Mitochondrial transcription factor A regulates mtDNA copy number in mammals

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Mitochondrial DNA (mtDNA) copy number regulation is altered in several human mtDNA-mutation diseases and it is also important in a variety of normal physiological processes. Mitochondrial transcription factor A (TFAM) is essential for human mtDNA transcription and we demonstrate here that it is also a key regulator of mtDNA copy number. We initially performed \textit{in vitro} transcription studies and determined that the human TFAM protein is a poor activator of mouse mtDNA transcription, despite its high capacity for unspecific DNA binding. Next, we generated P1 artificial chromosome (PAC) transgenic mice ubiquitously expressing human TFAM. The introduced human \textit{TFAM} gene was regulated in a similar fashion as the endogenous mouse \textit{Tfam} gene and expression of the human TFAM protein in the mouse did not result in down-regulation of the endogenous expression. The PAC-TFAM mice thus had a net overexpression of TFAM protein and this resulted in a general increase of mtDNA copy number. We used a combination of mice with TFAM overexpression and TFAM knockout and demonstrated that mtDNA copy number is directly proportional to the total TFAM protein levels also in mouse embryos. Interestingly, the expression of human TFAM in the mouse results in up-regulation of mtDNA copy number without increasing respiratory chain capacity or mitochondrial mass. It is thus possible to experimentally dissociate mtDNA copy number regulation from mtDNA expression and mitochondrial biogenesis in mammals \textit{in vivo}. In conclusion, our results provide genetic evidence for a novel role for TFAM in direct regulation of mtDNA copy number in mammals.

INTRODUCTION

The regulation of mammalian mitochondrial DNA (mtDNA) expression and copy number is poorly understood, despite the profound importance of these processes in human physiology and disease. We have recently made progress in this area by reconstituting the basal human mtDNA transcription machinery in a pure \textit{in vitro} system (1). A combination of three different nucleus-encoded proteins, i.e. mitochondrial RNA polymerase (POLRMT) (2), mitochondrial transcription factor A (TFAM) (3) and mitochondrial transcription factor B1 or B2 (TFB1M or TFB2M) (1), is sufficient to obtain specific initiation of transcription from linear DNA templates containing the heavy and light strand promoters (HSP and LSP) of human mtDNA. TFAM fulfills the definition of a \textit{bona fide} transcription factor because it displays sequence-specific binding to HSP and LSP (4) and it activates mtDNA transcription in a concentration-dependent manner \textit{in vitro} (1,4). In addition, import of recombinant TFAM into isolated rat liver mitochondria stimulates \textit{in organello} transcription of mtDNA (5).

Mammalian TFAM proteins have a molecular weight of \textasciitilde25 kDa and consist of an amino-terminal HMG domain, a basic linker region, a second HMG domain and a basic carboxy-terminal tail (3,6). The TFAM protein is synthesized as a precursor form with a mitochondrial targeting peptide, which is removed by processing after import to mitochondria (3). Biochemical characterization of human TFAM has demonstrated that it can efficiently bind, unwind and bend DNA without sequence specificity, similar to other proteins of the HMG domain family (7). The yield of TFAM from biochemical purification procedures was used to estimate that there is a minimum of \textasciitilde1 TFAM molecule per 1000 bp of mtDNA in

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human tissue culture cells (8). This estimate has been questioned by others who have reported a molar ratio of ~1 TFAM molecule per 10 bp of mtDNA (9,10). This very high molar ratio suggests that TFAM may fully coat mtDNA in mammalian cells (9,10), similar to the situation in budding yeast (11,12) and chicken cell lines (13). Biochemical fractionation and immunocytochemistry experiments have provided support for the idea that human TFAM is tightly associated with mtDNA and is involved in packaging mtDNA into protein–DNA aggregates referred to as nucleoids (10,14).

We have previously disrupted the Tfam gene in the mouse germ line and demonstrated that heterozygous knockouts (Tfam+/−) have ~35–40% reduction in mtDNA copy number, whereas homozygous knockouts (Tfam−/−) die in midgestation due to lack of mtDNA and severe respiratory chain deficiency (15). We have also exploited the conditional knockout system (cre–loxP) to disrupt Tfam in a variety of cell types in the mouse, e.g. cardiomyocytes (16,17), skeletal muscle cells (18), pancreatic β-cells (19) and pyramidal neurons (20). In all of these cases, tissue-specific depletion of TFAM protein leads to depletion of mtDNA, depletion of mitochondrial transcripts (mtRNA) and severe respiratory chain deficiency (16–20). These mouse knockout studies have thus led to the conclusion that TFAM is necessary for mtDNA maintenance in vivo. However, it has been unclear if TFAM regulates mtDNA copy number, because it can be anticipated that loss of any critical component of the mtDNA maintenance machinery will result in loss of mtDNA. We have now addressed this issue by creating transgenic mice containing large fragments of genomic DNA harboring the complete human TFAM gene. We chose to express the human TFAM protein in the mouse because it poorly activates transcription from mouse HSP and LSP in vitro despite its strong DNA binding activity. We have utilized a combination of mice with overexpression of TFAM and knockout mice with reduced TFAM expression to demonstrate that mtDNA copy number is directly proportional to the total TFAM protein levels in a variety of differentiated mouse tissues and in embryos. Our results also show that it is possible to dissociate mtDNA copy number control from mtDNA expression as the transgenic mice have increased mtDNA copy number without any increase in mitochondrial mass or respiratory chain capacity. These findings demonstrate that the TFAM protein is a key factor involved in directly regulating mtDNA copy number in mammals.

RESULTS

The human TFAM protein is a poor activator of mouse mtDNA transcription in vitro despite its highly non-specific DNA binding activity

We developed a pure in vitro transcription system for mouse mtDNA by expressing histidine-tagged versions of mouse POLRMT, TFB2M and TFAM in the baculovirus system. The proteins were purified to homogeneity and were >95% pure as determined by Coomassie staining of SDS–polyacrylamide gels. Similar to the human system, a combination of mouse POLRMT, TFB2M and TFAM was required for specific initiation of transcription from linear mouse mtDNA templates containing LSP and HSP (Fig. 1A). However, we obtained much lower transcription activation in the mouse in vitro transcription system when we used equal molar amounts of human TFAM instead of mouse TFAM (Fig. 1A). We also found that the addition of increasing amounts of human TFAM in the presence of mouse TFAM only had a mild stimulatory effect on transcription from LSP (Fig. 1B) and HSP (Fig. 1C) in vitro. Next, we mixed recombinant human and mouse TFAM with plasmid DNA and found that both proteins had a similar high DNA binding capacity (Fig. 1D). We also performed footprint analyses and found that the human TFAM protein, in
In contrast to the mouse TFAM protein, did not specifically bind mouse LSP (data not shown). In summary, our results show that human TFAM is a poor activator of mouse mtDNA transcription in vitro, despite its high capacity for non-specific interaction with mtDNA. These features make it attractive to express human TFAM in the mouse to determine whether its non-specific DNA binding activity is involved in mtDNA maintenance in vivo.

Generation of transgenic mice containing large genomic fragments harboring the human TFAM gene

We used the human TFAM cDNA to screen a human genomic P1 artificial chromosome (PAC) library and identified 33 positive clones. We used PCR analysis and found that 14 of these clones contained both the first and last exons of the TFAM gene. The size of the insert in these clones was determined by pulsed-field gel electrophoresis. We used vectorette (bubble) PCR to clone the end-fragments from five clones and used these fragments as probes for Southern blot analyses of DNA from the 14 PAC clones. These results allowed us to construct a contig of overlapping PAC clones each containing the human TFAM gene. We selected three clones with different flanking sequences (PAC2, PAC9 and PAC19; Fig. 2A) to make transgenic mice by the pronuclear injection technique. Transgenic founders from all three PAC clones were identified by Southern blot analysis of tail DNA. A probe for the human TFAM gene demonstrated that the transgenic mice contained the same genomic fragments as those found in human DNA (Fig. 2B). We determined that the PAC2 transgenic line had the lowest human TFAM gene copy number by comparing the hybridization signal from the human TFAM cDNA probe with a loading control consisting of a mouse 18S rRNA gene probe (Fig. 2B).

PAC transgenic mice have ubiquitous net overexpression of TFAM

We performed western blot analyses of total tissue protein extracts from wild-type and PAC transgenic mice by using polyclonal antibodies against mouse and human TFAM. The human TFAM protein was ubiquitously expressed in the PAC transgenic mice (Fig. 3A). We quantified the levels of human and mouse TFAM protein in relation to total protein content in heart, kidney and skeletal muscle of PAC2, PAC9, PAC19 and wild-type mice (Fig. 3B). Both the human and mouse TFAM proteins had rather similar patterns of expression with high levels in heart and kidney and low levels in skeletal muscle (Fig. 3B). This suggest that the regulatory sequences of the human TFAM gene are functional in the mouse and generate an expression pattern similar to the pattern observed for the endogenous mouse Tfam gene. We also isolated mitochondria from heart, kidney and skeletal muscle from PAC19 mice and found that the levels of human and mouse TFAM protein were ~2–4 ng per μg of total mitochondrial protein (Fig. 3C and data not shown), which corresponds to ~50–100% increase of total TFAM protein levels. We found that the levels of the mouse TFAM protein levels were very similar in mitochondria from wild-type and PAC19 mice, which demonstrates that the expression of the human TFAM protein does not affect the expression of the endogenous mouse TFAM gene (Fig. 3C).

We performed additional experiments to calculate the ratio of TFAM protein to mtDNA in isolated kidney mitochondria by using western and Southern blot analyses together with recombinant TFAM protein and plasmid DNA standards (data not shown). We found a molar ratio of total TFAM protein to mtDNA of ~977±454 (mean±standard deviation of the mean) in wild-type mice and 938±272 in PAC19 mice. Our data thus show both wild-type and transgenic mice have a fairly constant ratio of one TFAM molecule per ~15–20 bp of mtDNA, which is in good agreement with previous estimates (9,10).

Mitochondrial DNA copy number is increased in TFAM transgenic animals

We performed Southern blot analyses to assess mtDNA levels in total DNA extracts from heart, kidney and skeletal muscle from wild-type, PAC2, PAC9 and PAC19 animals (Fig. 4A) and found a clear increase of mtDNA levels in all investigated tissues from the transgenic animals (Fig. 4B). The overall increase in mtDNA was ~30–40% in PAC2 and ~40–70% in PAC9 and PAC19 animals (Fig. 4B). The mtDNA copy number
could thus be correlated with the copy number of the introduced *TFAM* gene (Fig. 2B), but not with the presence of specific flanking DNA sequences (Fig. 2A). We searched the Celera database and found no known mitochondrial genes in the 200 kb 3' and 5' flanking regions of human *TFAM*. These findings demonstrate that the introduced human *TFAM* gene is responsible for up-regulating mtDNA copy number in the transgenic mice. We also quantified the proportion of displacement loop (D-loop) strands, referred to as 7S DNA, in relation to total mtDNA and found no difference between wild-type and PAC19 mice (Fig. 4C and D). The increased amount of mtDNA in PAC19 mice thus results in an up-regulation of the absolute amount of 7S DNA so that a constant ratio between 7S DNA and mtDNA is maintained.

**Unaltered respiratory chain function and mitochondrial mass despite up-regulation of mtDNA copy number in TFAM transgenic mice**

We isolated total RNA from kidney from wild-type, PAC2, PAC9 and PAC19 mice and performed northern blot analysis of steady-state mitochondrial transcript levels (Fig. 5A and data not shown). The NADH dehydrogenase subunit 6 (ND6)
transcript encoded on the light (L) strand was increased by ~50–60% in PAC9 and PAC19 animals and remained unchanged in PAC2 animals (Fig. 5B). In contrast, the levels of both cytochrome c oxidase subunit I (COXI) and NADH dehydrogenase subunit 4 (ND4) transcripts encoded on the heavy (H) strand were not changed (Fig. 5B and data not shown). In agreement with this observation we found similar levels of the mtDNA-encoded cytochrome c oxidase subunit II (COXII) protein in kidney mitochondria (Fig. 3C) as well as in heart, kidney and skeletal muscle tissue homogenates (data not shown) from wild-type and PAC19 mice. We assessed respiratory chain function in wild-type, PAC9 and PAC19 mice by measuring the activity of complex IV (cytochrome c oxidase), where all subunits are encoded by mtDNA, and complex II (succinate dehydrogenase), where all subunits are nucleus encoded. Neither of these two complexes demonstrated any significant changes in activity in transgenic mice in comparison with wild-type mice (Fig. 6B). We performed volume density measurements of mitochondria on electron micrographs of heart tissue and found no significant difference between wild-type and PAC19 animals (Fig. 6C). The mitochondrial morphology also appeared normal (Fig. 6C). It can thus be concluded that expression of human TFAM in the mouse does not increase overall mtDNA expression, respiratory chain function or mitochondrial mass.

The human TFAM gene does not rescue Tfam knockout mouse embryos

We performed mouse crosses to investigate whether the human TFAM gene could rescue the embryonic lethality encountered in homozygous Tfam knockout embryos. We crossed heterozygous Tfam knockout mice (Tfam<sup>+/−</sup>) with mice that were heterozygous for both the Tfam knockout and the PAC–TFAM transgene (Tfam<sup>−/−</sup>, PAC–TFAM). This cross should theoretically result in 12.5% animals that are homozygous for the Tfam knockout and heterozygous for the PAC–TFAM transgene (Tfam<sup>−/−</sup>, PAC–TFAM). However, no pups with the Tfam<sup>−/−</sup>, PAC–TFAM genotype were born when we performed this cross with PAC2 (n = 38 pups born and genotyped), PAC9 (n = 68) or PAC19 mice (n = 78), whereas all of the other expected genotypes (except the Tfam<sup>−/−</sup> genotype known to be embryonic lethal) were recovered at expected Mendelian frequencies (data not shown). In contrast, we recovered Tfam<sup>−/−</sup>, PAC–TFAM embryos at the expected frequency when we collected E8.5–E9.5 embryos from the same mating. These embryos had a gross morphological appearance that was very similar to the previously described mutant phenotype of E8.5 Tfam<sup>−/−</sup> embryos. We performed Southern blot analysis of individual E8.5–E9.5 embryos of different genotypes (Fig. 7A) and found a clear correlation between mtDNA copy number and Tfam/TFAM gene dosage. Surprisingly, the Tfam<sup>−/−</sup>, PAC–TFAM embryos contained ~30% of wild-type mtDNA levels (Fig. 7A and B), but were nevertheless not able to survive embryonic development. We analyzed mitochondrial transcripts and found low levels of the ND6 transcript and very low levels of the COXI transcript in Tfam<sup>−/−</sup>, PAC–TFAM embryos (Fig. 7C). We also performed western blots to confirm that the human and mouse TFAM proteins were expressed as expected in embryos of different genotypes (Fig. 7D). In summary, Tfam<sup>−/−</sup>, PAC–TFAM E8.5–E9.5 embryos contain mtDNA but it is poorly transcribed and can therefore not rescue the embryonic lethality.

**DISCUSSION**

TFAM regulates mtDNA levels in mammals

We have demonstrated that mtDNA copy number regulation in vivo is directly dependent on the TFAM protein levels in a variety of differentiated mouse tissues and in mouse embryos. The transcriptional coactivator PGC1 acts upstream of TFAM and has the capacity to increase cellular mtDNA levels as well as mitochondrial mass in tissue-culture cells and in transgenic mice (21–23). The expression of human TFAM is also dependent on the nuclear respiratory factors 1 and 2 (NRF1 and...
and NRF2) (24). PGC1 has the dual action to stimulate induction of NRF1 and NRF2 expression and to coactivate the transcriptional function of NRF1 (22). It was previously thought that the induction of TFAM expression mainly would stimulate mtDNA transcription (24). However, our data show that the TFAM protein levels directly regulate mtDNA copy number

We utilized the finding that the human TFAM protein poorly activates transcription from mouse LSP and HSP in vitro despite its high non-specific DNA binding capacity, and found that expression of human TFAM in the mouse increases mtDNA copy number without altering respiratory chain capacity or mitochondrial mass. It is thus possible to experimentally dissociate mtDNA copy number regulation from regulation of oxidative phosphorylation capacity.

No feedback regulation of TFAM expression by mtDNA

The human and mouse TFAM protein was expressed at similar relative levels in different mouse tissues, indicating that important regulatory regions are conserved between the human TFAM gene and the mouse Tfam gene. The transgenic mice expressing human TFAM protein had ~40–70% increase of mtDNA copy number, but this did not result in down-regulation of the expression of the endogenous mouse TFAM protein. It can thus be concluded that the mtDNA copy number per se does not affect Tfam gene expression. Previous studies have shown that human cells lacking mtDNA (ρ0 cells) express TFAM mRNA but lack TFAM protein (25). It is thus likely that the interaction between TFAM and mtDNA is dynamic and that the presence of one component increases the stability of the other component. This dynamic interaction is probably beneficial from a regulatory point of view because small changes in TFAM protein levels or mtDNA levels result in rapid adjustment to maintain a constant optimal ratio between TFAM and mtDNA.

TFAM may provide a scaffold for nucleoid formation

TFAM is abundant in mammalian mitochondria and our measurements demonstrate a molar ratio of TFAM to mtDNA of ~900–1000:1, which is in good agreement with other estimates (9,10). It is thus likely that mammalian mtDNA, similar to yeast (11,12) and vertebrate (26) mtDNA, is fully covered with TFAM protein. The most straightforward interpretation of our findings is therefore that TFAM increases mtDNA copy number by directly binding and stabilizing
mtDNA. There are several reports presenting biochemical evidence that TFAM together with other proteins, e.g. mitochondrial single-strand DNA binding protein, the adenine nucleotide translocator 1, the lipoyl-containing E2 subunits of pyruvate dehydrogenase, branched chain α-ketoacid dehydrogenase and prohibitin 2, directly interacts with mtDNA to form nucleoids (9,10,14,27). Here we have shown that increased expression of the TFAM protein is sufficient to increase steady-state levels of mtDNA, which suggests that the TFAM protein is the limiting factor of the mtDNA-stabilizing protein scaffold constituting the nucleoid.

TFAM may also have a direct role in activating mtDNA replication

It is also possible that TFAM, in addition to the DNA packaging function, has a direct role in replicating mtDNA according to the strand-asymmetric model of mtDNA replication (4). This model is supported by biochemical evidence showing that an RNA primer synthesized by transcription from LSP is required for initiation of mtDNA replication at O_H (4). A role for TFAM in mtDNA replication is further supported by the observation that import of TFAM into isolated mitochondria stimulates the synthesis of nascent DNA strands initiated at O_H (28). However, this strand-asymmetric model has been questioned because two-dimensional agarose gel electrophoresis analyses indicate that a strand-coupled mode of mtDNA replication dominate in mammalian mitochondria (29,30). Recent data have even questioned the role of O_H as a distinct initiation site for mtDNA replication (31). The different replication models are not easy to reconcile and there is currently an intense ongoing debate about mechanisms for mammalian mtDNA replication (32–34). We found a correlation between increased levels of mtDNA and increased levels of the ND6 transcripts encoded on the L strand in the transgenic animals. In addition, the absolute levels of nascent H strands (D-loops) were increased in PAC transgenic mice so that a constant ratio of nascent H strands to mtDNA was maintained even though mtDNA copy number was increased. We will further address the important question of mtDNA replication mechanisms by reconstituting the mtDNA replication machinery in vitro to determine whether the replication can be coupled to transcription initiation. Characterization of the involved enzymes will also clarify if they can perform strand-coupled DNA replication. In addition, we will disrupt mouse genes encoding different components of the basal mtDNA transcription machinery to determine whether mtDNA can be maintained in the absence of mitochondrial transcription from LSP.

MATERIALS AND METHODS

Cloning, expression and purification of recombinant proteins

PCR amplification of cDNA templates was used to generate DNA fragments encoding mouse POLRMT, TFAM and TFB2M and these fragments were cloned into the vector pBacPAK9 (Clontech). In addition, pBacPAK9 plasmids encoding POLRMT with a 10xHis-tag at the N-terminus, TFAM with a 6xHis-tag at the C-terminus and TFB2M with a
6xHis-tag at the C-terminus were generated. The constructs for expressing human TFAM were as previously described (1). The plasmid constructs were used to generate Autographa californica nuclear polyhedrosis recombinant viruses as described in the BacPAK manual (Clontech). The recombinant proteins were expressed in Spodoptera frugiperda (SF9) cells and whole cell protein extracts were generated as described (1). Mouse POLRMT, TFB2M and TFAM proteins were purified following the previously published protocols for the corresponding human proteins (1).

**In vitro transcription**

DNA fragments containing LSP and HSP, corresponding to nucleotides 15942–16260 and 16206–16341 of the mouse mtDNA sequence (35) were cloned into the pCR4 vector with the TOPO cloning kit (Invitrogen). The pCR4–LSP and pCR4–HSP plasmids were linearized with the PstI restriction endonuclease and used as templates for run-off transcription assays under the same conditions as previously described (1). Individual reaction mixtures (25 µl) contained 85 fmol of linearized DNA template, 10 mM Tris–HCl (pH 8.0), 10 mM MgCl2, 1 mM DTT, 100 µg/ml bovine serum albumin, 400 µM ATP, 150 µM CTP and GTP, 10 µM UTP, 0.2 µM [α-32P]UTP (3000 Ci/mmol), 4 U of RNasin (APBiotech), 300 fmol POLRMT protein and 300 fmol TFB2M protein and varying concentrations (0.025–25 pmol) of human and mouse TFAM proteins.

**Gel retardation**

A DNA fragment of 584 bp was isolated by SspI–HindIII restriction enzyme digestion of the pUC18 plasmid and end-labeled at the HindIII site using [α-32P]dCTP (3000 Ci/mmol). The radioactively labeled DNA fragment (10 fmol) was incubated with increasing concentrations (0.1–25 pmol) of mouse or human TFAM proteins. The individual reaction mixtures (15 µl) contained 10 mM Tris–HCl (pH 8.0), 10 mM MgCl2, 1 mM DTT, 100 µg/ml bovine serum albumin, 400 µM ATP and 10% glycerol. Reactions were incubated for 15 min at room temperature and subsequently analyzed by electrophoresis in 1% agarose gel with 1 x TBE at 100 V for 90 min. The dried gels were used for autoradiography of films in cassettes with an intensifying screen.

**Generation, genotyping and breeding of PAC transgenic mice**

A human genomic PAC-library (RPCI) was screened with a human TFAM cDNA probe. A total of 33 positive clones were identified and 14 of these clones contained both the first and last exons of the human TFAM gene as determined by PCR analysis. PAC DNA from these 14 clones was prepared with CsCl2 density gradient centrifugation. Vectorette PCR was used to clone the end-fragments of five of the 14 PAC clones thus generating 10 different end-fragments. The end fragments were radioactively labeled with [α-32P]dCTP with the random-labeling method and used to probe Southern blots containing EcoRI-digested PAC DNA from the 14 clones. Insert sizes of the clones were determined with pulsed-field gel electrophoresis. These data were used to create a physical map of the extension of the 14 overlapping clones. Three clones with different flanking sequences (RPCI 1-107B19, RPCI 1-51L14 and RPCI 4-766D17), designated PAC2, PAC9 and PAC19, were chosen for pro-nuclear injection of FVB/N embryos. Founder mice were identified with Southern blot analysis of EcoRI-digested tail DNA probed with a [α-32P]dCTP-labeled human TFAM cDNA probe. Germ line transmission was established from the founder mice and the offspring was kept as heterozygous stocks by breeding to wild-type FVB/N mice. A PCR assay was developed for rapid genotyping of transgenic mice by utilizing oligonucleotide primers (TFAM7A, CAAATTGTAGAAGCCACTGGGTTGTTGT and TFAM7B, GGGCATATGTGGGCTGTCTCTTGA) specifically amplifying exon 7 of human TFAM.

**Southern blot analyses**

DNA was prepared by SDS and proteinase K treatment as previously described (16). The DNA was purified by phenol:chloroform extraction, precipitated with ethanol and dissolved in TE pH 8.0. Total DNA (2 µg) from heart, kidney and skeletal muscle of adult mice (>10 weeks of age) and from whole E8.5–E9.5 embryos was digested overnight with the PstI restriction enzyme, precipitated with 0.2 M NaCl and ethanol and separated on 0.8% agarose gels. Kidney mitochondrial DNA was heated to 95°C for 10 min to dissociate the 7S DNA and separated on a 1% agarose gel. Separated DNA was transferred to nitrocellulose membranes by Southern blotting. The membranes were probed with [α-32P]dCTP random-labeled COXI or 7S DNA probes. Phosphorimager analyses were used to assess total mtDNA levels normalized to the nuclear 18S rRNA gene and 7S DNA levels normalized to total mtDNA.

**Biochemical measurement of respiratory chain enzyme activities**

The activities of the respiratory chain complexes as well as citrate synthase were measured in frozen samples of heart, kidney and skeletal muscle from transgenic and control adult mice as described previously (36).

**Isolation of mitochondria**

Kidneys were collected from adult mice and crude mitochondrial preparations were performed as described (37). Briefly, two kidneys were homogenized in 6 ml buffer (0.32 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4) using a teflon pestle homogenizer. Debris was pelleted for 5 min at 1000 g and supernatants were collected and centrifuged for 2 min at 13 000 rpm in Eppendorf tubes. Mitochondrial pellets were washed three times in fresh homogenization buffer and resuspended in 20% sucrose–TE buffer. Samples were loaded on a 1.5/1.0 M sucrose gradient and centrifuged for 20 min at 22 000 rpm using a SW28 rotor.

**Isolation of proteins and western blot analyses**

Tissue homogenates, mitochondrial preparations and E8.5 embryos were suspended in protein buffer [equal parts of suspension buffer (0.1 M NaCl, 0.01 M Tris–HCl, pH 7.6,
aldehyde, carbon dioxide and the hearts were dissected. Small pieces of heart tissue were pooled with 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) post-fixed in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer (0.1 M Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT). Each sample was boiled for 10 min, sonicated and the supernatants containing the proteins were collected after centrifugation for 10 min at 10 000 g. Western blot analyses to determine protein levels of mouse and human TFAM, mouse COXII and mouse β-actin were performed as described (15). We quantified human TFAM protein levels by using a polyclonal antiserum against human TFAM and a standard of recombinant human TFAM protein. We quantified mouse TFAM protein levels by using a polyclonal antiserum against mouse TFAM and a standard of recombinant mouse TFAM protein.

Northern blot analysis

RNA from kidney of adult animals was isolated with the Trizol Reagent (GIBCO/BRL, Life Technology). RNA from whole embryos was isolated with the RNeasy mini kit (Qiagen). An antisense RNA probe was used to detect ND6 transcripts. Briefly, a DNA fragment corresponding to ND6 (~0.5 kb) was generated by PCR and cloned into the pCR2.1-TOPO plasmid. The recombinant plasmid was linearized with the restriction enzyme SpeI and used as a template for in vitro transcription by T7 RNA polymerase to generate the corresponding RNA probe. Double-stranded DNA fragments corresponding to COX1 (~0.8 kb) and ND4 (~0.8 kb) were randomly labeled with [α-32P]-dCTP and used as probes to detect the transcripts. Phosphorimager analyses of northern blots were used to assess levels of mitochondrial transcripts in comparison with the nuclear 18S rRNA levels as described previously (20).

Transmission electron microscopy (TEM)

Wild-type (n = 4) and PAC19 (n = 4) animals were killed by carbon dioxide and the hearts were dissected. Small pieces of myocardium from the left ventricle were fixed in 2% glutaraldehyde +0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and 3 mM CaCl2, pH 7.4, at room temperature for 30 min followed by 24 h at 4°C. Specimens were rinsed in 0.15 M sodium cacodylate buffer containing 3 mM CaCl2, pH 7.4 post-fixed in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer containing 1.5 mM CaCl2, pH 7.4 at 4°C for 2 h, dehydrated in ethanol followed by aceton and embedded in LX-112 (Ladd, Burlington, Vermont, USA) (38). Ultrathin sections (~40–50 nm) from longitudinal parts were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 transmission electron microscope (Fei company, Eindhoven, The Netherlands) at 80 kV.

Volume density measurements

Digital pictures at a final magnification of 6200 × were randomly taken on myofibrils from longitudinal sections of the myocardium. Printed digital pictures were used and the volume density ($V_v$) of mitochondria was calculated using point counting using a 2 cm square lattice as described (39). A pilot study was performed to determine the number of blocks and pictures needed for an appropriate sample using cumulative mean plot for evaluation (39). Thus, two different blocks from one animal were sectioned and 10 randomly selected pictures were collected from each block. The point counting was done twice and the mean value from each picture was used.

Statistical analyses

Two-sided unpaired t-tests were used to assess statistical significance.

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