was separated by electrophoresis through 1% low melting point agarose, extracted, and sequenced directly. About 100 ng of amplified DNA was used for each sequencing reaction, as follows: after 10 min boiling in 1× Sequenase (USB) buffer (40 m*M* tris, *p*H 7.5, 20 m*M* $MgCl_2$, and 50 m*M* NaCl) in the presence of 5 pmol of one of the oligomer primers, annealing was allowed to proceed by slow cooling of the template-primer mixture to room temperature over 30 to 40 min. Sequencing was as above (*11*).
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Two Molecular Transitions Influence Cardiac Sodium Channel Gating

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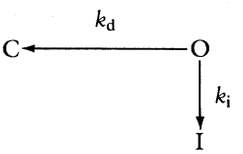
Sodium channels from diverse excitable membranes are very similar in their structure, yet surprisingly heterogeneous in their behavior. The processes that govern the opening and closing of sodium channels have appeared difficult to describe in terms of a single, unifying molecular scheme. Now cardiac sodium channels have been analyzed by high-resolution single-channel recordings over a broad range of potentials. Channels exhibited both complex and simple gating patterns at different voltages. Such behavioral diversity can be explained by the balance between two molecular transitions whereby channels can exit the open state.

VOLTAGE-DRIVEN CONFORMATIONAL changes that control the opening and closing of Na^+ channels form the molecular basis for membrane excitability. Despite the remarkable structural similarity of Na^+ channels from diverse sources (*1*), their functional gating properties have proven quite heterogeneous. Although Na^+ channels in some neuronal cells open briefly and only once with depolarization (*2*), the gating behavior in other neural preparations (*3, 4*) and heart (*5–8*) and skeletal muscle (*9*) is considerably more complex: channels open, close, and reopen many times before finally entering a long-lived inactivated state. We now report that single Na^+ channels from heart cells can exhibit either simple or complicated patterns of gating, both of which can be explained by a single gating paradigm.

To overcome the difficulties presented by inevitable recording system noise and diminishing open channel flux at voltages positive to −20 mV, we increased the Na^+ concentration in our pipettes ($[Na^+]_o$) from the usual 140 to 425 m*M* and thereby raised open channel conductance from ~10 to 25 pS. This enabled the resolution of brief unitary currents at voltages up to +20 mV (*10, 11*). We increased the osmolarity of the bath solution 1.5-fold to prevent patch rupture. Na^+ channel gating was not appreciably changed by the increase in permeant ion concentration, as judged by comparison of individual sweeps, ensemble current averages, and open time histograms. We used cell-attached patches to avoid modifications of gating behavior known to develop with patch excision (*12*).

The improvement in resolution reveals two prominently distinct patterns of gating at different membrane potentials. During depolarizing pulses to −50 mV (Fig. 1A), representative sweeps demonstrate the complicated pattern of multiple reopenings described previously in heart cells (*5–8*). The time to first channel opening is rather dispersed, and the lifetime of single openings does not parallel the decay of ensemble average current (Fig. 1A, bottom row). In contrast, voltage steps to −20 mV, or greater, elicit simple gating behavior (Fig. 1, B and C). Channels appear to open almost immediately on depolarization and once per depolarizing pulse. Consequently, the duration of single openings generally tracks the time course of declining ensemble current.

Does such dichotomous gating behavior arise from two conformationally different gating modes (*7, 9, 12, 13*) favored at different voltages? The analysis below argues against this notion by demonstrating that the two gating patterns are explained when the predictions of the standard gating model shown below (*2, 3, 14*) are considered in very different voltage ranges.



(1)

In this scheme there is a single open state (O), as required (*15*) by the predominantly single-exponential nature of open time histograms shown here and elsewhere (*2, 3, 5–9, 12*). In keeping with arguments presented elsewhere (*2, 3, 6, 16*), two principal pathways of exit from the open state are considered: one leading to a group of closed but available states (C) with rate constant k_d (deactivation) and the other to a group of absorbing, inactivated states (I) with rate constant k_i (inactivation).

Eyring rate theory enables us to predict the interaction of voltage with this model. This theory provides a simple link between the rate constants in Eq. 1 and the chemical and electrical components of the energy barriers encountered by channels leaving the open state (*17*). Thus,

$$k_d = k_d(0) \exp(+(Q_d V)/(RT)) \quad (2a)$$

$$k_i = k_i(0) \exp(+(Q_i V)/(RT)) \quad (2b)$$

where V is the transpatch voltage; $k_d(0)$ and $k_i(0)$ are the values of the respective rate constants at $V = 0$; Q_d and Q_i are the equivalent charge movements across the membrane (inside → outside) that occur as channels shift conformation from the open state to the transition state corresponding to deactivation or inactivation, respectively. Then, from Eq. 2 and elementary Markov theory (*15*), we can predict that the time constant of open time histograms (T_o) should be related to a biexponential function of V (*14*):

$$1/T_o = k_d(0) \exp(+(Q_d V)/(RT)) + k_i(0) \exp(+(Q_i V)/(RT)) \quad (3)$$

This first prediction of the gating scheme describes well the experimentally observed

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