ND5 is a hot-spot for multiple atypical mitochondrial DNA deletions in mitochondrial neurogastrointestinal encephalomyopathy

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive multisystem disorder associated with depletion, multiple deletions and site-specific point mutations of mitochondrial DNA (mtDNA). MNGIE is caused by loss-of-function mutations in the gene encoding thymidine phosphorylase (TP; endothelial cell growth factor 1). Deficiency of TP leads to dramatically elevated levels of circulating thymidine and deoxyuridine. The alterations of pyrimidine nucleoside metabolism are hypothesized to cause imbalances of mitochondrial nucleotide pools that, in turn, may cause somatic alterations of mtDNA. We have now identified five major forms of mtDNA deletions in the skeletal muscle of MNGIE patients. While direct repeats and imperfectly homologous sequences appear to mediate the formation of mtDNA deletions, the nicotinamide adenine dinucleotide dehydrogenase 5 gene is a hot-spot for these rearrangements. A novel aspect of the mtDNA deletions in MNGIE is the presence of microdeletions at the imperfectly homologous breakpoints.

INTRODUCTION

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder due to loss-of-function mutations in the gene encoding thymidine phosphorylase (TP; endothelial cell growth factor 1) (1–3). TP activity in buffy coats of MNGIE patients is <10% that of controls (3,4). The disease is characterized clinically by onset between the first to fifth decades, ptosis, progressive external ophthalmoplegia, gastrointestinal dysmotility, cachexia, peripheral neuropathy, myopathy and leukencephalopathy (1,5). MNGIE is associated with multiple deletions, depletion and site-specific point mutations of mitochondrial DNA (mtDNA) (1,6,7). In MNGIE patients, TP deficiency causes dramatically increased plasma levels of both thymidine (deoxythymidine, dThd) (4) and deoxyuridine (dUrd) (8). We have hypothesized that, in MNGIE, increased intracellular dThd and dUrd cause imbalances of mitochondrial nucleotide pools that, in turn, lead to the mtDNA abnormalities (7,9). In support of our hypothesis is the observation that mtDNA abnormalities are present in skeletal muscle (3). Normal muscle does not express TP, therefore loss of TP activity cannot account for the mitochondrial alterations in muscle of MNGIE patients. This ‘muscle paradox’ indicates that TP-deficiency causes mtDNA alterations indirectly through abnormal nucleoside metabolism. To characterize further the pathogenesis of MNGIE, we have analyzed the multiple deletions and depletion of mtDNA in MNGIE tissues.

RESULTS

MtDNA quantitation by Southern blot analysis

Total DNA was extracted from post-mortem brain cortex and white matter, liver, kidney and small intestine, and either autopsy or biopsy skeletal muscle from six MNGIE patients (MN1-1, MN2-1, MN2-2, MN3-1, MN4-2 and MN7-2) and was screened by Southern blot analyses (10). In addition to Southern blot signals from normal 16.6 kb mtDNA molecules, four bands corresponding to deleted mtDNA were visible in skeletal muscle DNA samples from five of the six MNGIE patients. While direct repeats and imperfectly homologous sequences appear to mediate the formation of mtDNA deletions, the nicotinamide adenine dinucleotide dehydrogenase 5 gene is a hot-spot for these rearrangements. A novel aspect of the mtDNA deletions in MNGIE is the presence of microdeletions at the imperfectly homologous breakpoints.

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mtDNA molecules were present at heteroplasmic levels ranging from <2% to 11% in five muscle DNA samples (MN1-1, MN2-1, MN3-1, MN4-2 and MN7-2). The Δ10.3 kb mtDNA molecule was identified at ≤2% heteroplasmy in four muscle DNA samples (MN2-1, MN3-1, MN4-2 and MN7-2) (Fig. 1A). Digestion of mtDNA with SnaBI and PvuII restriction enzymes did not reveal abnormal bands migrating above the 16.6 kb full-length mitochondrial genome indicating that mtDNA duplications were not present. In contrast to muscle, other tissues did not have abnormal mtDNA bands by Southern blot analyses, but had detectable deletions of mtDNA by polymerase chain reaction (PCR).

Real-time quantitative polymerase chain reaction

To confirm mtDNA depletion in samples of brain cortex and white matter, liver, kidney, small intestine and skeletal muscle from MNGIE patients, TaqMan® real-time quantitative PCR was performed with the ABI Prism 7000 Sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). In each sample, relative amounts of 12S ribosomal mtDNA and RNase P nuclear DNA (nDNA) were measured. As indicated in Figure 1B, the average of the mtDNA/nDNA ratios in the livers of MNGIE patients was significantly lower than in controls (decreased by 54%, \( P < 0.05 \)). In contrast, levels of mtDNA relative to nDNA were not significantly decreased in all of the other tissues from MNGIE patients.

Mapping of deletions in skeletal muscle DNA of MNGIE patients

To confirm that Southern blot bands in skeletal muscle DNA samples from MNGIE patients were multiple mtDNA deletions, we initially amplified nearly full-length mtDNA using long-PCR. In addition to the 16.3 kb wild-type fragment, numerous shorter mtDNA fragments were amplified (Fig. 2A). These PCR results were consistent with the results of the Southern blot hybridization of MNGIE skeletal muscle DNA. By long-PCR, we identified amplification products corresponding to the Δ5 kb, Δ7.7 kb, Δ8.1 kb and Δ10.3 kb species of mtDNA that we detected by Southern blot. We also identified a 9.5 kb deletion (Δ9.5 kb) band that was not visible in the Southern blot. We confirmed the identities of the deleted mtDNAs using a series of PCR and long-PCR reactions with...
shifted primers (Fig. 2B–E), and by sequencing of the PCR products.

Direct repeats at deletion junctions

PCR-amplified fragments corresponding to the Δ5 kb and the Δ7.7 kb were detected in all six skeletal muscle DNA samples, and after isolation from agarose gels, were sequenced directly (Fig. 2D and E). The upstream breakpoints of the Δ5 kb and Δ7.7 kb mtDNA fragments were in the nicotinamide adenine dinucleotide dehydrogenase (ND) 5 gene while the downstream breakpoints were in the genes encoding ATPase 8 (ATP 8) for the Δ5 kb and cytochrome c oxidase (COX) I for the Δ7.7 kb (Fig. 3). Both deletion junctions contained direct repeats (DRs): a 13 bp DR for Δ5 kb and 11 bp DR for Δ7.7 kb. In addition, immediately adjacent to the 11 bp DR was an imperfectly homologous sequence (9/14 bp identity between COX I and ND5) (Fig. 3). The Δ5 kb has been identified in numerous patients with single and multiple deletions of mtDNA and is known as the ‘common deletion’ (11). The Δ7.7 kb (12) had been identified previously in skeletal muscle of MNGIE patients.

Imperfectly homologous sequences at deletion junctions

By PCR, the Δ8.1 kb (Fig. 2A–C and E) was identified in skeletal muscle of all six MNGIE patients, the Δ10.3 kb was identified in four, the Δ9.5 kb was present in three and the Δ11.2 kb was identified in only one (Fig. 2A–C). By direct DNA sequencing of the Δ8.1 kb, Δ9.5 kb, Δ10.3 kb and Δ11.2 kb DNA fragments (Fig. 2B, C and E), it was not possible to define the junction of the deletions precisely because the PCR-amplified fragments contained mixed populations of DNA (data not shown). Therefore, we subcloned the PCR products spanning the deletions and sequenced ~10 cloned fragments of each amplified deletion junction.

The deletion junction of the Δ8.1 kb fragment contained an imperfectly homologous sequence (15/29 bp identity between tRNACys and ND5) (Fig. 3). Interestingly, the subcloned PCR product containing the Δ8.1 kb breakpoint revealed microdeletions of 2–24 bp within the homologous sequences (Fig. 3).

The breakpoints of the Δ9.5 kb mtDNA fragment were in tRNAMet and ND5, and were flanked by 31 bp imperfectly homologous sequences in the parental mtDNA sequences (13/31 bp identity) (Fig. 3). This deleted mtDNA fragment lacks the origin of mtDNA light (L)-strand replication. In addition, clones of these breakpoints showed microdeletions of 1–19 bp within the imperfectly homologous sequences.

The breakpoints of the Δ10.3 kb fragment were in the hypervariable segment 1 (HVS1) in the displacement loop (D-loop) of mtDNA and tRNA59r, and contained 40 bp imperfectly homologous sequences (18/40 bp identity) (Fig. 3). These junctions of this deletion were also heterogeneous and six of the seven sequenced molecules harbored microdeletions of 10–27 bp.

In contrast to the aforementioned mtDNA species containing a single deletion, the Δ11.2 kb DNA fragment harbored two deletions (Fig. 4). The first deletion was identical to deletion in the Δ10.3 kb fragment. The second deletion spanned Δ0.9 kb and contained a breakpoint with a 21 bp imperfectly homologous sequence (18/21 bp identity) with two variants (Fig. 3). This small deletion eliminated the origin of mtDNA heavy (H)-strand (OH) replication. Two breakpoint variants showed that the fourth base-pair of the homologous junction sequence (nt-16,263/nt-572) could be derived from either the upstream or downstream homologous sequence.

Curiously, ND5 of mtDNA is a hot-spot for deletion breakpoints (Δ5 kb, Δ7.7 kb, Δ8.1 kb and Δ9.5 kb species) in MNGIE skeletal muscle DNA (Fig. 5). The imperfectly homologous breakpoints revealed that, in the L-strand orientation, the 5‘-region of homologous sequences were derived from the upstream regions (blue in Fig. 3) while the 3‘-regions were derived from the downstream segments (red in Fig. 3).
We assessed the distribution of the major mtDNA deletions in post-mortem tissue samples (brain cortex and white matter, liver, kidney, small intestine and skeletal muscle) from four MNGIE patients by PCR. The Δ5 kb and Δ7.7 kb molecules, which contained breakpoint DR sequences, were identified in skeletal muscle, cerebral cortex and white matter, kidney and liver of the MNGIE patients (Fig. 6). In contrast, the Δ8.1 kb, Δ9.5 kb, Δ10.3 kb and Δ11.2 kb fragments, which harbor imperfectly homologous breakpoint sequences, were detectable in skeletal muscle, but not in other tissues. The Δ11.2 kb was presented in the skeletal muscle DNA sample from only one patient (MN7-2). In small intestine, no deletions of mtDNA were detected.

**Figure 3.** MtDNA deletion breakpoints in skeletal muscle from MNGIE patients. MtDNA L-strand sequences of the deletion junctions (a–g) and the regions flanking the upstream (A) and downstream (B) breakpoints in the parental molecules. Colored letters indicate homologous sequences in the parental mtDNA sequences upstream (blue), downstream (red), and perfect direct repeats (green). Gray letters indicate deleted sequences. Asterisks indicate alignments between the parental L-strand sequences. Underlined nucleotides indicate AT-rich, polypurine, and polypyrimidine regions that may form bent-DNA structures. A, missing nucleotide; -, nucleotide gap; ATP, ATPase; ND, nicotinamide adenine dinucleotide dehydrogenase; COX, cytochrome c oxidase; HVSI, hypervariable segment 1; and D-loop, displacement loop.

**Distribution of mtDNA multiple deletions in tissues from MNGIE patients**
DISCUSSION

In MNGIE, deficiency of TP alters dThd and dUrd metabolism (4,8). We have hypothesized that the increased circulating levels of dThd and dUrd cause imbalances of mitochondrial deoxynucleoside 5'-triphosphate (dNTP) pools (3,7). One of the factors contributing to the selective vulnerability of mitochondrial nucleotide pools is the separate and independently regulated dNTP pools within these organelles (13–15). Furthermore, because mitochondria are more dependent upon the dThd salvage pathway than nuclei, which rely upon the de novo dThd synthetic pathway, elevated levels of pyrimidine nucleosides are more likely to cause nucleotide pool imbalances in mitochondria rather than in nuclei (3,16). We have postulated that the imbalanced mitochondrial dNTP pools lead to impaired mtDNA maintenance and replication, and, in turn, generate somatic depletion, multiple deletions, and point mutations of mtDNA.

A detailed analysis of the mtDNA point mutations in MNGIE has generated hypothetical mechanisms that may account for high frequency of 5'-ATT to 5'-GTT somatic mutations (5'-AAT to 5'-AAC in the complementary strand) (7). The mutations may originate with mitochondrial DNA polymerase γ (Pol γ) misincorporation of a deoxyguanosine monophosphate opposite a template thymidine residue. Because deoxythymidine triphosphate and deoxyuridine triphosphate levels are increased, Pol γ may accelerate opposite the template 5'-AA sequence at the expense of exonuclease proofreading activity (‘next-nucleotide effect’) and the mutation becomes fixed (7,8). Although mitochondria have effective base-excision repair (BER) mechanisms for oxidatively damaged DNA, lack of BER mechanisms for mismatched nucleotides in mitochondria may also contribute to the accumulation of mtDNA point mutations (17). In addition, mitochondria do not have nucleotide excision repair mechanisms (17).

In contrast to the somatic point mutations of mtDNA in MNGIE, the deletions and depletion of mtDNA have not been characterized in detail. In this report, we have assessed tissues from MNGIE patients for quantitative defects and major rearrangements of mtDNA.

**MtDNA depletion in MNGIE**

Significant mtDNA depletion was detected in liver only, however, there were trends towards lower levels of mtDNA in all tissues tested except kidney. The lack of statistically significant depletion in these tissues may be due to the small number of available samples.

The mechanism responsible for the depletion of mtDNA in MNGIE is unknown but could be due to nucleotide pool imbalances similar to the hypothesized pathogenesis of mtDNA depletion syndromes (MDS) caused by mutations in the mitochondrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK) (18,19). In these forms of MDS, lack of the mitochondrial nucleoside kinases is thought to cause deficiency of
mitochondrial dNTPs, which impedes mtDNA replication. Alternatively, the depletion of mtDNA in MNGIE could be due to the accumulation of multiple point mutations in the D-loop (where mtDNA replication is thought to originate) (20,21). These mutations could impair mtDNA replication (7,22,23). A third possibility is that uracil DNA glycosylase initiated base excision of misincorporated deoxyuridine monophosphates in mtDNA could lead to degradation of mtDNA (8). Finally, the accumulation of deleted mtDNAs lacking origins of replication would prevent mtDNA from replicating and could cause mtDNA depletion. Thus, the depletion of mtDNA in MNGIE may be secondary to the mtDNA point mutations, multiple deletions, or both.

**Characteristics of the multiple mtDNA deletions in MNGIE**

In contrast to the depletion of mtDNA, which was significant in liver, multiple deletions were most prominent in skeletal muscle. Several features of the multiple deletions of mtDNA in MNGIE are noteworthy: (i) although most abundant in skeletal muscle, mtDNA multiple deletions were present at low levels (i.e. <2%) in kidney and brain; (ii) skeletal muscle harbored five major species of mtDNA deletions; (iii) ND5 was a hot-spot for mtDNA deletions; (iv) mtDNA molecules lacking O_l (Δ9.5 kb) and O_H (Δ11.2 kb) were identified; (v) the breakpoints of the deletions contained either perfect direct sequence repeats or imperfectly homologous sequences; (vi) microdeletions were identified at the breakpoints of the imperfectly homologous sequences; (vii) variations in the breakpoints of the imperfectly homologous sequences suggested that branch migration occurred; (viii) the junctions of the deletions did not show sequence specificity, however, the breakpoints of the imperfectly homologous sequences often included ends of stem structures in tRNA genes; and (ix) we did not observe any mtDNA duplications.

The multiple deletions of mtDNA were identified in skeletal muscle, brain and kidney, but not other clinically affected tissues such as small intestine. Presumably, post-mitotic cells accumulate mtDNA deletions while a selection bias eliminates replicating cells harboring mtDNA deletions as observed in patients with single mtDNA deletions (24). In contrast, in the small intestine which is severely affected in MNGIE patients, deletions and significant depletion of mtDNA were not detected, therefore, somatic point mutations of mtDNA may be responsible for the dysfunction of this organ (7). Alternatively, the abnormalities of mtDNA could accumulate predominantly in the autonomic nervous system of the intestines, thereby causing dysmotility without being detectable in total homogenized intestinal samples.

Although, we have not detected all of the deletions of mtDNA in skeletal muscle, we have identified five major species of mtDNA deletions: three (Δ5 kb, Δ7.7 kb and Δ8.1 kb) are present in all of the samples and two (Δ9.5 kb and Δ10.3 kb) in most. The fact that specific forms of deletions are reproducibly detectable in muscle samples from six MNGIE patients indicate that a specific mechanism causes specific large-scale rearrangements of mtDNA in this disease.

By PCR-mapping and DNA sequencing, we found that four of the five prominent mtDNA deletions contained a break-event in ND5 at nts-13,447, 13,994, 13,920, and 13,923 (Fig. 3); therefore, in MNGIE, ND5 appears to be a hot-spot for mtDNA deletions, including the common deletion. It is not clear why ND5 should be vulnerable to rearrangements of mtDNA because the gene is not involved in the initiation of replication or does not contain secondary structures like tRNA genes.

The existence of a Δ9.5 kb deletion lacking O_l is problematic because this molecule cannot replicate according to the strand-displacement model of mtDNA replication (20). Similar mtDNA molecules without O_l have been described in patients with inflammatory myopathies (25), normal individuals (26), patients with sporadic inclusion body myositis (27) and aged people (28). Even more surprisingly, the Δ11.2 kb mtDNA molecule, which contains two independent deletions, lacks O_H (Fig. 4); therefore, this molecule should not replicate according to both the strand-displacement and strand-coupled models of mtDNA replication (20,21). It is likely that the deletions arose sequentially, the Δ10.3 kb was generated first followed by a second Δ0.9 kb deletion event. Both the Δ9.5 kb lacking O_l and the Δ11.2 kb lacking O_H molecules were identified in the patients’ skeletal muscle by PCR only and were not detected in any other tissues; therefore, it is possible that these deleted molecules were spontaneously generated, but did not replicate. The replication incompetence of Δ11.2 kb mtDNA species may account for its low abundance.
Consistent with prior descriptions of single and multiple deletions of mtDNA, the breakpoints of the deletions contained direct repeat or imperfectly homologous sequences (11, 29–31) (Fig. 3). According to the nomenclature proposed by Mita et al. (31), the deletions with perfect direct repeats at the breakpoints are class I deletions and were found in the majority (71%) of their patients with chronic progressive external ophthalmoplegia (PEO) and a single mtDNA deletion. Class II deletions were not flanked by repeats or were flanked repeat elements that were not precisely at the breakpoints. The identification of class I and II deletions in MNGIE suggests that formation of the deletions involves aberrant base-pairing of the perfectly or imperfectly homologous sequences of mtDNA. The detection of microdeletions within the imperfectly homologous breakpoints is a unique observation. The microdeletions may originate during the formation of the deletions or may be due to secondary mutation events after the deleted molecule is generated.

The imperfectly homologous sequences revealed that the deletion breakpoints resided within the homologous sequences rather than immediately upstream or downstream of the homologous regions. Furthermore, the breakpoint of the 0.9 kb deletion showed variations suggesting that, after alignment of the homologous sequences, branch migration may alter the breakpoint (Fig. 3). In three deletions with imperfectly homologous sequences, the breakpoints involved tRNAs near the ends of the anticodon stem (Δ8.1 kb and Δ10.3 kb) or at the 3’ end of the gene (Δ9.5 kb). The anticodon stem of tRNA<sub>Cys</sub> ends at nt-5793 and the breakpoint of the Δ8.1 kb begins at nt-5795 while the breakpoint of the Δ10.3 kb begins at nt-5797 (Fig. 3). In contrast, tRNA<sub>Met</sub> ends at nt-4469 and coincides precisely with the breakpoint. The relationship between the tRNA genes and the breakpoints of the deletions suggests that the secondary structure of mtDNA might contribute to the formation of the deletions.

A priori, one might expect that imbalances of dNTP pools in mitochondria may cause deletions to occur at specific sequences. For example, if the deoxyribonucleotide triphosphate pools are decreased, one might expect to see deletion junctions near homopolymeric-C runs in the mtDNA because stalling of replication could predispose mtDNA to form deletion (9); however, the deletion junctions did not show sequence specificity analogous to the 5’-AAT sites that are hot-spots for somatic point mutations in MNGIE (7).

**Potential mechanisms for the formation of mtDNA deletions**

Since the recognition of autosomal dominant PEO (AD-PEO) as the first human disorder of intergenomic communication in 1989 (30), several autosomal dominant or recessive disorders associated with mtDNA alterations, such as multiple deletions have been described. AD-PEO is caused by nDNA mutations in the genes encoding adenine nucleotide translocator 1 (ANT1) (32), twinkle (a phage T7 gene 4-like protein) (33) and Pol γ (34). Autosomal recessive PEO (AR-PEO) is caused by TP gene mutations in MNGIE (3) and by Pol γ mutations (34).

The molecular mechanism responsible for generating mtDNA multiple deletions is not known (9). Mutations of the mitochondrial polymerase and a putative helicase (twinkle) causing AD- and AR-PEO suggest that the deletions may occur during replication of mtDNA. Two alternative mechanisms of mtDNA replication have been proposed. In the original strand-displacement (strand-asynchronous) model, replication of mtDNA begins RNA transcription at the L-strand promoter generating a RNA primer for initiation of heavy-strand synthesis at O<sub>H</sub> (20). Synthesis of most of the daughter H-strands is arrested prematurely, thereby generating a triple-strand D-loop structure. A small proportion of nascent H-strands continue to replicate beyond the D-loop and, after extending past O<sub>L</sub> (located about two-thirds of the genome from O<sub>H</sub>), synthesis of the mitochondrial L-strand begins. The alternative strand-coupled (strand-synchronous) model also proposes that mtDNA replication begins at O<sub>H</sub>, but posits that both strands are synthesized coordinately at one or more replication forks (21,35).

Within the context of the strand-asynchronous model of mtDNA replication, two hypothetical mechanisms for mtDNA deletions have been proposed: an illegitimate elongation model (36) and a slipped mispairing model (11,29). In the illegitimate elongation model (Fig. 7A), the deletion is formed as the H-strand replicates through an upstream direct repeat (DR1). The elongating (daughter) H-strand migrates to a downstream direct repeat (DR2) and binds to the complementary L-strand (Fig. 7A-3). The misalignment of the repeat sequences may be facilitated by stable secondary structures that bring the nascent H-strand closer to the downstream sequence (Fig. 7A-3). Elongation of the shortened daughter H-strand...
with subsequent replication of the L-strand produces a deleted mtDNA molecule.

In contrast, according to the slipped mispairing model, the upstream DR1 in the parental H-strand displaces the nascent daughter H-strand and base pairs with the complementary L-strand at the downstream DR2 (Fig. 7B-3) (11,29). The single-stranded parental H-strand breaks downstream of DR1 (Fig. 7B-3). Subsequence elongation of the truncated H-strand followed by replication of the L-strand would generate a mtDNA deletion. The slipped mispairing hypothesis is supported by the experiments demonstrating that parental H-strand can displace D-loop strands from the L-strand by branch migration (37).

In both the slipped mispairing and illegitimate elongation models, a pair of perfectly or imperfectly homologous sequences must exist in the mtDNA, mtDNA deletions are generated during H-strand replication, and homologous sequences should be derived from the H-strand. It is possible that in the setting of an imbalanced mitochondrial nucleotide pool, the relative paucity of a dNTP could lead to stalling of H-strand replication and enhance slip mispairing or illegimate elongation events. Ponamarev et al. (38) have proposed that in the setting of a Pol γ mutation, misincorporation of an incorrect nucleotide may enhance strand-slippage leading to formation of the common mtDNA deletion. Nevertheless, neither model can readily account for the presence of microdeletions within the

Figure 7. Hypothetical mechanisms for mtDNA multiple deletions in MNGIE. Illegitimate elongation (A), slipped mispairing (B) and homologous genetic recombination (C) models for formation of mtDNA deletions are depicted.
breakpoint junctions; therefore, an alternative mechanism for generating deletions in quiescent mtDNA must be considered.

Intra-molecular genetic recombination provides a third potential mechanism for mtDNA deletion formation (31). This process may begin with alignment of homologous segments of mtDNA, perhaps mediated by super-coiled circular genomes (Fig. 7C). One strand of each DNA is broken and joined to the other to form a Holliday intermediate structure (Fig. 7C-3). Heteroduplex DNA strands from different DNA molecules are paired and extended by branch migration (Fig. 7C-4). Two strands of the Holliday intermediate are cleaved by a resolvase and the breaks are repaired to form recombinant products (Fig. 7C-5). If cleaved 'vertically', the DNA flanking the heteroduplex region is recombined (Fig. 7C-5). Several reports have indicated that mtDNA recombination occurs in Meloidogyne javanica (39), mussels (40) and humans (26,41); however, the existence of mtDNA recombination in mammals is still controversial. In vitro experiments with human cells harboring partially duplicated mtDNA have demonstrated that after prolonged culture, equimolar levels of wild-type and deleted mtDNA are generated presumably by intramolecular recombination events (42). Because homologous recombination can occur in non-replicating mtDNA, this mechanism can generate deleted mtDNA molecules lacking O H. Such recombinations must have occurred many times to attain detectable levels. The aligned imperfectly homologous sequences (blue and red in Fig. 7C-6) could be unstable and asymmetric branch migration could account for the microdeletions within the aligned sequences. In addition, as proposed by Schon et al. (11), ATrich and polypurine/polypyrimidine tracts flanking the flanking DR and imperfect homologous sequences may generate bent-DNA regions that may form a triple helix with a displaced single-strand which may facilitate homologous recombination events (Fig. 3).

The positions of deletion breakpoints also support the homologous recombination model. According to the illegitimate elongation and slipped mispairing models, deletion junctions in the H-strand must migrate and elongate, therefore, imperfectly homologous sequences at the junctions must be derived from the upstream H-strand. However, the 0.9 kb deletion contains an imperfectly homologous sequence that originates upstream from O H; therefore, illegitimate elongation of the daughter H-strand or slipped mispairing of the displaced H-strand cannot account for this deletion.

Concluding comments

We have analyzed the major AmountDNA molecules and mtDNA depletion in MNGIE tissues, and have identified five major mtDNA deletions in MNGIE skeletal muscle. In this disease, the gene encoding ND5 appears to be a hot-spot for deletions, which accumulate most prominently in skeletal muscle. An unusual feature of the deletion breakpoints in MNGIE is the high frequency of microdeletions. DRs and imperfectly homologous sequences are clearly important in deletion breakpoint formation, perhaps through aberrant events like slipped mispairing or illegitimate elongation during replication or through intramolecular homologous recombination in non-replicating molecules. The positions of the breakpoints within the imperfectly homologous sequences suggest that branch migration occurs and implies that homologous recombination is more likely than strand slippage events. In addition, the relationship between stem structures of tRNA genes and the deletion breakpoints suggests that secondary structure of mtDNA may contribute to the formation of the deletions. Our results indicate altered dThd and dUrd metabolism in MNGIE produces specific mtDNA multiple deletions with atypical features.

MATERIALS AND METHODS

Patients and normal control subjects

We identified homozygous or compound-heterozygous TP gene mutations in 13 MNGIE patients (six families) from diverse ethnic groups (3,5), including: Ashkenazi Jewish [MNGIE family 1 (MN1)], German American [MNGIE family 2 (MN2)] (1), Puerto Rican [MNGIE family 3 (MN3)], African American [MNGIE family 4 (MN4)], Puerto Rican [MNGIE family 6 (MN6)], and mixed European [MNGIE family 7 (MN7)]. Numbers following MN refer to family followed by individuals (e.g. MN1-1). Normal control autopsy tissues were obtained from New York Presbyterian Hospital and the Brain and Tissue Bank, University of Maryland under Columbia University Institutional Review Board approved protocols.

Southern blot analyses

Autopsy tissues (brain cortex and white matter, liver, kidney and small intestine) were obtained from three MNGIE patients (MN3-1, MN4-2 and MN7-2) and 15 age-matched control subjects. In addition, biopsied skeletal muscles from six patients (MN1-1, MN2-1, MN2-2, MN3-1, MN4-2 and MN7-2) and seven age-matched control subjects (33–53-year-old) with neuromuscular diseases other than mitochondrial disorders. Total DNA samples were extracted by standard procedures (10). Approximately 5 µg of total DNA was digested with either the restriction enzymes PvuII or SnaBI (Roche Diagnostic Corp., Indianapolis, IN), which linearize mtDNA by cutting the circular molecule at unique sites [nts-2652 (PvuII) and 10736 (SnaBI)]. The DNA samples were electrophoresed through a 0.8% agarose gel and transferred to nylon membrane (Zeta-probe GT genomic testing blotting membranes, Bio-Rad, Hercules, CA). The PCR-amplified template for the hybridization probe corresponding to nt-1690 to 4207 of mtDNA (10), was labeled with α-32P-dATP (Random Primed DNA Labeling Kit, Roche Diagnostic Corp.). The signals were analyzed in the Molecular Imager System GS-363 (Bio-Rad) to quantitate the percentages of the deleted molecules.

Real-time quantitative PCR to estimate the amount of mtDNA

To quantitate mtDNA relative to nDNA in tissues from MNGIE patients, real-time quantitative PCR assays were carried out using TaqMan® probe system and the 7000 sequence detection system (Perkin-Elmer Applied Biosystems). A mtDNA 12S ribosomal RNA TaqMan probe (Perkin-Elmer Applied
Biosystems) and primers were used as described (43). RNase P nuclear primers and probes were contained in TaqMan pre-developed assay reagent (PDARs) (Perkin-Elmer Applied Biosystems). Standard curves for mtDNA and nDNA were generated using human blood DNA (Perkin-Elmer Applied Biosystems). Relative levels of mtDNA and nDNA were estimated in the tissue DNA samples using the standard curves. Statistical significance of the mtDNA/nDNA ratio between tissue DNA samples from MNGIE patients and normal controls was assessed by a Mann-Whitney _U_ non-parametric test because of the low number of samples (\(n < 10\)).

**Cloning the PCR fragments**

The deletion breakpoints were amplified by PCR from DNA using Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The PCR cycle conditions were: one cycle at 94°C for 2 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and an extension cycle at 72°C for 7 min. The PCR products were isolated by electrophoresis in a 2% agarose gels. Each of the PCR fragments was extracted from the agarose gel using the QIA quick gel extraction kit (Qiagen), ligated into pCR II-TOPO vector and subcloned using TOPO TA Cloning Kit (Invitrogen). Approximately 10 cloned plasmids of each PCR product were purified using High Pure Plasmid Isolation kit (Roche Diagnostic Corp.), and sequenced directly in an ABI Prism 310 Genetic Analyzer following standard procedures (45).

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