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MOLECULAR INSIGHT INTO THE ASYMMETRIC DISTRIBUTION OF PATHOGENETIC HUMAN MITOCHONDRIAL DNA DELETIONS

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SUMMARY: **Pathogenetic human mitochondrial DNA deletions occur very rarely in the minor region between the origins of replication. In order to understand the molecular basis of this asymmetry, we analyzed the structure of such a 4.680 kilobase deletion (position 471 - 5151). Directly repeated sequences (12/13 nucleotides) are present in the deletion junction, both promoters of heavy chain replication and both ribosomal RNA genes are deleted, and the 5' extent further narrows the absolute limits of mitochondrial DNA deletions. Several factors are identified that may contribute to the paucity of minor region deletions.**

Human mitochondrial DNA (mtDNA) is a 16.569-kb double-stranded circular molecule that encodes 13 protein subunits of oxidative phosphorylation, as well as the 22 transfer RNAs and 2 ribosomal RNAs required for their translation (1). Deletions of mtDNA have recently been etiologically linked to several human diseases, including the mitochondrial myopathies (2-18).

mtDNA is a circular molecule and two large regions could theoretically harbor viable deleted mtDNA: the counterclockwise region ("minor region") between the origin of heavy chain replication and the origin of light chain replication (map positions 191 - 5730) and the remaining region ("major region") between these sites (map positions 5760 - 190) (Figure 1). Deletion of either origin of replication renders the molecule replicatively incompetent and prevents its propagation. To date all mtDNA deletions studied at the molecular level spare these origins of replication, as well as the light and heavy chain promoters.

The original description of pathogenetic human mtDNA deletions included one deletion in the minor region (2), but since then none of the 125 deletions reported have

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The abbreviations used are: mtDNA, mitochondrial DNA; kb, kilobase; WISP-PCR, widely-interspaced primer polymerase chain reaction; ND-1, ND-2, ND-6, 1st, 2nd, and 6th subunits of NADH dehydrogenase; D-loop, displacement loop.

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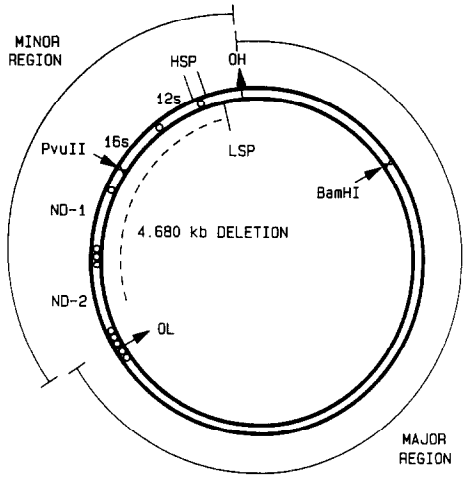

Figure 1. Schematic of Human mtDNA. The position of the patient's deletion is indicated by the inner, interrupted arc. HSP refers to the heavy chain promoters and LSP refers to the light chain promoter. OH and OL refer to the origins of replication of the heavy and light strands, respectively. 12s and 16s are the ribosomal RNA genes, ND-1 and ND-2 are the 1st and 2nd subunits of NADH dehydrogenase. The small circles interspersed between the genes represent transfer RNAs. The location of the recognition sites of the restriction enzymes PvuII and BamHI are as indicated. Only the genes encoded by the minor region of mtDNA are shown.

been identified in this region (3-18). Thus, despite the fact that the minor region contains over one-third of the total mtDNA, fewer than 1% of deletions described to date have been mapped to this location.

Sequence analysis of major region pathogenetic human mtDNA deletions has revealed a number of features crucial to understanding their biology (6-8,10-12,16,19-20). To help understand why mtDNA deletions show such an asymmetric distribution, we analyzed the sequence of a pathogenetic human mtDNA deletion found in the minor region between the origins of heavy and light chain replication.

PATIENT AND METHODS

Patient. A 55 year-old Turkish man had a 10 year history of bilateral ptosis, chronic progressive external ophthalmoparesis, and proximal limb weakness. Laboratory studies were remarkable for elevated creatine kinase, normal serum lactate level, negative anti-acetylcholine receptor antibody, and a negative edrophonium test. Electromyography demonstrated a myopathy, and a deltoid muscle biopsy showed numerous ragged red fibers.

Southern blotting. Total DNA was extracted from a skeletal muscle biopsy and Southern blotting was performed as previously described, after digestion with both BamHI and PvuII, by use of a cloned human mtDNA probe that spans the origin of heavy chain replication (6,7) (Figure 2).

WISP-PCR analysis. Widely-interspaced primer polymerase chain reaction (WISP-PCR) analysis of the deletion was carried out as described previously (6) with cycle times of denaturation 1 min at 94°C; annealing 1 min at 55°C; and extension 1 min at 72°C. The

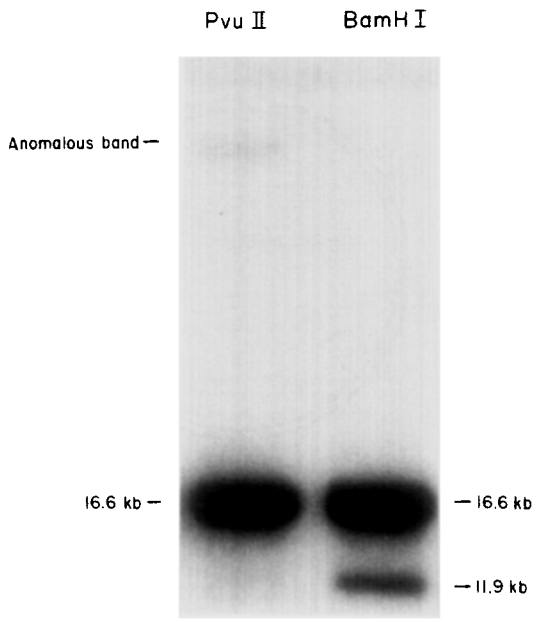

Figure 2. Southern blot analysis of mtDNA deletion. PvuII and BamHI refers to patient DNA cut with those restriction endonucleases. The linearized, normal mtDNA forms the 16.6-kb band in both lanes and the linearized, deleted mtDNA forms the 11.9-kb band in the BamHI lane. The anomalous band of apparent higher molecular weight in the PvuII lane is due to circular, deleted mtDNA which is missing the PvuII recognition site.

designation, location, and sequence of the WISP-PCR primers are given in Table 1 and the primer combinations and size of resultant products are described in Table 2. After the location of the deletion was approximated by the size on Southern blotting and the presence or absence of various primer sites, the boundaries were further narrowed by restriction enzyme analysis of the WISP-PCR products.

DNA sequencing. The WISP-PCR product was sequenced directly with three [32P]end-labelled primers, DRJ-41, DRJ-44, and DRJ-45 by dideoxy chain termination as previously described (6,7).

RESULTS

Southern blot analysis revealed a heteroplasmic mtDNA population after digestion with BamHI: the normal 16.6-kb band and a minor 11.9-kb band (which constituted 21%

TABLE 1. WISP-PCR PRIMERS

DESIGNATION	STRAND	5' - 3' SEQUENCE	POSITION
DRJ-13	L	TCA ACC AGT AAC TAC TAC T	14,200-218
C-3	L	CCA TAA ACA AAT AGG TTT GG	639-658
DRJ-44	L	CAG GCG AAC ATA CTT AC	182-198
DRJ-45	L	GGC GGT ATG CAC TTT	409-423
DRJ-38	H	TAG GAA TGC GGT AGT AGT	5120-103
DRJ-41	H	TAG GAG TAG CGT GGT AAG	5480-463

L = Light strand, H = Heavy strand of mtDNA. The sequences are numbered according to Anderson et al (1).

TABLE 2. RESULTS OF WISP-PCR ANALYSIS

5' PRIMER	3' PRIMER	WILD-TYPE DISTANCE	OBSERVED SIZE OF WISP-PCR PRODUCT
DRJ-13	DRJ-41	8.109 kb	3.4 kb
DRJ-13	DRJ-38	7.749 kb	NONE
C-3	DRJ-41	4.841 kb	NONE
DRJ-44	DRJ-41	5.298 kb	O.6 kb
DRJ-45	DRJ-41	5.071 kb	0.4 kb

of total mtDNA), consistent with a 4.7-kb interstitial deletion. Analysis after PvuII digestion showed a normal 16.6 kb-band without evidence of deleted bands; however, a band of apparently higher molecular weight was noted (Figure 2). These data indicated a 4.7-kb deletion that included the PvuII site (position 2652 in 16s ribosomal RNA) but that spared the BamHI site (position 14,258 in ND-6). WISP-PCR analysis confirmed a 4.7-kb deletion in the minor region between the origins of heavy and light chain replication, with a 5' boundary of 423 (+) and 639 (-) and a 3' boundary of 5120 (-) and 5480 (+). Restriction analysis of the WISP-PCR product verified deletion of the PvuII site and further bracketed the deletion junctions: positions 526 (Fnu4HI) and 585 (AluI) were deleted at the 5' end, and position 5269 (TaqI) was intact at the 3' end.

Direct sequencing of the WISP-PCR product revealed a 4.680-kb deletion (map position 471 - 5151) which joined the non-coding displacement loop (D-loop) region to the distal half of ND-2, and which deleted the major and minor heavy chain promoters, 6 transfer RNAs (Phe, Val, Leu(UUR), Ile, Gln, f-Met), the 12s ribosomal RNA, the 16s ribosomal RNA, ND-1, and most of ND-2 (Figure 1). The deletion encroaches upon the light chain promoter (map position 392-435) but does not directly alter it. The deletion junction occurred at a directly repeated sequence of 12 out of 13 nucleotides with a perfect direct repeat of 8 nucleotides (TAC TAC TA) (Figure 3). The actual cross-over point is

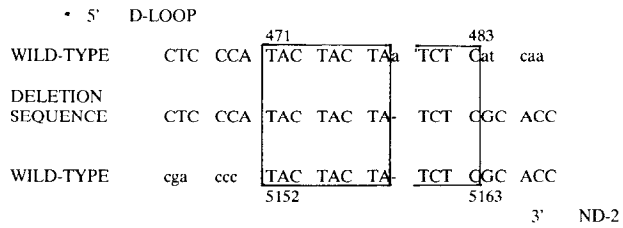

Figure 3. Sequence analysis of the deletion junctions of a 4.680 kb pathogenetic human mtDNA deletion. The upper case letters indicate the actual deletion sequence and the corresponding matching nucleotides in the 5' and/or 3' flanking sequences. Mismatched nucleotides in the flanking sequences are indicated by lower case letters. The directly repeated sequences are contained within the boxed regions. D-loop = Displacement loop, ND-2 = 2nd subunit of NADH dehydrogenase. The sequences are numbered according to Anderson et al (1).

within this perfect repeat and analysis of the entire sequence indicated deletion of the 5' imperfect direct repeat.

DISCUSSION

Molecular analysis of a pathogenetic human mtDNA deletion in the minor region between the origins of heavy and light chain replication reveals several important structural features. Direct repeats similar to those in our case have been shown to be important in most mtDNA deletions that occur in the major region of mtDNA (7-8,10-12,16,19-20). There are many fewer direct repeats of 10-13 nucleotides in the minor mtDNA region (20 in 5.3 kb) versus the major region (172 in 11 kb). Therefore the same fundamental mechanism of deletion formation may be operable in the minor region, but the 9-fold quantitative difference in the number of direct repeats may partly explain the paucity of deletions mapped there. However this accounts for less than one-tenth of the difference in the number of deletions found in the two regions.

The 8-nucleotide perfect direct repeat noted is part of a larger, imperfect direct repeat, and we can infer that the 5' repeat was deleted. In the analysis of a case of Kearns-Sayre syndrome, apparent deletion of the 5' repeat was promulgated as evidence for the "slip-replication" model of deletion formation (8). The deletion sequence in our case is also consistent with such a mechanism, but does not rule out a recombination event.

Deletions in the minor region may have been missed previously owing to exclusive use of the restriction enzyme PvuII. As shown in Figure 2, the anomalously migrating band (which represents circular, uncut deleted mtDNA that is lacking the PvuII site) could be easily missed without a second digestion with BamHI. Thus, deletions in the minor mtDNA region may be underrepresented in studies that rely solely on PvuII restriction digestion.

The absolute limits of human mtDNA deletions are unknown. This case establishes that both the major and minor heavy chain promoters can be deleted in a viable mtDNA deletion. This deletion also encroaches very closely upon the light chain promoter (within 35 nucleotides of it) but does not remove any of the 44-nucleotide structure required for optimal activity (21). It is unlikely that a replicatively viable mtDNA deletion could include the light chain promoter since it primes heavy strand replication as well as transcription of the light strand (22). Therefore this deletion is very close to the theoretical 5' limit of a human mtDNA deletion.

Virtually all of mtDNA encodes gene products (i.e., there are no introns), which is in sharp contrast to nuclear DNA. The only major exception is the D-loop, which has a central role in the regulation of transcription and replication (21,22). Pathogenetic mtDNA

deletions between the D-loop and coding regions have been demonstrated in a single family with autosomal dominantly inherited mtDNA deletions (10), but ours is the first such observation in a sporadic case of mitochondrial myopathy. Moreover, our deletion involves the D-loop on the opposite side of the origin of heavy chain replication, and thus the non-coding region is at the 5' end of the deletion junction. This case also provides evidence that the primary nucleotide sequences in the deletion junctions are crucial determinants of deletion formation, regardless of their coding status.

Deletion of transfer RNAs may be the central pathogenetic feature of human mtDNA deletions. The phenotypic similarity of this patient (chronic progressive external ophthalmoplegia and limb myopathy) to other mitochondrial myopathy patients with deletions in the major mtDNA region is consistent with this hypothesis. Transfer RNA deletions are the common structural features of deletions in the minor and major regions. Deletion of the heavy strand promoters and ribosomal RNAs in the minor region does not seem to alter the clinical phenotype and it is unlikely that they are crucial in pathogenesis.

In summary, this paper provides insight into the marked infrequency of pathogenetic deletions in the minor region of mtDNA between the origins of heavy and light chain replication. Other factors are likely to be involved (e.g. structural constraints on the deletion intermediates) and complete understanding of the marked discrepancy in mtDNA location will require further molecular study of these rare patients.

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