Replication-Competent Human Mitochondrial DNA Lacking the Heavy-Strand Promoter Region

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We identified two patients with progressive external ophthalmoplegia, a mitochondrial disease, who harbored a population of partially deleted mitochondrial DNA (mtDNA) with unusual properties. These molecules were deleted from mtDNA positions 548 to 4,442 and encompassed not only rRNA sequences but the heavy-strand promoter region as well. A 13-bp direct repeat was found flanking the breakpoint precisely, with the repeat at positions 535 to 547 located within the binding site for mitochondrial transcription factor 1 (mtTF1). This is the second mtDNA deletion involving a 13-bp direct repeat reported but is at least 10 times less frequent in the patient population than the former one. In situ hybridization studies showed that transcripts under the control of the light-strand promoter were abundant in muscle fibers with abnormal proliferation of mitochondria, while transcripts directed by the heavy-strand promoter, whether of genes residing inside or outside the deleted region, were not. The efficient transcription from the light-strand promoter implies that the major heavy- and light-strand promoters, although physically close, are functionally independent, confirming previous in vitro studies.

Mitochondrial diseases include myopathies and multisystem disorders that are defined by biochemical or morphological abnormalities of mitochondria in affected tissues (6). Since the initial description of heteroplasmic muscle mitochondrial DNA (mtDNA) deletions in patients with mitochondrial diseases in 1988 (14), more than 100 patients with such genetic abnormalities have been described (13, 16-18, 21, 23, 24, 28, 29, 34, 35). Patients with heteroplasmic muscle mtDNA deletions invariably have paralysis of the extracocular muscles, called progressive external ophthalmoplegia, and abnormal mitochondrial proliferation in muscle (21). About 70% of these patients seem to have unique deletions, while the remaining 30% of the cases have an identical 4,997-bp deletion flanked, in normal mtDNA, by a 13-bp direct repeat (26). Of the two pairs of 13-bp direct repeats in the human mitochondrial genome that do not flank the origins of heavy- or light-strand replication, only the one flanking the common deletion has previously been associated with the genesis of mtDNA deletions (15, 26).

We describe here two nonrelated patients with deletions involving the second pair of 13-bp direct repeats. The deletions were identical in both patients and were located in a region of the mtDNA usually found intact in these disorders, encompassing the heavy-strand promoter (HSP) region and part of its upstream regulatory sequences.

The human mtDNA main promoters have been defined as two closely spaced but functionally independent domains, primarily by studying the abilities of mitochondrial extracts or purified factors to transcribe cloned mitochondrial promoters in vitro (3, 5, 12, 30, 31). The HSP is responsible for transcription of the 16S and 12S rRNAs, 12 of the 13 protein-coding genes, and most of the tRNA genes. Transcription of rRNAs and structural genes has also been attributed to a second HSP (HSP2), located 90 to 100 bp downstream of the HSP (20). The light-strand promoter (LSP) transcribes a limited number of structural genes and tRNAs but has a key role in mtDNA replication by directing synthesis of an RNA primer (4).

Our observation that light-strand genes are expressed efficiently in mutant genomes lacking HSP sequences provides the first in vivo demonstration that the human mtDNA LSP is functionally independent of the HSP.

MATERIALS AND METHODS

The restriction enzymes used were from Boehringer Mannheim and New England BioLabs. The Klenow fragment of Escherichia coli DNA polymerase I was from Boehringer Mannheim. RNase A and chemicals were from Sigma. [32P]dATP and [35S]dATP (800 Ci/mmol) were from New England Nuclear. Single-stranded oligonucleotide primers were synthesized by Genetic Designs (Houston, Tex.). The sequences were as follows: primer Ball 321-F, 5'-CCCCGGCCTTGGCCACAGC-3' at positions 311 to 332 (positions according to reference 1); primer HaeII 4529-B, 5'-GTGGCGAAGCTTACGCCTGAT-3' at positions 4,544 to 4,523.

Patients. Patient 1, a 59-year-old woman, had exercise intolerance since adolescence and, since age 43, progressive limitation of eye movements (progressive external ophthalmoplegia) and droopy eyelids (ptosis). Neurological examination also showed generalized weakness. There was no clinical or laboratory evidence of involvement of tissues other than muscle. Family history was negative. Muscle biopsy showed abnormal mitochondrial proliferation, seen as scattered ragged red fibers by the modified Gomori trichrome stain (7).

Patient 2, a 36-year-old man, had transient exercise intolerance, difficulty in swallowing, and blurred vision. Liver function tests were abnormal, and hepatitis was diagnosed. All symptoms except blurred vision cleared in about 6 months; it was noted that the patient had bilateral ptosis and progressive external ophthalmoplegia. Family history was
negative. Myasthenia gravis was excluded. Muscle biopsy showed ragged red fibers and many cytochrome c oxidase (COX)-negative fibers.

**Southern blot analysis.** Total DNA was isolated from 50 mg of frozen muscle obtained by biopsy. DNA was purified as previously described (35). Southern analysis was performed by using two \(^{32}\)P-labeled probes. Probe A was purified mtDNA isolated from normal liver (35), and probe B was a cloned fragment of human mtDNA from nucleotide positions 1,229 to 2,560 (1) subcloned into bacteriophage M13. The proportions of normal and mutated mtDNA genomes were estimated by densitometry of X-ray film in a DU-70 spectrophotometer (Beckman). The approximate site of the deleted region was determined by restriction mapping with enzymes PvuII, HindIII, PstI, XbaI, SacI, AvaI, and EcoRI (35).

**PCR amplifications and sequencing.** Polymerase chain reaction (PCR) amplifications were performed with the Ampli-Taq kit from Perkin Elmer-Cetus (25). About 0.5 \(\mu\)g of total muscle DNA was used in the amplification reaction. All other conditions were as recommended by the manufacturer. We performed 33 cycles of amplification (1 min at 55°C, 3 min at 72°C, and 1 min at 94°C). The fragment obtained was reamplified and purified from a 1.5\% agarose gel with the Gene-Clean kit (Bio 101). Double-strand direct sequencing was performed as previously described (32). The Sequenase kit (U.S.B.) and about 1.5 \(\mu\)g of DNA template were used in the sequencing reactions.

**Histochemistry and in situ hybridization.** Eight-micrometer-thick muscle sections of patient 2 and a control mounted on the same slide were used in all experiments. COX and succinate dehydrogenase (SDH) staining was performed as previously described (27). The in situ hybridization procedure with \(^{35}\)S-labeled probes has been described in detail elsewhere (19).

**Preparation of mtDNA probes.** Specific mtDNA regions contained in M13 clones (a kind gift of G. Attardi and M. King) were used as templates. A 1-\(\mu\)g sample of a single-stranded M13 clone containing a strand-specific insert was mixed with 0.5 pmol of universal M13 primer (U.S.B.) in 1\(\times\)M buffer (Boehringer Mannheim) and incubated at 55°C for 5 min and then at 37°C for 20 min. After annealing of the primer to M13, 1 \(\mu\)l each of dCTP, dGTP, and dTTP (each at a concentration of 0.5 \(\mu\)M) and 5 \(\mu\)l of \(^{35}\)S-dATP or \(^{32}\)P-dATP were added to the mixture. One microliter of the Klenow fragment of *E. coli* DNA polymerase I (5 U/\(\mu\)l) was added, and the extension reaction was allowed to proceed for 20 min, after which the extension strand was chased with 1 \(\mu\)l of dATP (0.5 \(\mu\)M). The Klenow enzyme was inactivated by heating at 68°C for 10 min. A 1.5-\(\mu\)l volume of H buffer (Boehringer Mannheim), 1 \(\mu\)l of EcoRI (16 \(U/\mu l\)), and 1 \(\mu\)l of HindIII (20 \(U/\mu l\)) were added to the mixture to excise the labeled mtDNA insert. After 1 h of incubation at 37°C, the mixture was electrophoresed through a 1.5% low-melting-point agarose gel in the presence of 0.5 \(\mu\)M ethidium bromide. A gel slice containing the insert was excised, and the labeled insert was purified by using the Gene Clean kit (Bio 101). Specific activities were about 10\(^{9}\) cpm/\(\mu\)g.

The mtDNA cloned fragments in M13 were as follows: clone OP2, light-strand positions 2,441 to 2,954 within the 16S rRNA region (positions according to reference 1); clone 66-21, light-strand positions 7,658 to 8,287 within the COXII region; clone OP10, heavy-strand positions 7,658 to 8,287 within the COXII region; clone M8.9.8, heavy-strand positions 1 to 740 within the 7S RNA region. The labeled insert from clone M8.9.8 was excised with HindIII and BalI (there is an internal BalI site at mtDNA position 322) instead of HindIII and EcoRI. This modification was necessary to remove insert sequences downstream of the HSP.

**RESULTS**

**Mapping and sequencing of mutant mtDNAs.** Both patients had progressive external ophthalmpoplegia and abnormal mitochondrial proliferation in their muscle biopsies. Total DNAs were purified from the muscle biopsies and analyzed for the presence of mtDNA deletions by Southern blot hybridization using total mtDNA (Fig. 1, probe A) as the probe. When digested with PvuII, which cuts only at position 2,652 in human mtDNA, we detected, in addition to a population of linearized 16.6-kb molecules, a large population of hybridizing undigested molecules migrating in the gel as aberrantly large fragments (Fig. 1). Digestion with other restriction endonucleases provided an approximate location of the deletion and confirmed that these latter mtDNAs were resistant to digestion with PvuII because the region containing the PvuII site had been deleted in a subpopulation of mtDNA molecules. The mapping results were confirmed by hybridizing the same blot with a probe extending from positions 1,229 to 2,560 (Fig. 1, probe B). This probe was homologous to the deleted region and was therefore unable to hybridize with the population of mutant mtDNAs. Densi-
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FIG. 2. PCR amplification and sequence analysis of the deletion breakpoint region. (A) Total DNAs from the two patients (lanes 1 and 2) and two controls (lanes 3 and 4) were amplified by PCR using oligonucleotide primers homologous to regions flanking the breakpoint (see Materials and Methods). A small portion (5%) of the PCR reaction analyzed by agarose gel electrophoresis is shown. The approximate size of the PCR product (330 bp) is indicated at the right. Lane M contained molecular weight markers. (B) DNA sequence of the amplified fragments. The autoradiographed sequence (dideoxy reactions G, A, T, and C) is that of the light strand reading 5’ to 3’ from left to right. The 13-bp direct repeat flanking the deleted region is overlined. The sequences in brackets (positions 548 to 4,442) were deleted. The HSP is boxed. (C) Linearized representation of human mtDNA showing the location of the HSP− deletion. OH and OL, Origins of heavy- and light-strand replication; LSP, HSP, and HSP2, promoters of transcription; 12 S and 16 S, ribosomal RNA genes; ND1 to ND6, genes corresponding to NADH-coenzyme Q oxidoreductase subunits 1 to 3; CO1 to CO3, genes corresponding to COX subunits 1 to 3; Cyt b, cytochrome b gene; ATPases 6 and 8, genes corresponding to ATP synthetase subunits 6 and 8. The 3,895-bp deleted region is shown below the map.

tometric analysis showed that the population of partially deleted mtDNA accounted for 50 and 60% of the total muscle mtDNAs in patients 1 and 2, respectively. Guided by the mapping information, we designed oligonucleotide primers complementary to regions flanking the putative deletion breakpoint and subjected total muscle DNAs from two controls and the two patients to amplification by PCR. No specific fragment was amplified with the DNAs from normal muscle, but the DNAs from both patients generated a 330-bp fragment spanning the deletion breakpoint (Fig. 2A).

Sequence analysis of the PCR fragments revealed identical 3,895-bp deletions in both patients with the breakpoint at the left side between nucleotides 547 and 548 upstream of the HSP, and on the right side between nucleotides 4,442 and 4,443 within the tRNA^Met^ gene (Fig. 2B and C). The deletion was flanked by a perfect 13-bp repeat; one repeat was found immediately before the left deletion breakpoint, and an other 13-bp repeat was found (in normal mtDNA) at the extreme 3’ end of the deleted region (Fig. 2B). We did not establish which repeat was retained and which was deleted; therefore, the convention of Mita et al. (18), in which the left-side repeat is retained and the right-side repeat is deleted, was adopted. Because of its unusual location, encompassing the HSP, we designated this specific deletion the HSP− deletion.

Transcriptional analysis using in situ hybridization. The fact that HSPs were absent in the deleted molecules raised a question about the transcription efficiency of the LSP. On the basis of in vitro studies (3, 30), the functional boundaries of these oppositely oriented promoters, including regulatory sequences, are only about 70 nucleotides apart. To study the expression of both promoters and because of limited tissue availability (small muscle biopsies), we took advantage of previous reports showing that partially deleted mtDNAs are abundant in muscle fibers with abnormal mitochondrial proliferation (19, 29). In agreement with previous observations, we found that muscle fibers with abnormal mitochondrial proliferation—as recognized by intense staining with SDH, a respiratory chain complex encoded exclusively by nuclear DNA (e.g., the fiber marked with a star in Fig. 3A)—were devoid of COX (Fig. 3B). In situ hybridization studies with strand-specific DNA probes (see Materials and Methods) showed that these fibers contained high levels of transcripts under control of the LSP (2; Fig. 3C and F). Although the probe used in the experiment shown in Fig. 3F detects exclusively light-strand transcripts, the probe used in the experiment shown in Fig. 3C detects both light-strand transcripts and extruded single-stranded D-loop DNA (4). As expected, 16S rRNA (a heavy-strand transcript) was not overexpressed in these fibers, because this region is missing in mutant mtDNAs (Fig. 3D). COXII RNA, a heavy-strand transcript not encompassed by the deletion, was also not overexpressed in these fibers, implying that overall heavy-strand transcription of mutant genomes was impaired (Fig. 3E). The intensity of hybridization signals of the various probes agreed well with the reported steady-state levels of mitochondrial transcripts (2), and different exposure times were necessary to obtain detectable signals with the different probes (Fig. 3, legend). The distribution of deleted mtDNAs along a muscle fiber has been shown to be segmental, i.e., some regions are almost completely devoid of mutant mtDNAs, while others contain large proportions of such molecules (19). Because of this phenomenon, we analyzed serial sections only in fibers in which the first and the last sections showed the same intense SDH staining, indicating that we were studying a single domain characterized throughout by abnormal mitochondrial proliferation. The results shown in Fig. 3 were found in several other fibers. Specifically, of 305 fibers examined in a muscle section, 70 (23%) had abnormal mitochondrial proliferation as measured by SDH staining. All 70 fibers had elevated levels of light-strand transcripts, and none had elevated levels of heavy-strand transcripts. No fibers were found in control muscle with either proliferated mitochondria or aberrant levels of transcripts. We also observed several muscle fibers with COX deficiency and abnormally low levels of transcripts originating from normal genomes (e.g., the fiber marked with an open circle in Fig. 3; see below).
FIG. 4. Location of one of the 13-bp direct repeats within the mtTF1 binding site. The nucleotide sequence is numbered as in reference 1. mtTF1, mtTF1 binding site. The deleted region is in lowercase letters.

DISCUSSION

Participation of direct repeats in the genesis of human mtDNA deletions. We identified two patients with heteroplastic populations of partially deleted muscle mtDNAs in whom the deletions, which were identical in both patients, had an unusual location in the mitochondrial genome. Only one deletion between positions 0 and 5,700 has been reported (14). Although the sequence of that particular deletion breakpoint is not available, the published mapping information placed that breakpoint between positions 36 and 1,301 at the left side and between positions 5,274 and 5,789 at the right side, thus distinguishing it from the deletion reported here (548 to 4,442).

Of the approximately 100 human mtDNA deletions reported, about one-third are identical, involving a 13-bp direct repeat at positions 8,470 and 13,447 (designated the common deletion; 26). The high incidence of this specific deletion was ascribed, at least in part, to the length of the direct repeat as a target site for a putative recombination event. However, the relative paucity of the HSP -deletion, also involving a 13-bp direct repeat, suggests that a long direct repeat per se is not sufficient to create a “hot spot” for human mtDNA deletions. This is also supported by the fact that besides the common deletion, two other deletions, involving shorter repeats, have been reported more than once; i.e., a deletion involving an 11-bp direct repeat was reported in two patients (17, 18), and a second involving a 10-bp direct repeat was reported in four patients (18, 34).

The mechanism responsible for the generation of mtDNA deletions remains unknown, but a model involving slipped mispairing has been favored (15, 26, 28). This mechanism, hypothesized mostly on the basis of observations on the common deletion, requires that at some point both direct repeats be present as complementary single strands, thus allowing pairing and recombination to occur. This mechanism has also been supported by the observation of a familial case of mitochondrial myopathy with multiple mtDNA deletions, in which at least a dozen different deletions started within a small region in the D loop (between positions 16,068 and 16,679) and extended to small direct repeats scattered throughout the structural gene region between positions 7,440 and 8,630 (36; but see reference 33 for multiple deletions in non-D-loop regions). One problem with this model is that because replication of mammalian mtDNA is an asynchronous and asymmetric process, the repeats that are in a single-stranded state at any time are direct (i.e., not complementary). However, it has been pointed out that some regions of mtDNA may be susceptible to formation of bent DNA, thus possibly allowing discrete double-stranded DNA segments to expose small single-stranded regions containing the complementary repeat (26 and references therein).

In the case of the HSP -deletion, however, both repeats are contained within regions of duplex DNA; i.e., the repeat at position 535 to 547 is adjacent to but not within the single-stranded D-loop region (4), while the repeat at position 4,430 to 4,442 is behind the origin of the light-strand replication and is therefore not subjected to displacement of single-stranded DNA during synthesis of daughter strand DNA primed from the origin of heavy-strand replication.

An additional obstacle to the presumed pairing of the HSP -direct repeats during an intermediate phase of the recombination event is that the repeat at position 535 to 547 lies within a 25-bp sequence which has been recognized as the binding site for mitochondrial transcription factor mtTF1 (Fig. 4) (9, 11). Specific initiation of transcription by mitochondrial RNA polymerase requires mtTF1 (8, 12), a 25-kDa protein monomer that is capable of activating transcription of both the LSP and HSP in vitro (9, 10). The presence of mtTF1 on its natural binding site is an obvious obstacle to any kind of DNA-DNA interaction involving this region. However, the affinity of mtTF1 for the HSP binding site is...
severalfold weaker than it is for its second functional binding site at the LSP (11; Fig. 4). This low affinity may render the mtTFI binding site at the HSP unprotected for a time sufficient to allow, at low frequency, participation of the 13-bp repeats in deletion formation.

Taken together, these two features—the location of both of the HSP" repeats within regions of always-duplex DNA and the presence of mtTFI as an impediment to recombination—probably explain the rarity of this particular deletion in the patient population.

The LSP is independent of the HSP in vivo. Another aspect of the unusual mtDNA deletion reported here is that it encompasses HSP sequences (3, 20), including the transcription initiation sites. In vitro studies have shown that these sequences are essential for normal transcription of the heavy strand (3, 30). The mitochondrial major promoters are clustered in a 150-nucleotide region within the D loop (3), and in theory, a single bidirectional promoter could ensure complete expression of the mitochondrial genetic information. However, in vitro studies have indicated that mammalian mtDNAs have evolved independent, predominantly unidirectional promoters for transcription of the heavy and light strands. It has been speculated that spatial and functional separation of heavy- and light-strand transcription initiation could be demanded by the mode of replication of animal mtDNA (11). Although heavy-strand transcripts participate only in protein synthesis, transcription initiation at the LSP is required to generate primers for initiation of replication of the heavy strand. The fact that the mutant mtDNA described here is replication competent implies that the LSP still provided a correct RNA primer. Moreover, our in situ hybridization results clearly showed that light-strand transscripts were abundant in fibers with abnormal proliferation of mitochondria, while heavy-strand transcripts were not. It must be emphasized, however, that this abundance of light-strand transcripts is not due to up regulation at the LSP but merely reflects the abundance of organelles in these fiber domains. In agreement with previous in vitro work, our results provide an in vivo demonstration that the LSP is functionally efficient in the absence of D-loop sequences upstream of position 547, including the HSP region. LSP directionality has also been maintained in the mutant molecules, as significant levels of transcripts of heavy-strand genes were not detected in muscle fibers with abnormal mitochondrial proliferation. The LSP is capable of generating heavy-strand transcripts at low efficiency (5), but this bidirectionality was not enhanced in mutant mtDNAs, as the retention of LSP in HSP" mtDNAs did not restore normal levels of heavy-strand transcripts. The absence of significant levels of COXII transcripts also indicates that the deletion did not reveal or create a cryptic HSP. The mtTFI binding site is almost intact in mutant molecules (only the last 2 of 24 nucleotides are missing in the binding site), and mtTFI may still be able to bind to the deletion breakpoint. However, mtTFI binding in deleted genomes is not expected to promote heavy-strand transcription, as the 20-bp region (positions 4,443 to 4,463) downstream of the 13-bp repeat brought into proximity to the mtTFI site has only 5% identity with HSP sequences known to be indispensable for transcription. Furthermore, the mitochondrial promoter consensus sequence (5'-CAGCAAGCACAAAGAYA-3') does not have a significant degree of sequence similarity to any other mtDNA sequence, with the exception of a conserved sequence box (CSB-3) that is unable to promote transcription (3).

Pathogenesis of mtDNA deletions. The clinical manifestations in both patients (i.e., progressive external ophthal-moplegia and ptosis) were different from those in patients with deletions located in structural genes (21). Because the HSP" deletion is certainly a null mutation for most of the mtDNA information, our results reinforce the concept that the location of the deletions has no correlation to the clinical phenotype or the biochemical defect (19, 22, 35). The most likely explanations for this phenomenon would be either a lack, or unbalanced ratios, of tRNA molecules in organelles containing exclusively mutant or normal-plus-mutant genomes.

The mosaic expression of the respiratory chain defect in muscle fibers is a key aspect in the development of these diseases (19, 29). Shoubridge et al. (29) reported that lack of COX activity in certain muscle fibers is likely to be caused by a dominant effect of the abundant mutant mtDNAs upon wild-type mitochondrial genomes, which are present at normal levels. However, we detected a number of muscle fibers with profound COX deficiency (fiber marked with a circle in Fig. 3B) and a clear reduction in normal mitochondrial transcripts (most clearly seen in the analogous fiber in Fig. 3D). It is noteworthy that transcription of mutant genomes was not particularly abundant in these fibers (Fig. 3C and F). This observation suggests a complex interaction between normal and mutant genomes in causing a respiratory chain defect. Further work is needed to clarify this issue.

As illustrated here, patients with mitochondrial diseases harbor naturally occurring mtDNA mutations that are informative with respect to not only mechanisms leading to a pathological state but also mtDNA topology, biogenesis, and gene expression. Such natural mutations are particularly useful in studying mtDNAs of higher eucaryotes, in which genetic manipulation in intact mitochondria is not feasible.

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