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Intragenic Inversion of mtDNA: A New Type of Pathogenic Mutation in a Patient with Mitochondrial Myopathy

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We report an unusual molecular defect in the mitochondrially encoded ND1 subunit of NADH ubiquinone oxidoreductase (complex I) in a patient with mitochondrial myopathy and isolated complex I deficiency. The mutation is an inversion of seven nucleotides within the ND1 gene, which maintains the reading frame. The inversion, which alters three highly conserved amino acids in the polypeptide, was heteroplasmic in the patient's muscle but was not detectable in blood. This is the first report of a pathogenic inversion mutation in human mtDNA.

Introduction

Molecular characterization of mitochondrial encephalomyopathies has progressed rapidly, and many pathogenic mutations have been described (Schon et al. 1997). Maternal inheritance and multisystemic involvement are characteristic of mitochondrial disorders associated with point mutations in mtDNA (DiMauro and Bonilla 1997). Recently, however, we described a sporadic patient with isolated myopathy and complex I deficiency due to a nonsense mutation in the ND4 gene of mtDNA (Andreu et al. 1999*c*). This finding prompted us to revisit a patient, reported elsewhere (Bet et al. 1990), with the same clinical and biochemical phenotype. In this patient, we found an unusual molecular defect—an inversion of seven nucleotides within the ND1 gene.

Patient and Methods

Clinical History

This 43-year-old man, originally reported in 1990 (Bet et al.), had complained, since childhood, of severe exercise intolerance and myalgia. Morphological and biochemical studies of muscle showed 40% ragged-red fibers and a marked reduction of complex I activity (~40% of normal). At age 43 years, he still complains of exercise intolerance, and a neurological examination showed mild proximal limb weakness but was otherwise normal. His family history is noncontributory: his mother is alive and has always been a very active woman, and neither of his two siblings complains of exercise intolerance.

Molecular Genetic Analysis

Muscle DNA was extracted by standard methods. To sequence the mtDNA genes encoding the seven subunits of NADH oxidoreductase (ND), we amplified 11 overlapping mtDNA fragments spanning all six mtDNA-encoded ND genes, using a set of primers reported elsewhere (Rieder et al. 1998). Direct sequencing of PCR products was performed with an automatic sequencer (ABI Prism 310, PE Biosystems), by means of the manufacturer's dye terminator cycle sequencing kit. To determine the proportion of mutant and wild-type mtDNA, we performed RFLP analysis on mtDNA amplified by PCR, using a mismatched forward primer extending from nucleotide (nt) 3874 to 3901 (Anderson et al. 1981), but with the T at nt 3899 being replaced by a G, and a reverse primer extending from nt 4203 to 4180. The mismatch primer creates a restriction site for the enzyme *Fnu4HI* (recognition sequence 5′-GC↓NGC-3′) at the 5′ boundary of the inversion; this site is not present in the wild-type mtDNA. The PCR fragment was labeled with [a-32P]dATP (3,000 Ci/mmol, Amersham) in the last PCR cycle (Moraes et al. 1992). The products of the digestion were separated by electrophoresis through a 12% nondenaturing polyacrylamide gel, and the intensities of the labeled fragments were quantitated with a Phosphor-Imager (BioRad) and analyzed with appropriate software.

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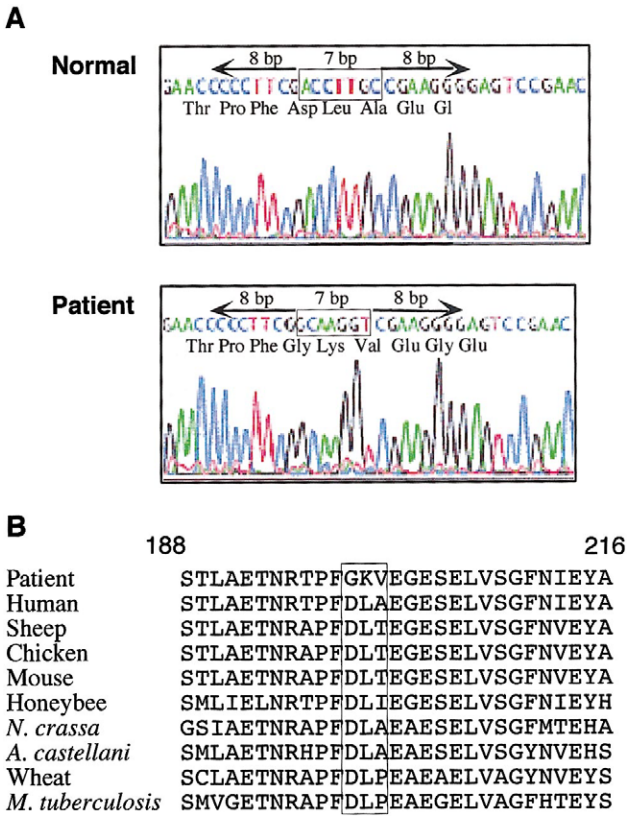

Figure 1 Detection of the inversion in human mtDNA. *A*, Electropherogram of the ND1 gene showing the DNA and deduced amino acid sequences from a normal subject and from the patient. The 7-bp complementary inversion (boxed), flanked by the 8-bp inverted repeats (arrows), are shown. *B*, Position of the three amino acid changes conceptually encoded by the inversion, and interspecies homology in this region of the ND1 polypeptide (boxed).

Results

We sequenced the PCR-amplified region of mtDNA from the patient's muscle containing all seven ND genes (i.e., ND1, ND2, ND3, ND4, ND4L, ND5, and ND6). We found mutations that differed from the "Cambridge" sequence (Anderson et al. 1981) at four positions: T3338C, G3423T, T11335C, and A11467G (not shown). All four were deemed to be neutral polymorphisms, because all four were found by others in normal individuals and were homoplasmic in the patient.

Sequencing of the ND1 region, however, revealed an inversion of seven nucleotides, located between positions 3902 and 3908 (fig. 1*A*). These seven nucleotides are flanked by two 8-bp inverted repeats. Thus, the sequence in this region is 5′-AACCCCCTTCG**ACC TTGC**CGAAGGGGAGT-3′ (inverted target in bold, flanking inverted repeats underlined) in normal mtDNA, whereas it is 5′-AACCCCCTTCG**GCAAGGT** CGAAGGGGAGT-3′ in the mutated mtDNA. The inversion causes an in-frame substitution of three amino acids (D199G, L200K, and A201V) in the ND1 protein (fig. 1*B*). RFLP analysis revealed that the mutation was heteroplasmic (80%) in muscle, but was not detectable in blood (fig. 2).

Discussion

We report the first example of an inversion of human mtDNA, in a patient with isolated mitochondrial myopathy and complex I deficiency (Bet et al. 1990). The mutation is 7 bp in length and is flanked by a pair of 8-bp inverted repeats located exactly at the boundaries of the inversion (see fig. 1*A*).

We believe, for a number of reasons, that the inversion is pathogenic. First, the mutation is heteroplasmic, a common feature of pathogenic mtDNA mutations. Second, the mutation is consistent with the biochemical

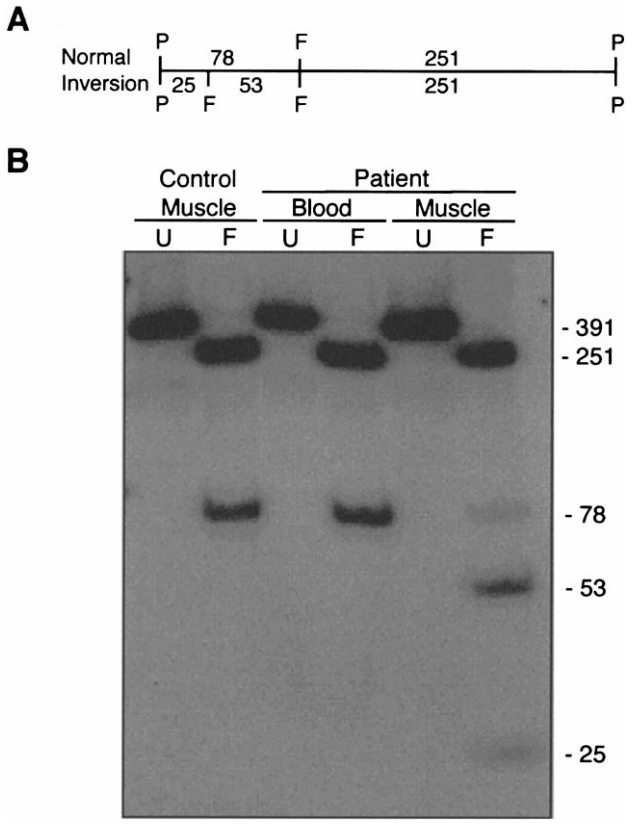

Figure 2 RFLP analysis. *A*, Map of the relevant region in the ND1 gene. *Fnu4HI* cuts the normal PCR-amplified fragment (329 bp, amplified with primers P) into two fragments of 251 and 78 bp. In the mutant, a mismatched forward primer (P′) creates a new restriction site for *Fnu4HI* that cuts the 78-bp fragment into two fragments of 53 and 25 bp. *B*, DNA fragments produced by the RFLP analysis, visualized in a nondenaturing polyacrylamide gel. U, uncut amplified fragment; F, *Fnu4HI* digestion. Fragment sizes, in base pairs, are at right.

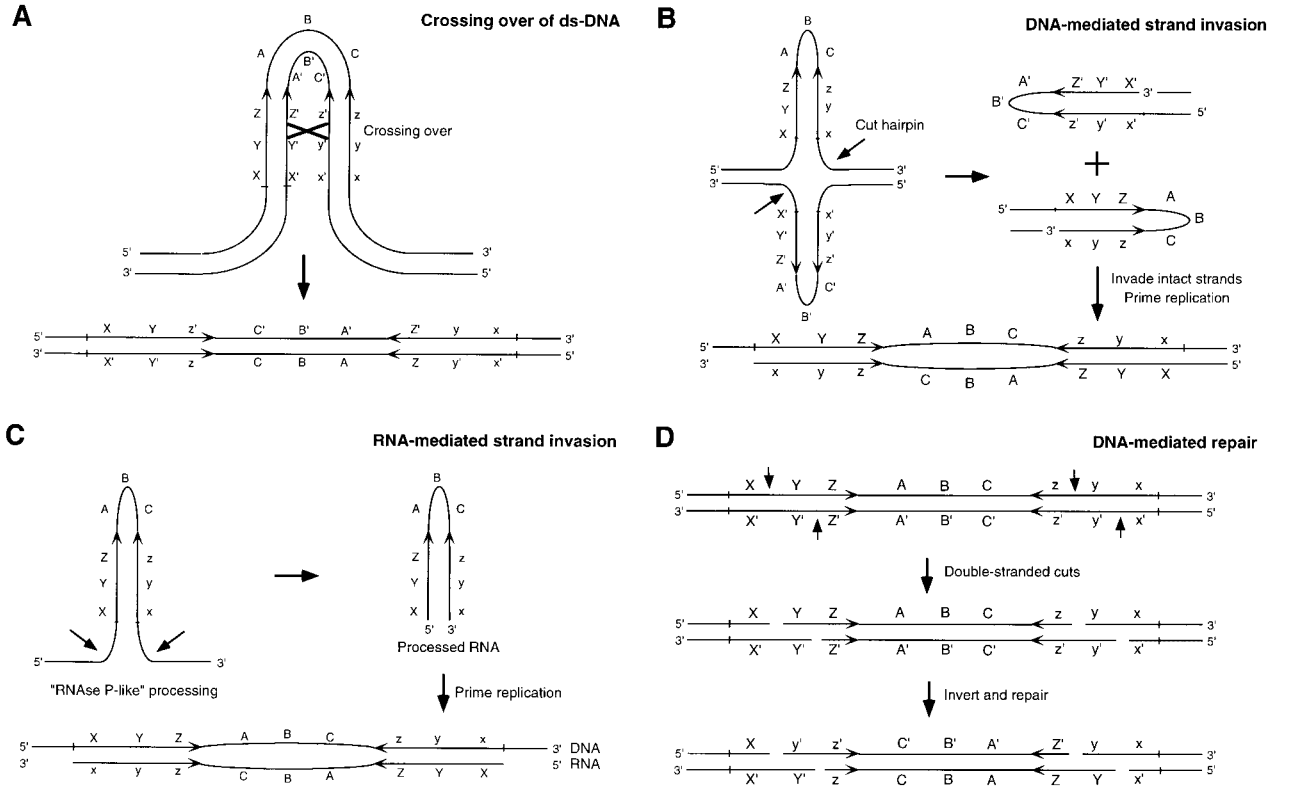

Figure 3 Scenarios of some possible mechanisms by which the inversion in mtDNA could occur. *A,* Crossing over of double-stranded DNA; *B,* DNA-mediated strand invasion; *C,* RNA-mediated strand invasion; *D,* DNA-mediated repair. See text for details.

defect in muscle (i.e., isolated complex I deficiency). Third, the mutation, which converts three consecutive amino acids from Asp-Leu-Ala at amino acid positions 199-201 to Gly-Lys-Val, involves a highly conserved region of the ND1 gene (fig. 1*B*), which has been postulated to play a role in ubiquinone binding (Zickermann et al. 1998). The replacement of the negatively charged Asp-199 with Gly (a neutral residue, and a helix breaker as well), and of the hydrophobic Leu-200 with a basic Lys residue, would seem particularly deleterious.

Pathogenic mutations in mtDNA restricted to skeletal muscle are being described with increasing frequency. They can affect tRNA genes or, more commonly, protein-coding genes, including genes encoding subunits of complexes I, III, and IV (Andreu et al. 1998*a,* 1998*b,* 1999*a,* 1999*b,* 1999*c*). Patients with mutations in protein-coding mtDNA genes are often sporadic and almost always have isolated myopathy, with the mtDNA mutation present only in skeletal muscle. This segregation pattern of mutant mtDNA could result from the gradual accumulation of the mutation in postmitotic muscle fibers and from the loss of mutant mtDNA in mitotic cell populations, such as leukocytes (Bouzidi et al. 1998). However, studies of muscle cultures from patients harboring such mutations failed to detect mutant mtDNA in myoblasts derived from satellite cells, even at early passages, which suggests that the mutations may have arisen de novo in a subgroup of myoblasts during embryogenesis.

Interestingly, the muscle biopsy from the patient reported in this study showed ragged-red fibers that stained intensely for cytochrome *c* oxidase activity (COX-positive RRF), in contrast with the COX-negative RRF seen in most patients with mutations in tRNA genes (DiMauro and Bonilla 1997). The only major exception to this rule is the MELAS syndrome, in which RRF are usually COX positive. We have also seen COX-positive RRF in one patient with exercise intolerance and a nonsense mutation in the ND4 gene and in patients harboring pathogenic mutations in the cytochrome b gene (Andreu et al. 1998*a,* 1998*b,* 1999*a,* 1999*b,* 1999*c*).

The generation of an inversion at a site flanked by perfect inverted repeats suggests that it arose via a homologous recombination event mediated by those repeats. The mechanism by which the inversion occurred is unknown, but a number of scenarios are conceivable (fig. 3). One possibility is simple crossing over between the inverted repeats. For example, if the inverted repeats formed a stem-loop structure (fig. 3*A*), crossing over

would invert the sequence in the hairpin. However, this mechanism is unlikely, owing to the intrinsic stiffness of double-stranded DNA: the repeats are so closely spaced that the DNA could not bend sufficiently to form the hairpin (Shore et al. 1981). Moreover, this mechanism does not appear to operate in *E. coli,* even with much longer inverted repeats (Lyu et al. 1999).

A second possibility is extrusion of a cruciform at the inverted repeat region. This creates, for example, a potential substrate that looks like a Holliday junction recombination intermediate. Cleavage of the intermediate would result in two hairpin molecules that could be processed further to the inverted structure shown in fig. 3*B*. Nicking of the single-stranded open area on opposite strands, invasion of the free end into the corresponding region on another mtDNA, and, finally, priming of replication or replicative repair by means of the homology will result in an inversion. In this scenario, there is a perfect match between the now-inverted single strands in the repeat region, but the intervening DNA (the seven-nucleotide region in our case) is not complementary to itself and exists as a "bubble." After one round of replication, however, one of the two daughter strands will fix the inversion. The DNA-mediated strand invasion event could arise from either a double-stranded (i.e., cruciform) or a single-stranded (i.e., hairpin) source. Since the ND1 gene, however, is located in the "minor arc" of the genome, which does not exist as single-stranded DNA during replication (Clayton 1982), a mechanism based on extrusion of a cruciform from double-stranded DNA is more likely.

Strand invasion can also be mediated by RNA. An RNA-based mechanism is particularly attractive for the inversion of mtDNA because precursor transcripts of human mtDNA are processed to release the mature transcripts (rRNAs, tRNAs, and mRNAs) by endogenous nucleases (Clayton 1982; Attardi and Montoya 1983). In particular, tRNA genes are located between most of the polypeptide-coding genes, and it is thought that the mature mRNAs are released from the precursor by cleavage of the single-stranded precursor transcript at the tRNA/mRNA junctions, by RNAse P and enzymes similar to it. The "8-7-8" motif of the inverted region is highly reminiscent of the inverted symmetry in the stem-loop regions of tRNA genes. Thus, it may be that, in rare cases, the inversion extrudes a hairpin on the precursor transcript that is recognized by the processing machinery and is cleaved (fig. 3*C*). Strand invasion and repair during replication would be as above.

Yet another possibility is repair after a double-stranded break in DNA. For example, if double-stranded, staggered cuts occurred at identical positions within each repeat, the intervening DNA could be inverted at the complementary overhangs; ligation at the nicks would then repair the breaks (fig. 3*D*). This type of mechanism implies that the inverted repeats are nicked in a sequence-specific manner, presumably by an endonuclease with sequence specificity. In fact, the sequence of the inverted repeat (5-CCCCTTCG-3′) points toward such a candidate nuclease—endonuclease G. Endo G is a 29-kDa endonuclease that is targeted to both the nucleus and to mitochondria (Cote and Ruiz-Carrillo 1993; Gerschenson et al. 1995). This enzyme, which has a nicking activity and which can cleave both single- and double-stranded DNA, has a strong preference for the sequence (dC)n.(dG)n. In addition, a second Endo G-like protein, 55 kDa in size, is also present in mitochondria and has a preference for (dC)n.(dG)n (Ikeda et al. 1996). Thus, the fact that there are four consecutive Cs in the 8-bp inverted repeat (and a fifth C immediately 5′ to these four) could render this sequence susceptible to staggered nicking. Furthermore, since the repeats contain homopurine-homopyrimidine tracts (8 bp at the 5′ repeat and 9 bp at the 3′ repeat) they also have the potential to form bent DNA and to extrude a single-stranded bubble in a triple-helical DNA (Lyamichev et al. 1986). Since Endo G has a preference for (dC)n over (dG)n in single-stranded DNA (Cote and Ruiz-Carrillo 1993), Endo G cleavage at these repeats would cut in the predicted staggered fashion.

Irrespective of the precise molecular etiology, intragenic inversions in mtDNA should now be added to the already rich repertoire of pathogenic mutations in mtDNA.

Acknowledgments

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References

Anderson S, Bankier AT, Barrel BG, DeBruijin M, Coulson AR, Drouin J, Eperon IC, et al (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457–465

Andreu AL, Bruno C, Dunne TC, Tanji K, Shanske S, Sue CM, Krishna S, et al (1999*a*) A nonsense mutation (G15059A) in the cytochrome b gene in a patient with exercise intolerance and myoglobinuria. Ann Neurol 45:127–130

Andreu AL, Bruno C, Shanske S, Shtilbans A, Hirano M, Krishna S, Hayward L, et al (1998*a*) Missense mutation in the mtDNA cytochrome b gene in a patient with myopathy. Neurology 51:1444–1447

Andreu AL, Hanna MG, Reichmann H, Bruno C, Penn AS, Tanji K, Pallotti F, et al (1999*b*) Exercise intolerance due to

mutations in the cytochrome b gene of mitochondrial DNA. N Engl J Med 341:1037–1044

Andreu AL, Krishna S, Bruno C, Shtilbans A, Shanske S, Brown RH, DiMauro S (1998*b*) A novel G15762A mutation in the mitochondrial cytochrome b gene. Neurology 50:A24

Andreu AL, Tanji K, Bruno C, Hadjigeorgiou GM, Sue CM, Jay C, Ohnishi T, et al (1999*c*) Exercise intolerance due to a nonsense mutation in the mtDNA ND4 gene. Ann Neurol 45:820–823

Attardi G, Montoya J (1983) Analysis of human mitochondrial RNA. Methods Enzymol 97:435–469

Bet L, Bresolin N, Moggio M, Meola G, Prelle A, Schapira AH, Binzoni T, et al (1990) A case of mitochondrial myopathy, lactic acidosis and complex I deficiency. J Neurol 237:399–404

Bouzidi MF, Poyau A, Godinot C (1998) Co-existence of high levels of a cytochrome b mutation and of a tandem 200 bp duplication in the D-loop of muscle human mitochondrial DNA. Hum Mol Genet 7:385–391

Clayton DA (1982) Replication of animal mitochondrial DNA. Cell 28:693–705

Cote J, Ruiz-Carrillo A (1993) Primers for mitochondrial DNA replication generated by endonuclease G. Science 261: 765–769

DiMauro S, Bonilla E (1997) Mitochondrial encephalomyopathies. In: Rosenberg RN, Prusiner SB, DiMauro S, Barchi RL (eds) The molecular and genetic basis of neurological disease. Butterworth-Heinemann, Boston, pp 201–235

Gerschenson M, Houmiel KL, Low RL (1995) Endonuclease G from mammalian nuclei is identical to the major endonuclease of mitochondria. Nucleic Acids Res 23:88–97

Ikeda S, Tanaka T, Hasegawa H, Ozaki K (1996) Identification of a 55-KDA endonuclease in rat liver mitochondria with nucleolytic properties similar to endonuclease G. Biochem Mol Biol Int 38:1049–1057

Lyamichev VI, Mirkin SM, Frank-Kamenetskii MD (1986) Structures of homopurine-homopyrimidine tract in superhelical DNA. J Biomol Struct Dyn 3:667–669

Lyu YL, Lin CT, Liu LF (1999) Inversion/dimerization of plasmids mediated by inverted repeats. J Mol Biol 285: 1485–1501

Moraes CT, Ricci E, Bonilla E, DiMauro S, Schon EA (1992) The mitochondrial $tRNA^{Leu(UUR)}$ mutation in mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS): genetic, biochemical, and morphological correlations in skeletal muscle. Am J Hum Genet 50:934–949

Rieder MJ, Taylor SL, Tobe VO, Nickerson DA (1998) Automatic identification of DNA variations using quality-based fluorescence re-sequencing: analysis of the human mitochondrial genome. Nucleic Acid Res 26:967–973

Schon EA, Bonilla E, DiMauro S (1997) Mitochondrial DNA mutations and pathogenesis. J Bioenerg Biomembr 29:131–149

Shore D, Langowski J, Baldwin RL (1981) DNA flexibility studied by covalent closure of short fragments into circles. Proc Natl Acad Sci USA 78:4833–4837

Zickermann V, Barquera B, Wikström M, Finel M (1998) Analysis of the pathogenic human mitochondrial mutation ND1/3460, and mutations of strictly conserved residues in its vicinity, using the bacterium *Paracoccus denitrificans*. Biochemistry 37:11792–11796