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Down-regulation of mitochondrial transcription factor A during spermatogenesis in humans

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Mitochondrial transcription factor A (mtTFA) is a key activator of mitochondrial transcription in mammals. It also has a role in mitochondrial DNA (mtDNA) replication, since transcription generates an RNA primer necessary for initiation of mtDNA replication. In the mouse, testis-specific mtTFA transcripts encode a protein isoform that is imported to the nucleus rather than into mitochondria of spermatocytes and elongating spermatids. We now report molecular characterization of human mtTFA (h-mtTFA) expression in somatic tissues and male germ cells. Similarly to the mouse, analysis of cDNAs and Northern blots identified abundant testis-specific transcript isoforms generated by use of alternate transcription initiation sites. However, unlike the mouse, none of the testis-specific transcripts predicts a nuclear protein isoform, and Western blot analysis identified only the mitochondrial form of h-mtTFA in human testis. Immunohistochemistry and in situ hybridizations were used to compare the distribution of mtTFA protein, testis-specific mtTFA transcripts, mtDNA and mtRNA in sections of human testis. Our results show that mtTFA protein and mtDNA exhibit parallel gradients with high levels in undifferentiated male germ cells and low levels or an absence in differentiated male germ cells. Testis-specific transcripts exhibit the opposite pattern, suggesting that in both humans and mice, these testis-specific mtTFA transcripts down-regulate mtTFA protein levels in mammalian mitochondria. Our findings demonstrate that mtTFA does not have a critical role in the nucleus, suggest a mechanism for reducing mtDNA copy number during spermatogenesis and have implications for the understanding of maternal transmission of mtDNA.

INTRODUCTION

The oxidative phosphorylation capacity of human tissues is tightly regulated during development and in response to changing physiological demands (1). For example, neuronal tissue, skeletal muscle and spermatozoa each have specific respiratory requirements, some of which can be altered by environmental stimuli. Respiratory efficiency is determined by the interplay between nuclear and mitochondrial genes; mitochondrial DNA (mtDNA) encodes 13 proteins that are all components of the respiratory chain, whereas nuclear DNA encodes the majority of the respiratory chain proteins, all proteins that regulate replication and transcription of mtDNA, as well as proteins necessary for the biogenesis of mitochondria (2,3). Previous studies have found a correlation between oxidative phosphorylation capacity and mtDNA levels, indicating that mtDNA replication may be regulated in response to different energy demands (4).

A possible focal point for regulation of human mtDNA copy number is human mitochondrial transcription factor A (h-mtTFA), a high mobility group (HMG)-box protein that is a key activator of mitochondrial transcription (5). It is likely that h-mtTFA also has a role in mtDNA replication, since a transcript from a major mitochondrial promoter is used as an RNA primer for initiation of mtDNA replication (2). Recently we have reported that the mouse homolog of mtTFA (m-mtTFA) encodes two protein isoforms, one of which is imported to the nucleus rather than into mitochondria (6). The nuclear m-mtTFA isoform is absent from somatic cells, but in male germ cells the nuclear m-mtTFA isoform predominates and levels of the mitochondrial isoform are greatly reduced compared with somatic cells. It has been suggested previously that the nuclear isoform of m-mtTFA functions as a testis-specific transcriptional activator (7) or participates in nuclear chromatin condensation (8). However, an alternative possibility is that transcriptional interference between

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the promoters for the transcripts encoding the nuclear and mitochondrial m-mtTFA isoforms serves as a mechanism to down-regulate the mitochondrial m-mtTFA mRNA and protein levels in mouse testis, leading to a decrease in mtDNA copy number during spermatogenesis.

To investigate these possibilities, we have characterized the structure and regulation of h-mtTFA in human somatic tissues and male germ cells. Similarly to the mouse, alternate h-mtTFA transcript isoforms, generated by the use of alternate transcription initiation sites, are present in male germ cells and the levels of h-mtTFA protein are down-regulated during spermatogenesis. However, unlike the mouse, no nuclear mtTFA protein isoform is encoded by the testis-specific transcripts. These findings indicate that mtTFA does not play a critical role in the nucleus and, furthermore, suggest a mechanism for down-regulation of mtDNA during human spermatogenesis with implications for understanding uniparental transmission of mtDNA.

RESULTS

Cloning of testis h-mtTFA cDNAs

Previous studies of h-mtTFA have been based on cDNA clones isolated from a lymphocyte library, all of which encode a 246 amino acid residue protein with a cleaved mitochondrial import signal located at residues 1–42 (5). To investigate h-mtTFA gene structure and regulation in germ cells, we characterized cDNA clones from a human testis library. Thirteen independent clones were isolated; five of these had 5′ ends that were truncated, but eight clones were found to have 5′ sequences that differed from the widely expressed h-mtTFA transcript of ∼2 kb found in somatic cells (Fig. 1A–C). Sequence characteristics of these cDNAs call into question their protein-coding potential. Testis cDNAs 1–7 (1.1–1.5 kb) contain additional 5′ sequence (exon I') identical to the genomic region immediately upstream of exon I (9), which suggests that this class of cDNAs is produced by a testis-specific transcription initiation site located at least 280 bp upstream of the site used in somatic cells. The translation initiation site for h-mtTFA is located at nt 402–404 of exon I (Fig. 1B), but the additional 5′ sequence includes a potential upstream translational initiation codon (CGGA TG) (10) at nt 254–260 of exon I' (Fig. 1B), followed by a stop codon after 18 nucleotides. Testis cDNAs 1–2 use a polyadenylation signal, AATAA, at nt 834–839 [numbering according to previously published h-mtTFA sequence (5)] and therefore do not include the termination codon, TAA, at nt 871–873. Testis cDNAs 3–7 do include this stop codon, but have shorter 3′-untranslated regions (3′-UTRs) compared with the widely expressed transcript of ∼2 kb, due to the use of polyadenylation signals at nt 962–967 (GATAAA) and at 1407–1412 (TATAAA). Besides the seven testis cDNAs that begin with exon I', one cDNA (testis cDNA 8 of Fig. 1A and C) begins with an alternate first exon (exon I) that lies in the genomic region between exon I and II (9). Exon I does encode an open reading frame (ORF) of 21 amino acids spliced in-frame with exons II–VII, but this ORF does not contain a methionine codon and therefore is unlikely to be translated.

Northern blot analysis of h-mtTFA transcripts

To determine the extent to which cDNA cloning represented transcripts present in vivo, we performed Northern hybridization with isoform-specific probes. A probe containing exons I–VII detects four transcripts on Northern blots (Fig. 2): transcripts 1–3 were present in all tissues, whereas transcript 4 corresponds to a heterogeneous group of testis-specific transcripts. Analysis with strand-specific probes demonstrated that all transcripts are detected by antisense, but not by sense, RNA probes (Fig. 3).

The testis-specific transcripts are ∼1–1.5 kb in size, which corresponds with the size of the cloned testis cDNAs 1–8. Probes specific for exon I and exon I hybridize only with the testis-specific transcripts, whereas a probe containing the 3′-UTR of exon VII hybridizes weakly with the testis-specific transcripts (Fig. 2). The results from Northern blot analysis were thus consistent with the results from the cDNA cloning, and demonstrate that the testis-specific transcripts contain exon I and exon I and have truncated 3′ ends.

The transcripts 1–3 were found in every tissue we examined (Fig. 2). Transcript 1 is ∼5 kb in size and hybridizes with a probe containing part of the 3′-UTR of exon VII, but has not been characterized further. Transcript 2 is ∼2 kb in size, which corresponds to the size of the previously cloned h-mtTFA
Figure 2. Northern blot analysis of h-mtTFA transcripts in different human tissues. The different h-mtTFA transcript isoforms are indicated by numbers 1–4. The different DNA probes used are indicated by symbols to the right of the panels. The translational start and stop codons are indicated by short vertical bars. The black boxes indicate exons with testis-specific expression. The dashed box indicates the 5′-untranslated region of exon VII.

Figure 3. Northern blot analysis of liver and testis RNA with RNA probes corresponding to exons I–VII of h-mtTFA demonstrate that both the widely expressed and the testis-specific transcript isoforms of h-mtTFA are detected with the antisense probe (left panel) but not with the sense probe (middle panel). Northern blot analysis of RNA from cultured 143B human osteosarcoma cells and human sperm probed with a DNA probe corresponding to exons I–VII of h-mtTFA (right panel). Only the testis-specific h-mtTFA transcript isoforms are present in sperm.

Lymphocyte cDNAs (5). Transcript 3 is ∼0.5 kb shorter than transcript 2 and does not hybridize with a cDNA probe containing part of the 3′-UTR region of exon VII (Fig. 2). The cDNA sequence of h-mtTFA contains a perfect poly(A) consensus sequence (AATAAA) at nt 1516–1521, which is 0.4 kb upstream of the poly(A) consensus sequence (AATAAA) at nt 1916–1921 of transcript 2 (5). Transcript 3 is probably generated by use of this alternate poly(A) signal.

Down-regulation of h-mtTFA protein during spermatogenesis

We have shown previously that a widely expressed mitochondrial m-mtTFA isoform of 25 kDa is present in somatic mouse tissues, whereas a nuclear isoform of 26 kDa is present only in mouse testis (6). Western blot analysis of protein extracts from human liver and testis identified only a single isoform of 25 kDa (Fig. 4A), whereas the total levels of h-mtTFA transcripts were much higher in testis than liver (Fig. 2). The difference in immunodetectable levels of h-mtTFA between liver and testis is not due to large amounts of testis-specific proteins that co-migrate with h-mtTFA, and could conceivably interfere with protein transfer or detection, as shown by comparison of Coomassie blue-stained total protein between liver and testis (Fig. 4B). In addition, the difference in levels of h-mtTFA between liver and testis is not likely to be due to testis-specific post-translational modification that specifically affects immunodetection, since the testis and liver proteins appeared as co-migrating single bands under different fractionation conditions (Fig. 4A). Instead, the difference between h-mtTFA levels in liver and testis is likely to reflect a true down-regulation of h-mtTFA protein. This conclusion is also supported by measurements of h-mtTFA levels in muscle and different human cell lines, all of which have h-mtTFA transcript and protein levels similar to liver (data not shown).

Immunohistochemical staining of h-mtTFA gave further support for a down-regulation of h-mtTFA during spermatogenesis. Granular immunoreactive material corresponding to mitochondrial h-mtTFA was found in interstitial cells and in basal cells of the seminiferous tubules, but not in cells at later stages of spermatogenesis (Fig. 5B). No nuclear immunoreactive material was observed in spermatocytes, spermatids or any other cell type.
Figure 5. Sections of testis tissue. The letters indicate the lumen of seminiferous tubules (t), basal lamina of seminiferous tubules (b) and interstitial Leydig cells (i). The bars correspond to a distance of 40 µm. (A) Enzyme histochemical staining of succinate dehydrogenase demonstrating that cells of all layers of the seminiferous tubules and the interstitial Leydig cells have mitochondrial enzyme activity. The nuclei are unstained. (B) Immunohistochemical staining with a polyclonal rabbit antiserum to h-mtTFA. Immunoreactive material appears brown and nuclei are visualized with hematoxylin staining and appear blue. Basal cells of the seminiferous tubules and the interstitial Leydig cells contain immunoreactive material with a distribution corresponding to mitochondria. Cells close to the lumen of the seminiferous tubules show few mitochondria with immunoreactive material. There is no nuclear immunoreactive material in elongating spermatids or any other cell type. These results differ from the findings in mouse testis where high levels of m-mtTFA are found in the nuclei of elongating spermatids and spermatocytes. (C) In situ hybridization to detect mitochondrial transcripts shows an even distribution of mtRNAs in the different cell layers of the seminiferous tubules. (D) In situ hybridization to detect mtDNA shows predominance of mtDNA in basal cells of the seminiferous tubules.

in testis (Fig. 5B). Pre-immune serum gave no immunoreaction (data not shown). Enzyme histochemical staining of succinate dehydrogenase was performed to study the distribution of mitochondria in testis. Enzyme activity was present in interstitial cells as well as in cells of all layers in the seminiferous tubules, demonstrating that mitochondria are present throughout spermatogenesis (Fig. 5A).

By in situ hybridization, we demonstrated that mtDNA transcripts (mtRNAs) were abundant and evenly distributed in cells of all layers of the seminiferous tubules, whereas mtDNA was present mainly in basal cells of the seminiferous tubules (Fig. 5C and D). The peripheral location of mtDNA is not due simply to higher cell density, since there is an even distribution of nuclei throughout the different stages of spermatogenesis (Figs 5A and 6A). The results from in situ hybridizations were consistent with the Northern blot analysis demonstrating abundant mtRNAs in testis (Fig. 2). Both mtRNAs and mtDNA were present in interstitial Leydig cells (Fig. 5C and D).

We also used in situ hybridization to detect h-mtTFA transcripts in testis. Antisense RNA probes for exons I–VII (Fig. 6B), exon II′ (Fig. 6C) and exon I′ (Fig. 6D) hybridized strongly with cells close to the lumen of the seminiferous tubules, while the corresponding sense RNA probes gave no signal (Fig. 6E–G). These data demonstrate that the testis-specific h-mtTFA transcript isoforms are located in differentiated germ cells close to the lumen of the seminiferous tubules, which is in good agreement with the result from Northern blot analysis of sperm RNA demonstrating that only the testis-specific h-mtTFA transcript isoforms are found in fully differentiated male germ cells (Fig. 3).
DISCUSSION

We have demonstrated that h-mtTFA protein is down-regulated during human spermatogenesis, most likely due to the production of alternate testis-specific h-mtTFA transcript isoforms. Our findings also demonstrate that mtTFA does not play a critical role in the nucleus, and have implications for understanding transmission of mtDNA in human genetics.

Different mechanisms could account for the decreased levels of h-mtTFA protein in testis compared with somatic tissues. One possibility, for which there is no direct evidence at present, is that transcriptional interference between the promoters for the testis-specific transcript isoforms, beginning with exon I⁺ or exon II⁺, and the somatic transcript isoforms, beginning with exon I, may lead to decreased levels of the somatic transcript isoform and low levels of h-mtTFA protein in germ cell mitochondria.

Unlike the mouse, several findings suggest that the human testis-specific transcript isoforms containing exon I⁺ or exon II⁺ do not encode a functional protein. There is an inverse correlation between h-mtTFA transcript and protein levels in testis compared with liver as well as in undifferentiated germ cells compared with differentiated germ cells. The testis-specific transcripts have long 5′-flanking regions, which may prevent translation of the ORF encoding h-mtTFA. Translational regulation plays a prominent role in regulating gene expression during spermatogenesis (11) and long 5′-flanking regions have been shown to prevent translation of certain testis-specific transcript isoforms (11,12).

Surprisingly, several lines of evidence point to the lack of a nuclear isoform of h-mtTFA in human testis: (i) Sequence comparison demonstrated that mouse exon I⁺, which encodes the amino terminus of the nuclear isoform of m-mtTFA (6), is not conserved in the h-mtTFA genomic sequence (9). (ii) None of the eight cloned testis-specific cDNAs encodes a h-mtTFA isoform similar to the nuclear m-mtTFA isoform. (iii) Western blot analysis of protein extracts from human testis did not identify any additional h-mtTFA isoforms. (iv) Immunohistochemical staining with a polyclonal antiserum against h-mtTFA demonstrated only cytoplasmic immunoreactivity on tissue sections from testis. The possibility still exists that a nuclear isoform of h-mtTFA may be expressed during a specific developmental stage or certain physiological situations, although we think this is unlikely. A cDNA clone for Xenopus laevis mtTFA has been isolated recently (13), demonstrating that the principal mitochondrial form of mtTFA is conserved among vertebrates. Future studies are necessary to determine if the nuclear testis-specific mtTFA isoform is present in other vertebrates besides mouse.

It is likely that h-mtTFA is involved in regulating mtDNA replication, since biochemical studies have demonstrated that replication is linked to transcription of mtDNA and that h-mtTFA is essential for initiation of mitochondrial transcription in vitro (14,15). Support for the involvement of h-mtTFA in regulation of mtDNA copy number also comes from studies of patients with mitochondrial disorders (3). There is a correlation between the
levels of h-mtTFA and mtDNA in muscle (16,17); low levels of h-mtTFA are present in muscle fibers with mtDNA depletion and high levels are found in ragged-red muscle fibers with accumulation of mtDNA (16). Patients with infantile mitochondrial myopathy have a depletion of mtDNA and abundant mitochondria in muscle; the finding of low levels of h-mtTFA in muscle from these patients demonstrates that the levels of h-mtTFA correlate with the levels of mtDNA, but not with the abundance of mitochondria (16).

This study provides additional support for the hypothesis that h-mtTFA may be involved in regulating mtDNA copy number, since the down-regulation of h-mtTFA correlates with a reduction of mtDNA copy number during human spermatogenesis. We recently have demonstrated that the testis-specific transcript isoforms in the mouse are unlikely to encode the mitochondrial form of mtTFA and there are low levels of m-mtTFA in mitochondria isolated from mouse testis (6). Others have reported that there is a reduction of mtDNA copy number from $10^3$ to $10^2$ copies per cell during spermatogenesis in the mouse (18). These findings demonstrate that the down-regulation of the mitochondrial form of mtTFA and mtDNA is conserved in the mouse.

Interestingly, mitochondria are abundant and high levels of mtRNAs are present throughout human spermatogenesis. It has been demonstrated that mitochondria from rat male germ cells have a high oxidative phosphorylation capacity (19), perhaps because spermatogenesis requires high ATP production. Because mtDNA copy number is down-regulated, high levels of mtRNAs in human spermatogenic cells are probably caused by stability of existing mitochondrial transcripts, and may ensure that the oxidative phosphorylation capacity is maintained while mtDNA copy number is reduced.

In summary, our findings demonstrate that the nuclear form of mtTFA does not play a critical role during spermatogenesis in mammals. The principal function of the testis-specific transcript isoforms may be to down-regulate the levels of mtTFA in mitochondria during mammalian spermatogenesis and subsequently mtDNA copy number in sperm. The paternal inheritance of mtDNA is highly conserved (20), and reduction of mtDNA copy number during spermatogenesis together with other mechanisms (21) may prevent paternal transmission of mtDNA. It should be noted that our results do not exclude the possibility that down-regulation of mtTFA during spermatogenesis may be secondary to the down-regulation of mtDNA. However, this seems less likely since the production of alternate transcript isoforms provides a specific mechanism for down-regulating mtTFA during spermatogenesis in mouse and man. Definitive proof for the involvement of mtTFA in regulation of mtDNA copy number during spermatogenesis should be obtained by genetic experiments in the mouse, e.g. by tissue-specific knock-out of the m-mtTFA gene or overexpression of m-mtTFA during mouse spermatogenesis.

**MATERIALS AND METHODS**

**Isolation of testis h-mtTFA cDNA clones**

A human testis cDNA library in the ZAP Express vector (Stratagene) was screened with a h-mtTFA cDNA (5) probe by using standard protocols (22). Isolated cDNA clones were analyzed by cyclic sequencing with fluorescent labeled sequencing primers and the ABI 373A DNA Sequencer (Applied Biosystems).

**Northern blot analysis**

Northern blots containing poly(A)+ RNA from multiple human tissues were obtained from Clontech. Total RNA from human liver, testis, sperm and cell lines were electrophoresed in agarose gels containing formaldehyde and blotted to supported nitrocellulose as described (22).

**Western blot analysis**

Total protein extracts from human liver and testis were separated in 15 or 18% SDS–polyacrylamide gels and analyzed by Western blotting using a rabbit polyclonal antiserum to h-mtTFA as described (16).

**Enzyme and immunohistochemical staining**

Enzyme histochemical staining of succinate dehydrogenase activity and immunohistochemical staining of h-mtTFA were performed on cryostat sections of fresh frozen tissue specimens as previously described (16).

**In situ hybridization to detect mtDNA and mtRNA**

Paraffin-embedded formalin-fixed tissue specimens were used for in situ hybridization to detect mtDNA and mtRNA as previously described (23). A mixture of double-stranded human mtDNA fragments (24) labeled with $^{35}$S was used as probe.

**In situ hybridization to detect h-mtTFA transcripts**

Three-µm sections of formalin-fixed and paraffin-embedded tissue were mounted on Superfrost/Plus slides, deparaffinized and incubated in 25 mM HCl for 10 min. After rinsing in phosphate-buffered saline (PBS), the sections were incubated in 0.3% Triton X-100 in PBS for 15 min, rinsed in PBS and then treated with pronase E (Sigma), 0.1 mg/ml in 0.05 M Tris–HCl, 50 mM EDTA, pH 7.5 for 5 min. The sections were rinsed in PBS and incubated for 5 min in 0.1 M glycine in PBS and post-fixed in 4% paraformaldehyde in PBS for 5 min. After rinsing in PBS, the sections were acetylated by treatment with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min. After rinsing in water, the sections were dried in air and pre-hybridization was performed in 5× SSPE, 50% formamide, 1% PVP, 10% dextran sulfate, 5× Denhardt's solution, 0.5% SDS, denatured salmon sperm DNA (0.1 mg/ml), 0.1 M dithiothreitol (DTT) for 2 h at 42°C. Hybridization was performed overnight at 55°C in the same solution with the addition of $^{35}$S-labeled (10$^7$ c.p.m./ml) RNA probes. After hybridization, the sections were washed in 4× SSC with 10 mM DTT at 55°C for 1 h and then at 50°C for 30 min. The sections were then washed in 2× SSC with 50% formamide and 10 mM DTT for 20 min at 65°C. After rinsing in buffer (10 mM Tris–HCl, 1 mM EDTA, 0.5 M NaCl, pH 8.0) the sections were treated with RNase A (20 µg/ml) in the same buffer for 30 min at 37°C. After rinsing in RNase buffer and then in 0.1× SSC, the sections were dehydrated and dried in air.
 Autoradiography was performed with LM-1 emulsion (Amersham) and D19 developer (Kodak).

**RNA probes for in situ hybridization**

*In vitro* transcription with the plasmids pN33, pN59 and pN60 and [α-35S]UTP was performed to obtain antisense and sense probes corresponding to exons I–VII, exon II*F* and exon I*F* of h-mtTFA, respectively. The plasmid pN33 (a 1911 bp h-mtTFA cDNA clone in the pBK-CMV phagemid obtained from the testis cDNA cloning) was used to generate RNA probes corresponding to exons I–VII of h-mtTFA. Transcripts from pN33 were subjected to partial alkaline hydrolysis to obtain RNA fragments with an estimated average length of 0.3 kb prior to hybridization. The plasmid pN59 was constructed by cloning a 110 bp EcoRI fragment of exon II*F* (nucleotides 1–110 of Fig. 1C) into the corresponding site of the Bluescript KS II+ vector. The plasmid pN60 was constructed by isolating a 270 bp FokI (treated with Klenow polymerase to obtain a blunt end)–EcoRI fragment of exon I*F* (nucleotides 1–270 of Fig. 1B), which was cloned into the EcoRI and Smal sites of the Bluescript KS II+ vector.

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