Double-strand breaks of mouse muscle mtDNA promote large deletions similar to multiple mtDNA deletions in humans

Sarika Srivastava1 and Carlos T. Moraes1,2,*

1Department of Cell Biology and Anatomy and 2Department of Neurology, University of Miami School of Medicine, Miami, FL 33136, USA

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Mitochondrial DNA (mtDNA) deletions are a common cause of mitochondrial disorders and have been found to accumulate during normal aging. Despite the fact that hundreds of deletions have been characterized at the molecular level, their mechanisms of genesis are unknown. We tested the effect of double-strand breaks of muscle mtDNA by developing a mouse model in which a mitochondrially targeted restriction endonuclease (PstI) was expressed in skeletal muscle of mice. Because mouse mtDNA harbors two PstI sites, transgenic founders developed a mitochondrial myopathy associated with mtDNA depletion. The founders showed a chimeric pattern of transgene expression and their residual level of wild-type mtDNA in muscle was ~40% of controls. We were able to identify the formation of large mtDNA deletions in muscle of transgenic mice. A family of mtDNA deletions was identified, and most of these rearrangements involved one of the PstI sites and the 3′ end of the D-loop region. The deletions had no or small direct repeats at the breakpoint region. These features are essentially identical to the ones observed in humans with multiple mtDNA deletions in muscle, suggesting that double-strand DNA breaks mediate the formation of large mtDNA deletions.

INTRODUCTION

Mitochondrial DNA (mtDNA) deletions can present as a single clonal species or as multiple deletions. Multiple mtDNA deletions have been associated with mutations in the mtDNA polymerase gamma, adenine nucleotide translocator and the helicase twinkle (reviewed in 1). Low levels of multiple mtDNA deletions have also been associated with normal aging (2). The molecular features of multiple mtDNA deletions include the presence of very small (or no) direct repeats at the deletion breakpoint and a bias towards involving the end of the D-loop control region (3,4). Although slip-mispairing has been proposed to mediate mtDNA deletions (5,6), more recent findings suggest that double-strand breaks could be involved in the formation of deletions with small homologies at the breakpoint region (4). To better understand the consequence of double-strand breaks in animal muscle mtDNA, we targeted PstI to mouse skeletal muscle mitochondria. Our results provided clues to the formation of large-scale deletions in mammals.

RESULTS

Expression of Mito-PstI in transgenic mouse muscle

Because mouse mtDNA harbors two sites for the restriction endonuclease PstI, we attempted to cause double-strand breaks in muscle mtDNA by producing transgenic mice expressing PstI specifically in muscle mitochondria. A construct harboring a mammalianized (i.e. codon usage optimized for mammalian ribosomes) version of the bacterial PstI gene downstream of a mitochondrial targeting sequence and the human skeletal muscle actin promoter was used to produce transgenic mice (Fig. 1A). Three male PstI transgenic founder mice (Fd1, Fd2 and Fd3) were obtained. Both Fd1 and Fd2 mice showed growth defects, whereas Fd3 did not show such a defect. At ~3 months of age, both founders were growing slower than a control sibling of the same age (Fig. 1B). Fd1 and Fd2 mice showed growth defects, whereas Fd3 did not show such a defect. At ~3 months of age, both founders were growing slower than a control sibling of the same age (Fig. 1B).
Figure 1. Production of transgenic mice expressing a mitochondrial targeted PstI in skeletal muscle. (A) depicts the structure of the transgene construct containing a mammalianized version of the bacterial PstI gene fused in frame with an N-terminal mitochondrial targeting sequence of COX VIII. (B) shows founder 1 (Fd1) and a non-transgenic sibling. Extensive breeding of Fd1 and Fd2 failed to produce pups containing the transgene, whereas 50% of Fd3 pups had the transgene (C). Western blot analysis showed that mitochondria isolated from Fd1 and Fd2 skeletal muscle (fore limb and hind limb muscles) contained the transgene product. Muscle mitochondria from an Fd3 pup harboring the transgene did not express PstI (D). Muscle mitochondria isolated from Fd1, Fd2 and non-transgenic littermate controls were analyzed for the activity of complex I + III, II + III and IV by spectrophotometric assays (E). The activities were expressed as a ratio to citrate synthase activity to normalize the results to mitochondrial abundance. Complexes I and IV were significantly decreased in the transgenic Fd1 and Fd2. *P < 0.05; **P < 0.005; n = 3 independent assays of each mitochondrial preparation.
50% of F1 generation (Fig. 1C). However, the Fd3 progeny did not express the PstI protein in muscle mitochondria, whereas Fd1 and Fd2 did (Fig. 1D). At 6 months (Fd1) and 7 months (Fd2) of age, these mice developed a myopathy and had difficulties in moving (Supplementary Material, Movie S1). When the myopathy became severe, the mice were sacrificed for skeletal muscle analysis.

**Skeletal muscle of Mito-PstI transgenic mice showed mitochondrial respiratory chain defects**

Isolated mitochondria were used to measure the respiratory chain enzyme complex activities. Muscle mitochondria from both Fd1 and Fd2 mice showed a significant defect in the complex I + III and complex IV activity (P < 0.005, Fig. 1D). Morphological analyses showed mitochondrial abnormalities in muscle of Fd1 by electron microscopy (EM) and by histochemical staining. EM of Fd1 muscle showed mitochondrial proliferation, swelling and cristae disorganization (Supplementary Material, Fig. S2). Gomori trichrome stain showed the presence of ragged-red fibers, which were more evident in Fd1 than in Fd2 (Fig. 2A). Cytochrome oxidase (COX) deficiency was evident in both mice, but was not observed in all the fibers, suggesting chimerism (Fig. 2A). COX deficient fibers had increased succinate dehydrogenase (SDH) activity staining, confirming that mitochondria with mtDNA defects were present at increased amounts. Skeletal muscle sections from control, Fd1 and Fd2 mice were also analyzed for the levels of PstI and the mtDNA-coded CoxIp by immunocytochemistry. PstI immunostaining in both Fd1 and Fd2 mouse muscle revealed a chimeric pattern of PstI expression (Fig. 2B), which is compatible with the chimeric COX deficiency. This observation may also explain why Fd1 and Fd2 failed to transmit the transgene. It is likely that, if expressed in all fibers after germline transmission, Mito-PstI would cause an embryonic lethal phenotype. Muscle fibers expressing PstI had a significant decrease in CoxIp (Fig. 2B), implying damage to mtDNA molecules.

**MtDNA levels were decreased in the skeletal muscle of transgenic Mito-PstI mice**

To determine the mtDNA abundance, Southern blot membranes with muscle and liver DNA samples from control and transgenic founder mice were hybridized to mtDNA (COXI region) and a nuclear 18S rDNA probes (Fig. 3B–I). This analysis showed a significant depletion in the mtDNA levels in the skeletal muscle of both Fd1 and Fd2 mice. Fd1 and Fd2 mice showed 66% and 60% reduction of mtDNA levels, respectively, when compared with an age-matched control (Fig. 3J).

**Detection of large-scale mtDNA rearrangements in the skeletal muscle of transgenic Mito-PstI mice**

In addition to the band corresponding to the wild-type mtDNA, a relatively smaller size band (~9.3 kb) of weaker intensity was detected in the muscle samples from the transgenic mitochondrial PstI founders with the COXI probe. Such a band was absent from the control muscle samples (Fig. 3D) or liver samples (Fig. 3I). By re-probing the blot with the ND4 probe, the ~9.3 kb mtDNA species was no longer detected in the muscle samples from transgenic founders (Fig. 3E) suggesting that the free double-strand ends generated at the site of PstI digestion participated in recombination events, creating large-scale deletions. The COXI probe also detected a ‘broad hybridization signal’ in the 5–6 kb region (Fig. 3D). Although this signal was also observed in the control sample, it appeared slightly stronger in muscle of Fd1 and Fd2. At this point, we do not know whether this signal corresponds to additional mtDNA rearrangements. Undigested DNA showed the presence of abnormal bands in the Fd1 and Fd2 DNA, which could be relaxed circles of partially deleted mtDNAs. PstI-digested mtDNA should migrate as a 12.5 kb fragment, which also could be present at low levels, even though band identity of undigested DNA is difficult to prove (Fig. 3F).

To define the main mtDNA regions involved in recombination, we performed PCR with various sets of widely spaced primers distributed along the mouse mtDNA (see Materials and Methods). In standard PCR conditions, the primer regions are too far apart to generate a product. However, if deletions occur between the primers, smaller amplicons are obtained. As templates, we used total DNA isolated from skeletal muscle of control and transgenic founder mice. Most of the reactions did not yield a product, including reactions using primers flanking the two PstI sites (data not shown). However, PCR analysis with a forward primer flanking one of the PstI sites (8300-F) and the reverse primer in the mtDNA D-loop region (15 982-B) amplified a ~650 bp fragment in the skeletal muscle from both Fd1 and Fd2 mice, which was absent from the control skeletal muscle. This result suggested the presence of a large size deletion in the mtDNA of both transgenic founders (Fig. 4A and B). Cloning and sequencing analysis of individual ~650 bp inserts revealed the presence of a family of 6.9–7.0 kb size deletions in the skeletal muscle of both Fd1 and Fd2 mice (Fig. 4C), which are compatible with the size of the smaller band observed by Southern blot. Interestingly, the deleted molecules showed one of the breakpoints exactly at, or close to, the PstI site located at nucleotide position 8420 (i.e. distal from the D-loop). Some of the partially deleted molecules contained 2–4 bp homology repeats and the deletion breakpoints involved the nucleotide positions 8346–8420 on one end and nucleotide positions 15 368–15 433 on the other, suggesting that the free double-stranded ends generated by PstI cleavage recombinated with the 3’ end of the D-loop region.

An additional primer pair combination (8300-F and 13125-B) yielded a faint band after PCR (data not shown). After nested re-amplification, cloning and sequencing, we determined that the amplification corresponded to another group of deletions involving the same PstI site (at position 8420) and an mtDNA region between positions 12 957–13 094. The features of the breakpoints were essentially identical to the ones observed at the D-loop region (i.e. 0–3 bp homology repeats involved in the breakpoint). Therefore, although prevalent, deletions involving the free end and the D-loop are not the only ones, a finding that also parallels observations in muscle of patients with multiple mtDNA deletions.
Clonal expansion of mtDNA deletions in skeletal muscle

To test if deletions formed by double-strand breaks expand in aging muscle, we isolated single muscle fiber segments (20 μm) of COX-stained sections and PCR amplified the breakpoint of the previously observed mtDNA deleted region. Isolated fiber segments were subjected to the amplification of both an mtDNA region outside the observed deleted region (i.e. outside the nt 8420–15 500 region) and the deletion breakpoint region (using the same primers shown in Fig. 4). COX-positive fiber segments showed amplification of the wild-type region, whereas they did not show amplification of the deleted mtDNA breakpoints. Using the same conditions that allowed the amplification of wild-type mtDNA in COX-positive fibers, only 11 of 20 COX-negative fibers showed amplification of the wild-type mtDNA region, and the amplification product was weaker than the ones observed in COX-positive fibers (data not shown). These findings suggest that most COX deficient fibers have an mtDNA depletion, which would be compatible with the Southern blot results. Amplification of the deleted mtDNAs was observed in four of 20 COX-negative fibers.

Figure 2. Chimeric expression of the Mito-PstI in muscle of Fd1 and Fd2. (A) shows histochemical staining for cytochrome oxidase (COX) and succinate dehydrogenase (SDH) activities. Both Fd1 and Fd2 had COX deficiency but normal or elevated SDH activity. The COX deficiency was present in a mosaic pattern. The mosaic pattern of expression of Mito-PstI was also observed by immunocytochemistry using an anti-PstI antibody (B). Fibers expressing Mito-PstI had decreased levels of CoxIp, an mtDNA-coded polypeptide (B).
DNA fragments from three fibers were cloned into plasmids and restriction digestion and sequencing of the inserts showed that two of the fiber segments had only one type of deletion (type F or I, Fig. 4), whereas one fiber had a mixture of three different types of deletions (Fig. 5B). These findings indicate that individual muscle fiber segments were enriched for one subtype of mtDNA deletion, suggesting clonal expansion during mitochondrial proliferation.

DISCUSSION

Mito-Pst1 expressing muscle showed the presence of low levels of mitochondrial genomes with large-scale deletions. The free ends generated by the endonuclease were recombined with a region at the 3' end of D-loop, deleting a large fragment of mtDNA. The size of the deletions ranged from 6.9 to 7.0 kb and many deleted molecules contained a 2–4 bp homologies at the breakpoint region, suggesting the participation of homologous recombination (HR). It was surprising to observe that most double-strand breaks preferentially recombined with a specific region at the end of the D-loop. This could be due to the fact that the D-loop region has a loose triple-strand conformation that might allow an easy access for single strand annealing (SSA), exchange and recombination (7). It may also be related to recent observations, which suggested that the D-loop region mediates replication fork arrest (8). Replication fork arrest is known to stimulate recombination events (9). Such polymerase pauses may also contribute to recombination at the less abundant nt 13 000 region.

Although the presence of direct repeats at the breakpoint region of some mtDNA deletions suggests a slip-mispairing mechanism (5), the mechanism of genesis of mtDNA deletions without direct repeats at the breakpoint has been puzzling. Samuels et al. (6) studied the molecular features of 263 mtDNA deletions in humans and found that among deletions flanked by small direct repeats (<5 bp) or not flanked by direct repeats [also known as Class II deletions (10)], there was a remarkable bias for the 3' end of the deletion to be located at the very end of the D-loop region (positions 16 000–16 100 in the human mtDNA, which correspond approximately to positions 15 400–15 500 in the mouse mtDNA). If double-strand breaks are a major cause of Class II mtDNA deletions in human, how could they be generated? In the case of sporadic mtDNA deletions, double-strand
Figure 4. Characterization of mtDNA deletions associated with a double-strand break. Using oligonucleotide primers flanking a PstI site and the D-loop region (A, arrows), we amplified an approximate of 650 bp PCR fragment only in DNA extracted from muscle of Fd1 and Fd2 (B). The PCR products were cloned into plasmids and sequenced. Sequence analysis showed the presence of a family of mtDNA deletions involving the PstI site region and the end of the D-loop (C). Most deletions had small homologies at the breakpoint region (underlined nucleotides in C).
breaks could be caused by ionizing radiation or the collapse of replication forks when the replication machinery encounters single-stranded breaks in the template DNA (11). Reactive oxygen species, which are abundant in the vicinity of mtDNA molecules could cause single-strand or double-strand breaks through the formation of hydroxyl radicals (12). Double-strand breaks could also occur during normal mtDNA replication. Replication of the lagging strand (in a coupled replication model) could be influenced by polymerase gamma pauses in the D-loop region (4), triggering a replication-induced break/recombination event.

There are three known mechanisms associated with double-strand break repair: homologous annealing (HR), single-strand annealing (SSA) and non-homologous end-joining (NHEJ). HR and SSA are more prevalent in prokaryotic cells, whereas NHEJ is commonly associated with repair of eukaryotic cells (13). HR differs from SSA in that in the latter two internal repeats (present in a single-strand segments) recombine, deleting the distal segments. Moreover, continuing replication is not necessary in the SSA model (13). In the HR or SSA models, after a double-strand break, exonucleases produce an unpaired free 3’ end, which has been shown by Szostak et al. (14) to be an initial step in double-strand break repairs. The free 3’ end of the double-strand break, through small homologies, could anneal with the end of the unpaired D-loop strand and serve as a primer for replication (Fig. 6A), or simply as templates for repair by SSA (13). Single strand regions can also be created during coupled replication (Fig. 6B). In either case, repair could be achieved simply by extending the annealed 3’ end. However, if elongation does not proceed around the circle, a more complex mechanism, involving the resolution of a ‘Holliday junction-type’ structure would be required.

In the case of mtDNA multiple deletions, there is emerging evidence for the participation of double-strand breaks. The disease known as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is caused by mutations in the Thymidine Phosphorylase gene, leading to a defect in the thymidine nucleotide pool. Patients present with mtDNA deletion, point mutations and multiple deletions in muscle (15,16). A nucleotide pool unbalance has been hypothesized to lead to replication fork stalling (16). Wanrooj et al. (4) have proposed that a double-strand break mechanism could be associated with autosomal dominant multiple deletions (which are mostly Class II deletions). They based their suggestion on the molecular features of mtDNA deletions observed in muscle of patients with mutations in the helicase twinkle and the polymerase gamma. They found that replication stalling at homopolymeric runs, which are frequently present at deletion breakpoints associated with polymerase gamma mutations, are likely to promote double-strand breaks. They also noted that the higher proportion of spontaneously linearized full-length muscle mtDNA molecules in patients with polymerase gamma mutations supports the double-strand break model. Mutations in the helicase twinkle, which has been shown to control mtDNA copy number (17), could be directly involved in double-strand break repair, as has been shown for other helicases (18). Finally, we obtained direct evidence that double-strand breaks of mtDNA lead to the formation of Class II mtDNA deletions, with molecular features that are remarkably similar to the ones caused by mutations in the mtDNA replication/repair apparatus in humans. These findings suggest that double-strand breaks mediate the formation of class II mtDNA deletions in mammals.

We and others (19,28) have reported that mtDNA molecules linearized by restriction endonucleases are not stable and only traces of such molecules can be observed in Southern blots. It is likely that most of them are rapidly degraded by exonucleases, whereas a small fraction undergoes recombination. The presence of low levels of mtDNA deletions in muscle of an animal with homoplasmic levels of a susceptible mtDNA haplotype, suggests that mitochondria targeted restriction endonucleases could still be safe to shift mtDNA haplotypes in a heteroplasmic environment, as the repopulation with non-susceptible mtDNA haplotypes should staunch the accumulation of partially deleted mtDNA.

**MATERIALS AND METHODS**

**Production of transgenic *Pst*I mice**

A mammalian version (synthetic and codon optimized) of the bacterial *Pst*I gene (19) with a 5’ mitochondrial targeting sequence from human COX VIII (cytochrome c oxidase subunit VIII) and with a 3’ PolyA signal were cloned in a plasmid downstream of a human skeletal muscle actin promoter (pSMA), kindly donated by Dr Jeffrey Chamberlain, University of Washington. The skeletal muscle actin promoter was originally cloned in Dr Larry Kedes’ laboratory (20). The promoter fragment was characterized in Dr Hardeman’s laboratory in transgenic mice. She reported that in newborn
animals, the SMA promoter was expressed in fiber types in the following order: IIB > IIX > IIA > I (21). In adult animals, the expression of dystrophin under this promoter did not show fiber-type variation. It also did not show heart expression between 2 weeks and 2 years (J. Chamberlain, personal communication).

Briefly, the *Pst*I gene along with the targeting sequence was excised with *Eag*I from the plasmid pCDNA3PstI, described elsewhere and was cloned into an *Not*I site of pSMA (22). The final construct pSMA PstI was linearized with enzymes *Cla*I and *Pvu*I and the fragment was purified using elutip-D minicolumn (Schleicher and Schuell, Keene, N.H). The purified fragment was used for transgenic microinjection. Microinjection into B6/SJLF1 fertilized oocytes was performed by the transgenic core facility of the University of Miami School of Medicine. Out of 29 mice born from implantation of injected fertilized oocytes into pseudo-pregnant mothers, three were positive for the transgene by PCR of tail DNA. The sequence of the mouse mtDNA was obtained from GenBank accession no. AJ512208.

**Enzymes activity analyses**

For measuring the specific activity of the respiratory chain complexes, mitochondria were isolated, and assays were performed spectrophotometrically (DU-640 spectrophotometer, Beckman Instruments Inc., Fullerton, CA, USA) as described elsewhere (23). The activities of NADH cytochrome c oxidoreductase (complex I + III), succinate-cytochrome c reductase (complex II + III), cytochrome c oxidase...
were performed at 37°C in 1 ml medium (except the citrate synthase at 30°C).

Electron microscopy analysis
Muscle tissue was fixed overnight in phosphate buffer containing 2% paraformaldehyde and 2.5% glutaraldehyde. The tissue was rinsed in phosphate buffer, treated with 1% osmium tetroxide and processed for transmission electron microscopy (TEM) as described (24). TEM images were captured using JEOL CX 100 at the EM core facility of the University of Miami.

Histochemistry
Gomori trichome staining, succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) activities were determined using 6 μm thick muscle sections, as described by Sciacco and Bonilla (25).

Western blot and immunocytochemistry
For western blots, 30 μg of muscle mitochondrial protein was resolved on 15% SDS–PAGE and transferred to PVDF (polyvinylidene difluoride, Immobilon, Bio-Rad, Hercules, CA, USA) membrane. The membrane was blocked overnight in 5% milk and after washings in PBS–Tween, the blot was incubated with a polyclonal anti-PstI antibody (19) for 2 h at RT. The blot was washed and incubated with secondary antibody conjugated to HRP for 1 h at RT. Chemiluminescent substrate was used to detect the signal. For immunostaining, 6 μm thick muscle sections from transgenic mice were incubated overnight with a 1:100 dilution of a polyclonal anti-CoxIp (Molecular Probes, Eugene, OR, USA) antibody in a humid chamber at 4°C. Next day, the sections were washed with PBS–Tween and incubated with 1:200 dilution of secondary antibodies (anti-rabbit Alexa 488 and anti-mouse Alexa 594) for 2 h at RT in humid chamber. After 2 h, the sections were washed and mounted with Antifade mounting medium (Molecular Probes).

Southern blot analyses
To perform Southern blot analysis, total DNA (5 μg) was digested with NheI, electrophoresed on 0.7% agarose gel and transferred to a zeta-probe membrane (Biorad). To detect mtDNA, [α-32P] dCTP labeled probes were synthesized using random priming method. Two mtDNA probes were synthesized, one against the COXI region (nt positions 5227–5794) and the other against the ND4 region (nt positions 10 176–10 730) of mouse mtDNA (26). The COXI probe allowed the detection of both intact and partially deleted mtDNA molecules, whereas the ND4 probe allowed detection of intact mtDNA molecules only. The radioactive signal was quantitated using a Cyclone Phosphorimager (Perkin-Elmer, Boston, MA, USA). To determine the relative mtDNA abundance, the blots were stripped and hybridized with a nuclear 18S rDNA probe, obtained by PCR amplification of mouse DNA (primers spanning positions 1–574 according to GenBank accession no. X00686). The ratio of the mtDNA signal intensity to the respective 18S rDNA signal was obtained to estimate mtDNA abundance.

PCR analysis and sequencing
To search for mtDNA deletions, muscle mtDNA from control and transgenic Mito-PstI mice were PCR amplified using oligonucleotides corresponding to mtDNA positions (8300–8320:15 962–15 982). A PCR product of ~650 bp amplified from muscle samples of transgenic Mito-PstI mice (corresponding to deletion breakpoint regions) was gel purified and cloned in a plasmid ‘TOPOblunt’ (Invitrogen). After bacterial transformation of the plasmid, eight clones were picked and the PCR fragment was sequenced using T7 and M13 reverse primers. Additional PCR reactions were performed to search for mtDNA deletions. The primer pairs used were at the following mtDNA locations: (8300–8320:12 340–12 322), (8300–8320:13 125–13 101), (8300–8320:14 234–14 211), (8300–8320:15 198–15 176).

Single fiber analyses
COX-stained skeletal muscle sections (20 μm thick) were microdissected by laser capture microscope (LCM). Total DNA was isolated from the single fibers using alkaline lysis method as described elsewhere (27). For amplification of a non-deleted mtDNA region in the single fiber segment analyses, we used primers (5227–5250:5794–5771). Nested PCR was performed with primer pair (5310–5332:5690–5666). PCR for the deleted region was performed using the primer pair (8300–8320:15 962–15 982). Reactions were re-amplified with nested the primers 8321–8340 and 15 962–15 982. PCR products from the amplified fibers were cloned in a TOPO Blunt Vector (Invitrogen) followed by bacterial transformation. Five colonies from each transformation were subsequently analyzed by plasmid isolation and sequencing of the PCR product to determine the mtDNA deletion breakpoints.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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