

Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells

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ABSTRACT

Mitochondrial transcription factor A (TFAM) is an abundant mitochondrial protein of the HMG superfamily, with various putative roles in mitochondrial DNA (mtDNA) metabolism. In this study we have investigated the effects on mtDNA replication of manipulating TFAM expression in cultured human cells. Mammalian mtDNA replication intermediates (RIs) fall into two classes, whose mechanistic relationship is not properly understood. One class is characterized by extensive RNA incorporation on the lagging strand, whereas the other has the structure of products of conventional, strand-coupled replication. TFAM overexpression increased the overall abundance of RIs and shifted them substantially towards those of the conventional, strand-coupled type. The shift was most pronounced in the rDNA region and at various replication pause sites and was accompanied by a drop in the relative amount of replication-termination intermediates, a substantial reduction in mitochondrial transcripts, mtDNA decatenation and progressive copy number depletion. TFAM overexpression could be partially phenocopied by treatment of cells with dideoxycytidine, suggesting that its effects are partially attributable to a decreased rate of fork progression. TFAM knockdown also resulted in mtDNA depletion, but RIs remained mainly of the ribosubstituted type, although termination intermediates were enhanced. We propose that TFAM influences the mode of mtDNA replication via its combined effects on different aspects of mtDNA metabolism.

INTRODUCTION

In mammalian cells, mitochondrial DNA (mtDNA) was long believed to replicate by an unusual, strand-asymmetric mechanism (1). However, recent studies, using two-dimensional neutral agarose gel electrophoresis (2DNAGE), have revealed the presence, both in vertebrate tissues and cultured cells, of two classes of mtDNA replication intermediates (RIs) whose structures are not consistent with the strand-asymmetric model. Both classes are essentially duplex throughout their length, but differ in their ribonucleotide content (2,3). One class shows extensive RNA incorporation on the lagging strand [ERIOLS, Ref. (2)], whereas the other has structures fully consistent with conventional, strand-coupled DNA replication (3–5). ERIOLS intermediates are generally nicked or gapped on the RNA strand (2) and are hence labile to partial degradation during extraction.

The mechanistic relationship between RIs of the ERIOLS and strand-coupled types, as well as how they relate to the ‘orthodox’, strand-asymmetric replication model, are not properly understood. ERIOLS intermediates have been suggested to be processed to resemble those of the strand-coupled type via a maturation step (2). Different replication modes may also operate simultaneously in the same cell. In solid tissues, strand-coupled replication appears to initiate bidirectionally in a broad origin zone, spanning at least several kilobases downstream of the major non-coding region (NCR) of the genome (5,6). In cultured cells recovering from drug-induced mtDNA depletion, such initiation is confined to a much narrower region of the NCR (3). The initiation mechanism which gives rise to RIs of the ERIOLS type remains unclear. Initiation within the NCR can also give rise to the synthesis of 7S DNA (1), which establishes the characteristic D-loop form of mtDNA, although its relationship with productive replication of the genome remains enigmatic.

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The mitochondrial transcription factor TFAM, an abundant HMG-box protein of mitochondria, appears to have multiple functions in mtDNA metabolism (7). It was originally characterized by the absolute requirement for the protein for transcriptional initiation *in vitro* at either the heavy- or light-strand promoter of the genome (8). More recently, these findings were confirmed using a fully reconstituted system, containing mtDNA-derived templates, RNA polymerase and the additional transcription factor TFB1M or TFB2M (9). Transcription from the light-strand promoter is required to create the primer for heavy-strand mtDNA synthesis according to the orthodox, strand-asymmetric replication model. Therefore TFAM has been considered to be an essential protein also for mtDNA replication. Consistent with this view, abolition of TFAM expression using a conditional knockout strategy in the mouse showed clearly that TFAM is required for mtDNA maintenance as well as cellular function and survival (10). However, this finding is also consistent with TFAM protein having other essential roles in mtDNA metabolism.

TFAM has been proposed to play a structural role in the maintenance of the mitochondrial chromosome, independent of its transcriptional activity. It is highly abundant, is mainly (or entirely) complexed with mtDNA in nucleoid structures (11,12), shows significant, non sequence-specific DNA-binding (8) and promotes DNA compaction (13), leading to the suggestion that it coats the entire DNA in a manner similar to histones in the eukaryotic nucleus or the HU protein in bacteria (14). Its homologue in yeast, Abf2p, has been shown to induce compaction by introducing sharp bends into the DNA backbone (15) and is required for the stable maintenance of wild-type mtDNA (16).

Mammalian TFAM has preference for binding to branched DNA structures such as Holliday junctions (17) and to cisplatin-damaged or oxidized DNA (18). *In vitro*, TFAM promotes the resolution of D-loop forms (19). It also interacts physically with p53 [Ref. (20)], suggesting a possible function in DNA repair or other recombinational processes. Although it does not have a directly protective role, TFAM overexpression in rat myoblasts has been reported to accelerate the recovery of mtDNA levels after peroxide damage (21) and transgenic expression of human TFAM in mice mitigates mtDNA loss and other mitochondrial defects after cardiac ischemia (22). In yeast, Abf2p is required for recombination and segregation of mtDNA to daughter cells (23) and genetic evidence also implicates it in non-recombinational mtDNA repair pathways (24). All of these pieces of evidence point to TFAM and its homologues being key regulators of DNA transactions in mitochondria.

In organello, TFAM imported into rat liver mitochondria stimulates the synthesis both of mitochondrial RNAs (25) and 7S DNA (26). Transient overexpression of TFAM in cultured HEK cells also results in increased transcription, but with no change in mtDNA copy number (27). However, high levels of TFAM added exogenously *in vitro* (9,28,29), as well as prolonged over expression in HEKcultured cells (27), bring about a paradoxical decrease in transcription. This effect may be attributable to an over-condensed state in the template DNA. The transient increase in mitochondrial transcription brought about by TFAM over-expression in HEK cells is accompanied by an increased level of RNase

H-sensitive mtDNA species (27), which may correspond with RIs of the ERIOLS type.

Several lines of evidence suggest that TFAM regulates mtDNA copy number independently of its role(s) in transcription. Heterozygosity for TFAM knockout produces copy number depletion of ~40% in mice (10) and 50% in chicken cells (30), but with minimal effects on RNA levels. Although human TFAM has only a weak transcription-stimulatory effect on mouse mtDNA promoters *in vitro*, transgenic expression of human TFAM in mice produces a stoichiometric increase in mtDNA levels (31). Overexpression of a transcriptionally inert variant of TFAM in human cells also results in a proportionate copy number increase (14), whilst TFAM knockdown by RNA interference (RNAi) causes copy number depletion with only minor effects on transcription per template molecule (14). In chicken cells in which the endogenous c-TFAM gene has been disrupted, transgenic c-TFAM lacking the C-terminal tail region required for transcription-promoting activity is nevertheless able to support maintenance of mtDNA at 50% of wild-type levels (30).

The idea that TFAM regulates mtDNA copy number by a simple titration model is, however, contradicted by the observation that, following transient, ethidium bromide (EtBr)-induced mtDNA depletion in cultured cells, TFAM levels were observed to recover more slowly than mtDNA. This suggests that the packing ratio of TFAM on mtDNA can vary and may influence the rate of mtDNA replication (32).

In order to investigate further the effects and mode of action of TFAM on mtDNA replication and copy number modulation, we analysed mitochondrial RIs from cells overexpressing TFAM and from cells in which TFAM expression was knocked down by RNAi. We report here that overexpression of TFAM brings about a dramatic change in the relative abundance of strand-coupled versus ribosubstituted (ERIOLS-type) RIs, accompanied by systematic alterations in copy number, transcript levels and mtDNA topology. In contrast, TFAM knockdown results in copy number depletion, but with only minimal effects on mtDNA RIs. The findings are consistent with the idea that the synthesis of mtDNA is dependent on at least two different TFAM-influenced processes, one of which is transcription-associated, the other related to mtDNA organization.

MATERIALS AND METHODS

TFAM constructs

Full-length *TFAM* cDNA lacking its usual stop codon was amplified from a cDNA clone (27, kind gift of Dr R. Wiesner), using the following primers (restriction sites as indicated, underlined, start codon in bold italics):

5'-CCGGAATCCGCGATGGCGTTTCTCCGAAGC-3' (EcoRI) and 5'-CGCGGATCCACACTCCTCAGCACCAT-ATTTTCG-3' (BamHI)

The restriction-digested PCR product was ligated into EcoRI + BamHI-cut pcDNA3.1(-)Myc-HisA (Invitrogen) to create the construct mtTFA-myc, capable of directing the expression of C-terminally Myc-His epitope-tagged TFAM. The full-length TFAM cDNA, including the natural stop codon, was amplified using the following primers

(restriction sites as indicated, underlined, plus start and stop codons in bold italics):

5'-CCCAAGCTTGCGATGGCGTTTCTCCGAAGC-3' (HindIII) and 5'-CGCGGATCCTTAACTCCTCAGCACCATATTTTC-3' (BamHI).

The restriction-digested PCR product was ligated into HindIII + BamHI-cut pcDNA3.1(+) vector, to create the construct mtTFAPcDNA3.1 for transient expression.

In order to create cell lines inducibly expressing either natural TFAM or C-terminally Myc epitope-tagged TFAM, the two plasmids described above were digested with PmeI and the liberated inserts recloned into the vector pcDNA5/FRT/TO (Invitrogen), which was then transfected into the Flp-InTM T-RexTM-293 host cell line (Invitrogen) according to the manufacturer's recommendations. Full details of the use of this system to create cell lines inducibly expressing proteins involved in mtDNA metabolism will be published elsewhere (S. Wanrooij *et al.*, manuscript in preparation).

siRNAs for TFAM knockdown

TFAM siRNAs were synthesized using the *Silencer*TM siRNA construction kit (Ambion). Six putative TFAM-specific siRNA sequences were selected using the manufacturer's prediction programme (www.ambion.com/techlib/misc/siRNA_finder.html). After testing by transfection and western blotting (see below), two were found to be efficient. The sequences of the relevant mRNA targets were as follows. Si2: 5'-AAGTTGTCCAAAGAAACCTGT-3' (np 273–293 of the TFAM mRNA sequence, Genbank NM_003201) and Si4: 5'-AAGATGCTTATAGGGCGGAGT-3' (np 431–451, exon 4). See legend to Supplementary Figure 3 for details of other siRNAs tested in trial experiments.

Cell-culture and transfection

HEK293T and Flp-InTM T-RexTM-293 cells (Invitrogen) were cultured in DMEM containing 4.5 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/ml uridine and 10% fetal bovine serum, at 37°C in a humidified atmosphere with 5% CO₂ in air. No antibiotics were added for HEK293T cells, but transgenes in Flp-InTM T-RexTM-293 cells were maintained under selection with hygromycin and blasticidin according to the manufacturer's recommendations. All cell lines were routinely detached by pipetting alone and passaged at 1:10 dilution every 3–4 days. TFAM transgene expression was induced by adding 10 ng/ml doxycyclin (Sigma-Aldrich) to the culture medium for the times indicated in the Figures and legends. Transfections were carried out using TransFectinTM-lipid reagent (Bio-Rad), following the manufacturer's recommended procedure, with 12 µg of plasmid DNA and 40 µl of reagent, both in 1.5 ml of serum-free medium, per 10 ml plate.

For TFAM knockdown, HEK293T cells were transfected using LipofectamineTM 2000 (Invitrogen) and a final concentration of 20 nM of siRNA. To arrest mtDNA synthesis by chain termination, cells were treated for various times in medium containing 100 µM dideoxycytidine (Sigma-Aldrich). To suppress both mtDNA copy number and mitochondrial transcription, cells were treated with medium containing EtBr (50 ng/ml) for 72, after which cells were washed and replated in fresh medium, then cultured for a further 48 h.

DNA and RNA extraction and quantitation

For mtDNA copy number analysis, total cellular DNA was extracted using standard techniques (33). Copy number was assessed independently by two different methods, to minimize possible artefacts. For Southern blotting, total DNA was cut by EcoRI and analyzed as described in Ref. (34), with quantitation by phosphorimaging (Storm 840 scanner and ImageQuant 5.1 software, all from Molecular Dynamics). Copy number was also estimated by real-time quantitative PCR (35) with Taqman probes for mitochondrial cytochrome *b* and for amyloid precursor protein (APP), used as a single-copy nuclear DNA standard. Primers and probes were as follows (all 5'–3'): APP Forward: TTTTGTGTGCTCTCC-CAGGTCT, APP Reverse: TGGTCACTGGTTGGTTGGC, APP Probe (FAM+BHQ): CCCTGAACTGCAGATCACCA-ATGTGGTAG, Cyt-*b* Forward: GCCTGCCTGATCCTCC-AAAT, Cyt-*b* Reverse: AAGGTAGCGGATGATTAGCC, Cyt-*b* Probe (TET+BHQ): CACCAGACGCCTCAACC-GCCTT.

RNA extraction from cells, agarose or urea-PAGE and Northern hybridization were as described previously (34,36). Probes were ³²P end-labelled oligonucleotide as follows (5'–3'): for ND3 mRNA, GTCACATAGGCCAG-ACTT, for 5S rRNA (loading control), GGGTGGTATGGC-CGTAGAC, for tRNA^{Leu(UUR)} and tRNA^{Tyr} as described previously (37). Quantitation was by phosphorimaging as for mtDNA copy number.

For the preparation of mtDNA (mitochondrial nucleic acids) for analysis of RIs, mitochondria were isolated from cells essentially as described by Spelbrink *et al.* (38). Briefly, cells from 10 to 20, 14 cm plates were collected by pipetting in PBS, centrifugation at 400 *g*_{max} for 3 min at room temperature and transfer to ice. The cell pellet was resuspended by gentle pipetting in two volumes of ice-cold 0.1× homogenization buffer (4 mM Tris-HCl, pH 7.8, 2.5 mM NaCl, 0.5 mM MgCl₂), kept on ice for 5 min and homogenized in a glass homogenizer with 20 strokes of a tight-fitting pestle. Disruption of the cells was monitored by microscopy. One-ninth volume of 10× homogenization buffer was added and nuclei and cell debris were pelleted by sequential centrifugations at 1200 *g*_{max} for 3 min at 4°C until no pellet was visible. Mitochondria from the post-nuclear supernatants were recovered by centrifugation at 16000 *g*_{max} for 10 min at 4°C. The mitochondrial pellet was washed once in resuspension buffer (10 mM Tris-HCl pH 7.4, 0.32 M sucrose, 1 mM EDTA, 5 mM MgCl₂) and re-centrifuged at 16000 *g*_{max} for 10 min at 4°C. The pellet was placed immediately on ice and resuspended thoroughly in 500 µl of DNA extraction buffer (25 mM EDTA pH 8.0, 75 mM NaCl) followed by the addition of 50 µl 10% SDS with gentle mixing and incubation on ice for a further 10 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) was added and the tube was shaken gently overnight at 4°C on a rotatory shaker, then centrifuged at 5000 *g*_{max} for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and phenol extraction repeated several times until the interface was clear, after which 0.2 vol. 10 M ammonium acetate and 2 vol. 80% EtOH were added to the final aqueous phase. The solution was gently mixed, incubated on ice for 15 min and centrifuged at 5000 *g*_{max} for 15 min at 4°C. The precipitated nucleic acids were washed once with 80%

EtOH and centrifuged at 5000 g_{\max} for 5 min at 4°C min. The pellet was air-dried, dissolved in 80 μ l of TE buffer and stored at 4°C.

Enzymatic treatment of DNA

MtDNA samples were treated with the following DNA-modifying enzymes under conditions recommended by the manufacturers: T7 gp3 endonuclease (New England Biolabs), topoisomerase I (New England Biolabs) and topoisomerase IV (John Innes Enterprises).

Two-dimensional neutral agarose gel electrophoresis

One microgram of total mitochondrial nucleic acids was used per analysis. Restriction digestions were performed following manufacturers' recommendations, except for BclI which was carried out at 37°C for double the usual reaction time. If subsequent nuclease treatments were used, DNA was first recovered by ethanol precipitation and resuspended in the appropriate reaction buffer, before treatment with 50 U of RNaseI (New England Biolabs), 2 U of RNaseH (Promega), each for 1 h at 37°C or 50 U S1 Nuclease (Promega) for 30 s. Reactions were stopped by the addition of an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8.0) and immediately extracted. 2DNAGE was performed essentially as described in Kajander *et al.* (39). The first-dimension was run without EtBr in a 0.4% agarose gel in TBE buffer, 1.2 V/cm for 24 h at 4°C. The gel was stained with EtBr (300 ng/ml) in TBE. Individual lanes were cut out, rotated 90° and 1.0% agarose containing 300 ng/ml EtBr, precooled to 55°C, was cast around them. The second dimension was run at 6 V/cm for 5 h at 4°C with constant buffer recirculation. For analysis of high molecular weight fragments (e.g. 16.6 kb mtDNA linears), the first-dimension gels were 0.28% agarose, run at 1.4 V/cm for 24 h at room temperature, with the second dimension in 0.58% agarose, 300 ng/ml EtBr, run at 2.6 V/cm for 67 h at room temperature with constant buffer recirculation. Gels were processed for Southern blotting using standard procedures.

Radiolabelled probes and blot hybridization

For Southern hybridization, the following probes were created by *Pfu*-PCR, using cloned segments of human mtDNA as template and subsequently sequenced to confirm their identity: O_H (np 35–611, Anderson *et al.*, 1981), ND2 (np 4480–4988), A8-6 (np 8460–9107), ND4 (np 11 161–11 640) and ND5 (np 12 992–13 670). Probes were labelled using RediprimeTM II random prime labelling kit (Amersham) and [α -³²P]dCTP (Amersham; 3000 Ci/mmol).

Sub-fractionation of mitochondria

For assaying the localization of recombinant TFAM the mitochondrial pellet, prepared as above, was resuspended in 2 vol. of lysis buffer (0.25 M sucrose, 20 mM Tris–HCl, pH 7.6, 2 mM EDTA, 7 mM β -mercaptoethanol). Mitochondria were lysed by adding 20% NP40 to a final concentration of 0.5% (v/v) with incubation on ice for 1 h. After centrifugation at 16000 g_{\max} for 10 min at 4°C the pellet and supernatant fractions were processed for SDS–PAGE.

SDS–PAGE and western blotting

SDS–PAGE used 7.5–12% polyacrylamide (Laemmli) gels under standard conditions. Sample preparation, western blotting and immunodetection were carried out as described previously (38). Primary antibodies used were: mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals), 1:15 000 dilution of a 5 mg/ml stock, rabbit anti-human TFAM (kind gift of Dr R. J. Wiesner), 1:10 000 dilution and mouse anti- α -actinin monoclonal AT6/172 (Upstate) 1:5000 of a 1 mg/ml stock. Signals were quantified using a ChemiDoc XRS chemiluminescence detection instrument and associated QuantityOne software.

RESULTS

Effects on mitochondrial nucleic acids of modulating TFAM expression levels *in vivo*

We manipulated TFAM expression levels in cultured human cells using transient or inducible expression of epitope-tagged or untagged TFAM, as well as TFAM knockdown by RNAi (Figure 1, see also Supplementary Figures 1–3). Transient expression (data not shown) produced essentially the same effects on all parameters studied as inducible over-expression using the Flp-InTM T-Rex system, although with less quantitative reproducibility. Inducible over-expression of TFAM carrying a C-terminal MycHis tag produced qualitatively similar but quantitatively more dramatic effects, namely a small but transient increase in mtDNA copy number, followed by progressive mtDNA depletion. After 10 days of full induction (10 ng/ml doxycyclin, full details on the use of the induction system to be published elsewhere, Wanrooij *et al.*, manuscript in preparation), TFAM-MycHis expression resulted in the reduction of mtDNA levels to ~20% of control levels. Over-expression of TFAM with its normal stop codon (TFAM-stop) caused a decrease in mtDNA copy number of 40–60%. Copy number depletion was verified by two independent methods, real-time PCR and phosphorimaging of Southern blots (Supplementary Figures 1 and 2).

Although the absolute amount of TFAM protein did not increase markedly during induction, measured relative to a loading control (Supplementary Figures 1 and 2), the progressive drop in mtDNA copy number means that the amount of TFAM protein per mtDNA molecule did increase substantially and consistently during induction: in the case of TFAM-stop to over twice the endogenous level, in the case of TFAM-MycHis by an order of magnitude. Crude fractionation of mitochondrial protein lysates by sucrose density-gradient centrifugation (Supplementary Figure 2) showed that over-expressed TFAM-MycHis partitioned, like endogenous TFAM, mainly into the pellet fraction, forming high-molecular weight complexes in the same proportion as endogenous TFAM, indicating that it is likely complexed with mtDNA.

Previous experiments *in vitro* and *in organello* have suggested that a large excess of TFAM can suppress rather than activate transcription. We measured the steady-state levels of various mitochondrial transcripts using northern blot analysis (Supplementary Figures 1 and 2 and other

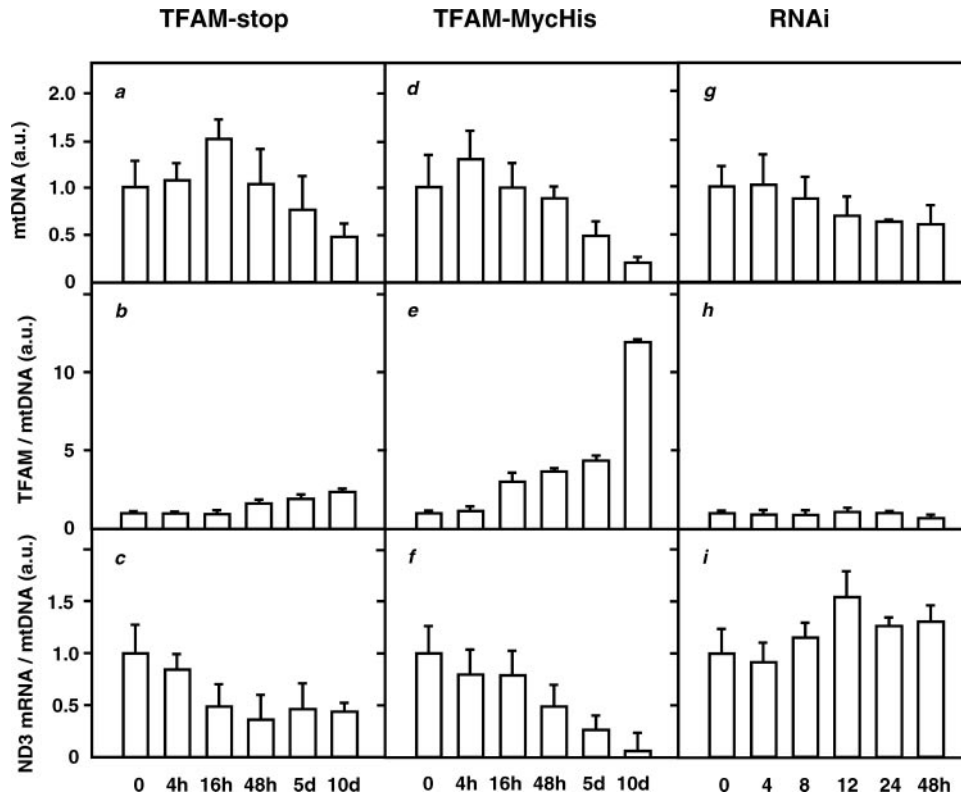


Figure 1. Effects of induced expression of TFAM-stop and TFAM-MycHis and of RNAi knockdown of TFAM expression. Mitochondrial proteins, DNA and RNA were analysed from Flp-InTM T-RexTM-293 cells stably transfected with the TFAM-stop (a–c) or TFAM-MycHis construct (d–f), induced over the times indicated or from HEK293T cells (g–i) following transfection with siRNAs Si2 and Si4 over the times indicated. In each case, error bars indicate means \pm SEs from at least three independent experiments. a.u., arbitrary units. Measurements of mtDNA levels (a, d and g) are arbitrarily normalized to the mean values for uninduced or untreated cells. For cells under TFAM induction, the measurements were made by two independent methods, Southern blotting and Q-PCR and the values plotted for each time point are the means of measurements by the two methods, shown in Supplementary Figures 1 and 2. (b, e and h) show TFAM protein levels normalized to the mtDNA levels shown in (a, d and g), then normalized against the level in uninduced or untreated cells. (c, f and i) show ND3 mRNA levels normalized first against the 5S rRNA loading control, then against the mtDNA levels shown in (a, d and g), then finally against the level in uninduced or untreated cells. Samples of the raw data and compiled data for TFAM protein, mtDNA and RNA levels on which this figure is based, are shown in Supplementary Figures 1–3.

data not shown), under conditions where over-expression of TFAM-stop or TFAM-MycHis was induced *in vivo*. The level of short-lived mRNAs such as ND3 showed a marked decline even relative to the decreased amount of mtDNA. Although modulation of post-transcriptional processing and RNA stability contribute to changes in the steady-state level even of short-lived transcripts, the drop in ND3 mRNA levels is consistent with a substantial drop in transcription per template molecule, under conditions of TFAM over-expression, which was especially marked for TFAM carrying the C-terminal MycHis tag. As a more rigorous and direct test of its effects on transcriptional activity, we also analysed the consequences of TFAM-MycHis expression on the rate of recovery in mitochondrial tRNA levels in cells treated for 72 h with EtBr, following removal of the drug (Supplementary Figure 4).

In conformity with the published literature, RNAi knockdown of TFAM produced a progressive reduction of TFAM protein levels and mtDNA copy number. A combination of two siRNAs was selected, based on preliminary trials, which decreased TFAM protein to low levels (<10%) during 7 or 14 days continuous culture (Supplementary Figure 3). Over shorter-time periods, both TFAM protein and mtDNA

depletion by RNAi were rather modest and the levels of TFAM protein or ND3 mRNA per template mtDNA molecule were almost unchanged from control cells (Figure 1).

Altered TFAM expression leads to systematic effects on mtDNA replication intermediates

The copy number depletion of mtDNA produced either by RNAi knockdown or by over-expression prompted us to investigate further the effects of these treatments on mtDNA replication, using 2DNAGE. Transient expression of TFAM-stop or inducible overexpression of either TFAM-stop or TFAM-MycHis, produced dramatic and systematic effects on the patterns of mtDNA RIs, which were essentially the same in all three cases (Supplementary Figure 5b). The most consistent effects on RIs were seen after 48 h of induced over-expression (or 48 h after transient transfection), when effects on mitochondrial transcripts were clearly evident and quite similar in all cases.

TFAM overexpression resulted in a substantially increased abundance of RIs relative to the unit-length restriction fragment (Figure 2; see Supplementary Figure 5 for further explanations and interpretations of the gel data). For example,

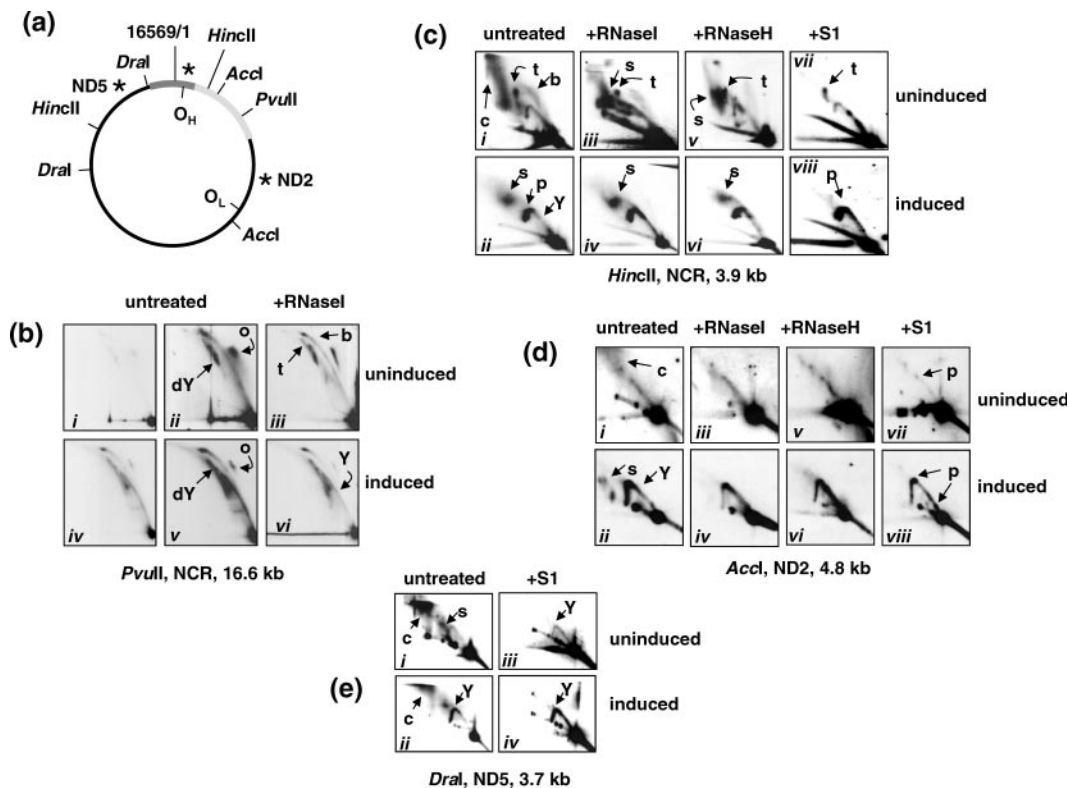


Figure 2. 2DNAGE analysis of mtDNA replication intermediates (RIs) in cells induced to overexpress TFAM-stop. (a) Diagrammatic map of human mtDNA, showing the origins of heavy- (O_H) and light-strand (O_L) replication according to the orthodox model, relevant restriction sites and probes for the three regions of the genome analysed (approximate location of probes indicated by asterisks). NCR shown as dark grey bar, rDNA as pale grey bar. (b–e) 2DNAGE of mtDNA from uninduced cells and from cells induced to express TFAM-stop for 48 h, analysed using the restriction digests and probes indicated, with or without additional enzymatic treatments as shown. In each panel, the various arcs and other salient features are denoted as follows: Y, standard Y arcs, dY, standard double-Y arcs, c, ‘cloud’ of RNase-sensitive material, o, circular molecules, b, standard bubble arcs, s, slow-moving Y-like arcs, t, termination intermediates lying on a portion of a standard X arc, p, prominent pause sites. See Supplementary Figure 5 for diagrammatic interpretations of the various arcs. Panels i and iv of part (b) and panels iii and iv of part (c) are equivalent exposures, for comparison. Other panels of uninduced cell mtDNA are 5- to 10-fold more exposed than induced cell material, in order to reveal the main features of the arcs. Note the general enhancement of RIs, relative loss of nuclease-sensitive species, of termination intermediates and of bubble arcs, following TFAM induction. The appearance of a complete or almost complete, Y arc in Figure 2b, panels iv–vi, is consistent with frequent strand breakage at O_H or with recombinational strand-switching (also generating a free end at O_H) or with frequent initiation far distant from the NCR.

using a restriction enzyme which cuts only once in the genome (PvuII, np 2560, Figure 2b), TFAM over-expression gave a clear enhancement of the clubheaded bubble arc indicative of initiation far upstream of the restriction site, as well as revealing a prominent simple Y-arc. In uninduced cells these RIs were either much fainter or absent. The main features were the clubheaded bubble arc and corresponding double-Y arc, as well as a number of species that were sensitive to or modified by, RNaseI treatment. The RNaseI-sensitive species included circular molecules, a short arc which corresponds with dimeric circles and/or broken theta forms (T. Yasukawa, personal communication), plus a diffuse cloud of material migrating in the region between circular molecules and the bubble arc. A putative termination arc was also visible only in uninduced cells, although this was revealed more clearly when other species were modified by RNaseI (Supplementary Figure 5).

In other digests, the exact transformations of RIs resulting from TFAM overexpression varied according to which region of the genome was being analysed (Figure 2c–e). In general, ERIOLS-type RIs, i.e. slow-moving arcs and ‘clouds’ of heterogeneous, nuclease-sensitive material were diminished and

migrated as more discrete entities. There was general enhancement of nuclease-resistant arcs, in particular those resistant to S1 nuclease. In addition, rather specific subtypes of RIs were seen to accumulate along the arcs, which differed from those seen in uninduced or untransfected cells.

Changes were most dramatic in the rDNA region, extending to O_L (Figure 2d). In uninduced cells, in contrast to the patterns of RIs from this region of the genome seen in solid tissues or in cultured cells recovering from drug-induced mtDNA depletion (2–4), complete Y-arcs of the strand-coupled type were not detectable, even on long exposure. Instead, a heterogeneous ‘cloud’ of complex, high-molecular weight material was seen, plus the ascending portion of a Y-like arc, ending in a faintly detected replication pause site within the ND1 gene. These forms were partially sensitive to or modified by nucleases. In TFAM overexpressing cells, a complete Y-arc was easily detected, even at relatively low exposure. The descending portion of this arc was now the most prominent, although this segment was relatively sensitive to nucleases. The cloud of heterogeneous material was replaced by at least two discrete, slow-moving Y-like arcs, which were also nuclease-sensitive. The replication pause

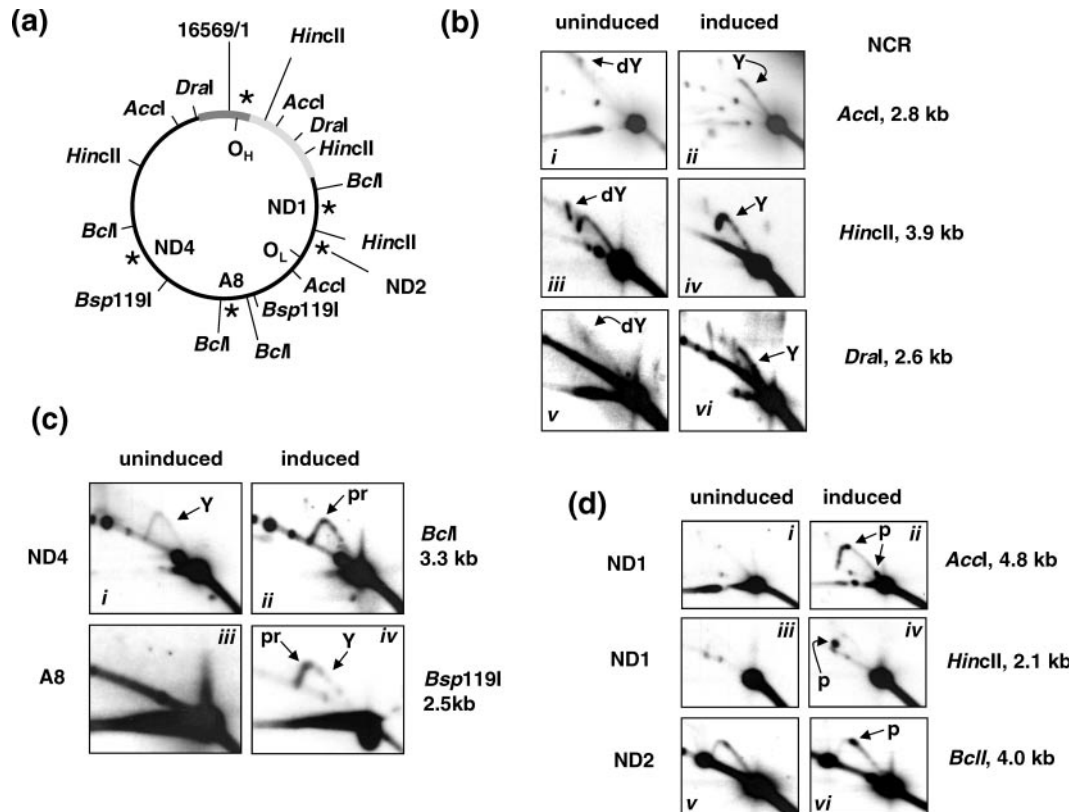


Figure 3. 2D-NAGE analysis of S1 nuclease-resistant mtDNA RIs in cells induced to overexpress TFAM-stop. (a) Diagrammatic map of human mtDNA, nomenclature as for Figure 2a. (b–d) 2D-NAGE of mtDNA from uninduced cells and from cells induced to express TFAM-stop for 48 h, analysed using the restriction digests and probes indicated. All samples were treated with S1 nuclease before electrophoresis. Nomenclature as for Figure 2, plus pr, pause region (replication slow-zone). See Supplementary Figure 5 for diagrammatic interpretations. Comparable exposures are shown, to illustrate the general enhancement of S1-resistant RIs, the strengthening of pause sites and regions and the decrease in termination intermediates. Note that bubble arcs are not visible at these exposures following S1 nuclease treatment.

in ND1 was prominently detected after S1 nuclease treatment.

The major NCR (Figure 2c), containing the principal sites of replication initiation and termination, showed more subtle changes, affecting most obviously the termination region. In uninduced cells, the most prominent species were termination intermediates lying on or close to the apex of the X-arc, plus a heterogeneous cloud of material migrating at high molecular weight which, after nuclease treatment, was either modified (RNaseI or H) or abolished (S1). The ascending portion was the most prominent portion of the partial Y arc and this was also sensitive to ribonucleases, as was the bubble arc. In TFAM overexpressing cells the bubble arc was barely visible even on long exposure and the descending portion of the partial Y arc, leading to the termination site, was strongly enhanced. However, termination intermediates lying on the X-arc were less prominent, especially after S1 nuclease treatment. Heterogeneous, high molecular weight, S1-sensitive material was less dispersed and its migration less affected by ribonucleases.

We next analysed the nature of fully double-stranded (i.e. S1 nuclease-resistant) RIs around the genome in further detail, under conditions of TFAM overexpression (Figure 3). Different restriction digests (Figure 3b) confirmed that TFAM overexpression led to an accumulation of material on the

standard Y arc in the region approaching the terminus at O_H , with a corresponding loss of termination intermediates in which the fork had entered the fragment from the other end and stalled at the terminus prior to resolution.

A number of pause sites or regions were strongly enhanced, in addition to the strong pause in ND1 (Figure 3d). These included the pause at O_L (Figure 3d), several discrete sites in the regions of ND4, ND3, COXIII, A6 and A8, most of the COXII gene (Figure 3c) and a broad region of the ND5 gene (Figure 2c). Conversely, the abundance of 7S DNA was diminished by TFAM overexpression (Figure 6).

RNAi knockdown of TFAM expression produced more subtle effects on RIs (Figure 4). RIs resistant to S1 nuclease were little altered (Figure 4c), but ribonuclease-sensitive ‘clouds’ of heterogeneous, nuclease-sensitive material were somewhat enhanced, especially in the rDNA region (Figure 4c) or showed altered mobility. The clearest transformation was seen in the origin/termination region encompassing O_H , where there was an increase in the abundance of double-Y termination intermediates lying on the X-arc, relative to the bubble and partial Y arcs (Figure 4b), an opposite result to that produced from TFAM overexpression, which diminished the abundance of termination intermediates (Figures 2 and 3).

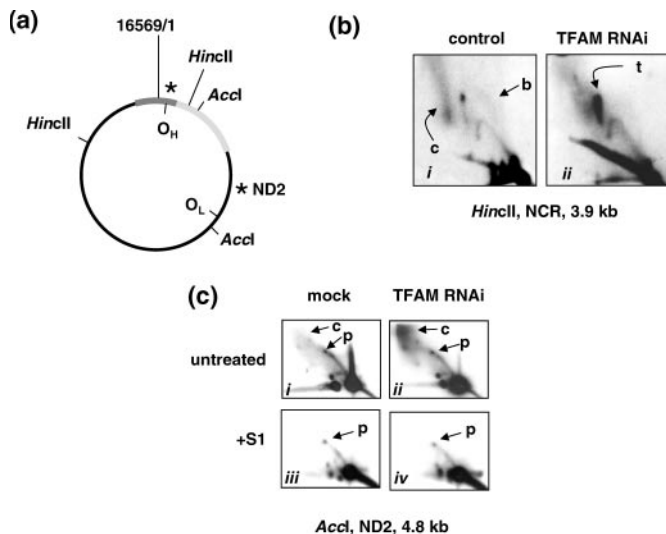


Figure 4. 2DNAGE analysis mtDNA RIs in cells treated with TFAM-specific siRNAs. (a) Diagrammatic map of human mtDNA, nomenclature as for Figure 2a. (b) and (c) 2DNAGE of mtDNA from control (or mock-transfected) cells and from cells treated with TFAM siRNAs for 24 h, using the restriction digests and probes indicated, with or without additional S1 nuclease treatment as shown. Nomenclature as for Figure 2. See Supplementary Figure 5 for diagrammatic interpretations. Comparable exposures are shown, to illustrate the general enhancement of termination intermediates and of the cloud of nuclease-sensitive material migrating at high molecular weight.

TFAM over-expression shows similarities with ddC treatment

Arcs of fully double-stranded, ribonuclease-insensitive RIs should be generated by true strand-coupled replication. However, they could also arise from maturation of ERIOLS type intermediates and, if so, should be enhanced where leading-strand synthesis has stalled randomly or has been drastically slowed (i.e. if the rate of lagging-strand maturation is now comparable with that of fork progression). The effects of TFAM overexpression could thus be interpreted either as a switch to strand-coupled-type replication or as a general slowing in progression of the replication fork, such that maturation of the lagging strand now occurred as fast as the fork progressed.

To address this issue we compared the effects on mtDNA RIs of TFAM over-expression with treatment of cells with the replication inhibitor dideoxycytidine, ddC (Figure 5). After conversion to ddCTP this drug produces repeated chain termination events during mtDNA replication, requiring removal of the incorporated dideoxynucleotide by exonuclease action or recombination, thus greatly slowing down the overall rate of fork progression and leading to mtDNA depletion. As shown in Figure 5, ddC treatment induced rather similar transformations in the pattern of mtDNA RIs as those brought about by TFAM overexpression: progressive and dramatic reduction in ERIOLS-type RIs, a corresponding increase in fully double-stranded RIs of the strand-coupled type (Figure 5d) and the disappearance of termination intermediates (Figure 5b, panels i–iii). Removal of the drug rapidly induced a burst of mtDNA replication but with intermediates remaining initially of the strand-coupled type and with a delayed re-appearance of termination intermediates

(Figure 5b, panel vii). However, both during and following ddCTP treatment, the enhancement of RIs of the strand-coupled type was more general than that resulting from enhanced TFAM expression, rather than being concentrated in the rDNA region and at replication pause sites (e.g. compare Figure 5e, panels ii and iii).

Alterations to TFAM expression modify mtDNA topology

The effects of TFAM overexpression on mtDNA replication might reflect TFAM-induced changes in transcription or in the overall organization of mtDNA. To test whether modulation of TFAM expression affects mtDNA topology or organization, we analysed uncut mtDNA from TFAM-induced and uninduced cells, both before and after treatment with various DNA-modifying enzymes (Figure 6). In uninduced cells, most of the mtDNA migrated either as relaxed circles or in high molecular weight catenated forms that were sensitive to topoisomerase IV but not topoisomerase I. A pronounced smear of material was also visible in the high molecular weight region of the gel. Induced TFAM over-expression resulted in a pronounced shift towards monomeric supercoils, with much less catenated mtDNA, including the high molecular weight smear, as well as creating novel junctional forms that were resolved by phage T7 gp3 endonuclease. Despite these differences, the residual products from combined treatment with topoisomerase IV and T7 gp3 were strikingly similar, when comparing TFAM-induced and uninduced cells. ddC treatment also resulted in a shift away from catenated forms in favour of monomeric circles, both relaxed and supercoiled (Supplementary Figure 6). TFAM knockdown by RNAi produced more subtle changes in mtDNA topology (Figure 6), with an increased level of one particular high molecular weight species (arrowed in Figure 6) and of linear molecules. The arrowed species corresponded in mobility with a catenated form which could be enhanced in control cell mtDNA by treatment with T7 gp3 endonuclease and thus might represent an abortive termination product.

Finally, we analysed the effects of various treatments on the steady-state level of 7S DNA. Both ddC treatment [(40); Supplementary Figure 6] and TFAM overexpression (Figure 6b) resulted in a substantial drop in the amount of 7S DNA relative to other forms of mtDNA, whereas TFAM knockdown resulted in a small increase in 7S DNA (Figure 6b).

DISCUSSION

In this study we investigated the effects on mtDNA replication of modulating TFAM expression in cultured human cells. Sustained over-expression, resulting in a >2-fold increase in the ratio of TFAM to mtDNA, greatly enhanced the steady-state levels of RIs of the strand-coupled type, with corresponding depletion of ribonucleotide-rich, ERIOLS-type RIs. This was accompanied by decreased mitochondrial transcription, depletion of 7S DNA and of replication-termination intermediates, reduced copy number and decatenation of mtDNA. TFAM knockdown in the same cell background also produced copy number depletion,

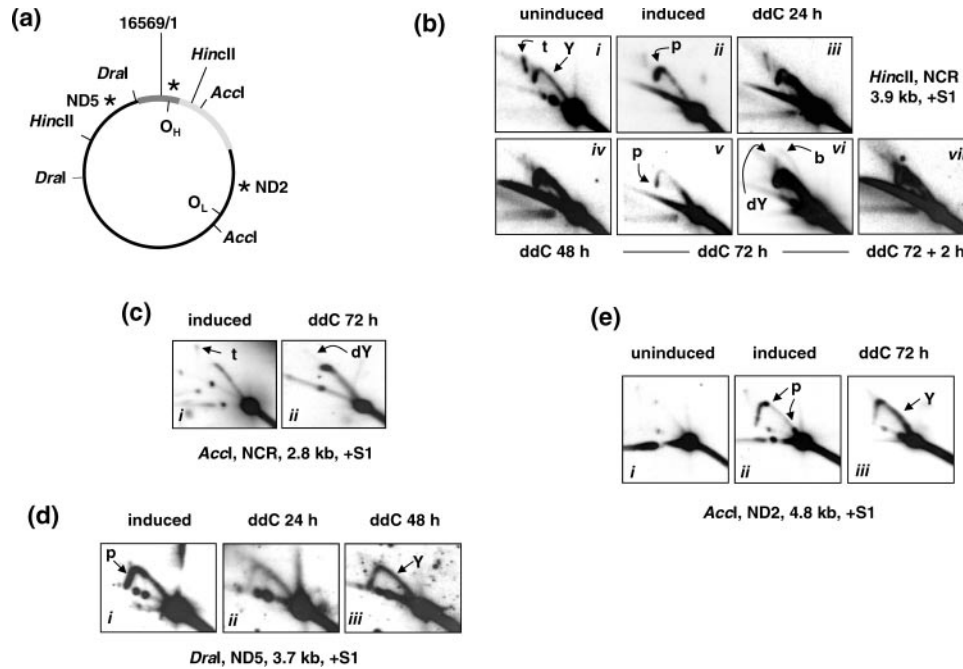


Figure 5. 2DNAGE analysis of RIs in cells induced to overexpress TFAM-stop or treated with ddC. (a) Diagrammatic map of human mtDNA, nomenclature as for Figure 2a. (b–e) 2DNAGE of mtDNA from uninduced cells, cells induced to express TFAM-stop for 48 h or HEK293T cells treated with ddC for the indicated times (72 + 2 h meaning 72 h of treatment followed by 2 h of recovery in fresh medium). Restriction digests and probes as indicated. All samples were treated with S1 nuclease before electrophoresis. Nomenclature as for Figure 2. See Supplementary Figure 5 for diagrammatic interpretations. Exposure times vary, as needed to reveal the main features of arcs of RIs. Note the similar effects of TFAM overexpression and ddC treatment: general enhancement of S1 nuclease-resistant RIs, suppression of termination intermediates. Panel vi of part (c) is a longer exposure of panel v.

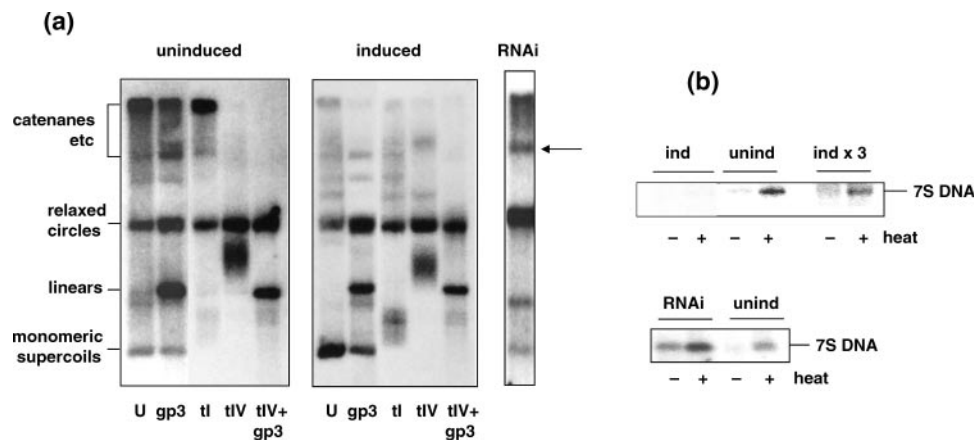


Figure 6. Effects of TFAM overexpression and knockdown on mtDNA topology. One-dimensional agarose gel blots, hybridized with O_H probe. (a) mtDNA from uninduced cells and from cells induced to overexpress TFAM-stop for 48 h, fractionated on a 0.4% agarose gel run in TBE. Only the high molecular weight portion of the gel is shown. Samples were either untreated (U) or treated with T7 gp3 endonuclease (gp3), topoisomerase I (tI), topoisomerase IV (tIV) or topoisomerase IV plus T7 gp3 endonuclease (tIV+gp3). Identity of the main topoisomers was inferred from enzymatic sensitivity and confirmed by other treatments (data not shown). DNA from cells treated with TFAM-specific siRNAs (RNAi) for 24 h was run on a separate gel. (b) MtDNA from TFAM-induced, uninduced and siRNA-treated cells, fractionated on 0.4% agarose gels run in TBE. Only the low molecular weight portion of each gel is shown. First 4 lanes of upper panel are equally exposed, whereas the right-most two lanes are ~3-fold overloaded, to reveal the presence of 7S DNA in induced cells. Samples were either heated for 2 min at 95°C (+) or left unheated, as indicated.

but with different alterations to mtDNA topology, no systematic effects on transcription and, apart from a strong enhancement of termination intermediates, virtually no change in the pattern of RIs. The findings suggest that TFAM can influence mtDNA replication and copy number in several different ways.

TFAM as a structural protein of the mitochondrial chromosome

Previous views of TFAM as a copy number regulator have assumed that the ratio of TFAM:mtDNA is invariant, so that changes in the rate of TFAM synthesis determine the amount of mtDNA present in the cell (10,14,30,31).

However, the observation of a reduced ratio of TFAM protein to mtDNA during mtDNA reamplification following EtBr treatment (32) suggests that the TFAM:mtDNA ratio is not invariant and may indeed be subject to regulation, affecting mtDNA copy number in ways distinct from a simple titration model.

The present study offers support to the latter view, by confirming that an increased ratio of TFAM protein to mtDNA can be sustained in human cells and has systematic, but opposite effects on mtDNA copy number than would be predicted by the simple titration model. Moreover, whereas most experiments hitherto conducted on TFAM have employed highly non-physiological tools, such as cells treated with EtBr, heterologous expression of a human protein in mice or of a truncated TFAM variant never seen in nature, we observed alterations in mtDNA replication and copy number resulting from increased expression of a protein identical to endogenous TFAM. The fact that we were able to achieve and sustain an increased ratio of TFAM to mtDNA contradicts the titration model, but is consistent with the results of studies using *in organello* footprinting (41–44), which indicate that protein binding to mtDNA is not uniform and thus that there are sites ordinarily unoccupied by protein where over-expressed TFAM can potentially bind.

The effects of TFAM over-expression on RIs were qualitatively very similar to those brought about by treatment with the drug ddC which, following conversion to ddCTP, is assumed to be a potent inducer of repeated replication stalling via premature DNA chain termination. ddC treatment also resulted in a large increase in the steady-state levels of nuclease-resistant RIs of the strand-coupled type from all around the mitochondrial genome, with concomitant loss of termination intermediates. Although the effects of these treatments were not absolutely identical, with TFAM over-expression generating a subtly different pattern of such RIs, especially in the rDNA region and in the vicinity of pause sites, their overall similarity strongly suggests that the main effect of TFAM is, like ddC, to provoke a substantial decrease in the net rate of DNA synthesis. This may reflect an increased compaction of mtDNA when the ratio of TFAM protein to mtDNA is increased, such that decondensation of the nucleoid becomes rate-limiting for fork progression. The TFAM-specific transformations of RIs may reflect different degrees of compaction and inhibition of fork progression in different regions of the mitochondrial genome, depending on their affinity for TFAM and other proteins.

The increased abundance of strand-coupled RIs may thus be due to maturation of ERIOLS-type intermediates, which we assume normally to be a slow step compared with the rate of fork progression. If the latter is slowed by increased compaction, the rate of maturation may become comparable with it. Alternatively, if the two classes of RI represent entirely different modes of DNA replication, ERIOLS type replication might be unable to use a highly compacted mtDNA template, leaving only strand-coupled replication (with a less discrete origin, as implied by Figure 2b). Initiation of bidirectional, strand-coupled replication in many bacterial plasmids is stimulated by or dependent on, DNA-bending proteins functionally related to the HMG superfamily (45). Excess TFAM may thus lead to copy number depletion by enforcing a switch to an inherently

slower replication mode or simply by suppressing the alternative mode.

Exactly the same transformations of RIs were produced by transient expression of TFAM as were produced by inducible expression of either natural or C-terminally MycHis-tagged TFAM. However, the two inducible variants brought about copy number depletion with markedly different kinetics (Figure 1). Copy number depletion may thus not be due entirely to a change in the rate of fork progression, but involve also another TFAM-related process with which the C-terminal tag may interfere. Such an effect could, however, be indirect. The amount of TFAM-MycHis protein rose much more steeply than TFAM-stop during induction (Figure 1, Supplementary Figures 1 and 2), suggesting that it may escape physiological turnover mechanisms. This is supported by the observation (Supplementary Figure 2) that TFAM-MycHis almost completely replaced endogenous TFAM during induction. The fact that sustained overexpression of natural TFAM is comparatively difficult to achieve may reflect a natural homeostatic mechanism, whereby TFAM levels can modulate copy number only within certain limits.

Another possibility is that, as for other HMG proteins (46), the C-terminal tail of TFAM recruits or interacts with other nucleoid proteins. It is already implicated in interactions with specific components of the transcriptional apparatus (47). The epitope tag may therefore disturb interactions with other nucleoid proteins involved in copy number homeostasis. Copy number derangement produced in cells or mice by C-terminally truncated or heterologous TFAM variants (14,30,31) may prove to be due to loss of such regulation, rather than by the titration model inferred previously.

The fact that RNAi knockdown of TFAM results in copy number depletion without substantial changes in the patterns of RIs is further evidence that at least one other TFAM-dependent process is critical for mtDNA maintenance. One obvious possibility already mentioned is the compaction of nascent mtDNA into TFAM-containing nucleoid structures, in the absence of which the newly replicated mtDNA may simply be unstable. Other possibilities are discussed below.

TFAM as a regulator of mitochondrial transcription

Although TFAM was originally identified and named on the basis of its being essential for mtDNA transcription, previous studies have shown that excess TFAM, supplied either to a reconstituted *in vitro* system (9) or produced by sustained overexpression in HEKcultured cells (27), results in a paradoxical suppression of transcription. Our own observations are consistent with this (Figure 1, Supplementary Figures 1 and 2).

A drop in transcriptional activity could, conceivably, underlie the switch in DNA replication mode that favours the generation of strand-coupled RIs. Although the decrease in transcriptional activity which accompanies TFAM overexpression appears modest, according to the data of Figure 1 (Supplementary Figures 1 and 2), the steady-state level of ND3 mRNA is a relatively insensitive measure of the actual transcription rate. The mechanism by which ERIOLS-type RIs are generated remains unknown. However, since on 2DNAGE they include heterogeneous ‘clouds’ of material sensitive to both RNaseI and RNaseH which are suppressed

by TFAM overexpression (Figure 2), it is possible that they could arise by a mechanism involving either preformed RNA or nascent transcripts. Transcriptional suppression by excess TFAM, rather than over-compaction of the nucleoid, may therefore be the mechanism driving replication towards the slower, strand-coupled mode. In support of this, the segment of the genome most affected by TFAM overexpression is also the most heavily transcribed region (rDNA), in which discrete RIs are ordinarily hard to detect (Figure 2d), but became prominent when TFAM was overexpressed.

Whereas copy number depletion brought about by TFAM overexpression was accompanied by a clear drop in transcription, transcription per template molecule appeared unchanged when copy number was depleted by TFAM knockdown. Copy number regulation may therefore be independent of transcription. However, transcription was more severely affected by overexpression of epitope-tagged TFAM than natural TFAM and also provoked a more rapid drop in mtDNA copy number. The issue of a relationship between the transcriptional activity of TFAM and copy number control thus remains open.

TFAM overexpression leads to reduced levels of 7S DNA, which might reflect decreased transcriptional activity at the light-strand promoter and/or enhanced resolution of D-loop forms *in vivo*, another known property of TFAM [(19), see also following section]. If D-loops represent a precursor step in DNA replication, as proposed by the orthodox model, their depletion by TFAM overexpression may be crucial in bringing about copy number reduction or in inducing a switch to strand-coupled replication. On the other hand, the increased level of 7S DNA resulting from TFAM knockdown shows clearly that the D-loop form is not sufficient to maintain a high copy number.

TFAM as an enhancer of replication pausing

Replication pauses are well documented in both bacteria and eukaryotes, as well as in plasmids and mtDNA (4,48). However, their roles in DNA homeostasis are unclear, except where they function as definitive terminators. In human mtDNA, prominent pauses occur at the so-called termination-associated site delimiting 7S DNA, at O_L and in the region immediately downstream of rDNA, within the ND1 gene (Figures 2 and 3). One possibility is that, as in yeast rDNA, these pauses are the signatures of proteins which bind at specific sites to facilitate the passage of oppositely moving replication and transcription complexes (49) or simply markers of collision sites, as in bacteria (50), at which replication finally resumes after dissociation of the transcriptional machinery (51). Although TFAM has only a low sequence-specificity for DNA-binding, it may enhance such pauses by promoting DNA-bending, as proposed for the protein Sap1p at one of the replication pause sites in *Schizosaccharomyces pombe* rDNA (52). Such bending may also facilitate the binding of other, more sequence-specific DNA-binding proteins, which directly function in fork arrest. Both copy number depletion and transcriptional inhibition following TFAM overexpression may therefore be due, at least in part, to its effect as a strong enhancer of replication pausing.

TFAM as a cofactor in junctional resolution

TFAM overexpression and TFAM knockdown produced opposite effects on the abundance of termination intermediates. Whereas these were depleted by TFAM overexpression (or ddC treatment), they were strongly enhanced by TFAM knockdown (Figures 3–5). A simple interpretation is that resolution of these forms is a late and slow step in mtDNA replication, which is in some way dependent on the supply of TFAM. Under conditions of TFAM overexpression, either that step is facilitated by increased loading of TFAM onto the nascent DNA or the rate of fork progression is slowed down so much that resolution of termination intermediates is no longer rate-limiting. Under conditions of TFAM ‘starvation’ this resolution step would conversely be inhibited and this may be the primary reason for copy number depletion following TFAM knockdown.

TFAM is already known to have binding preference for junctional structures (17), to facilitate the resolution of D-loops (19) and to bend DNA in a manner analogous with bacterial proteins such as HU and IHF. The exact molecular structure of mtDNA replication-termination intermediates is unclear, although it cannot simply comprise two oppositely moving forks that have almost met, since such a structure would be thermally unstable unless the unreplicated region between them were at least 50 bp, in which case they would not lie precisely on the X-arc on 2DNAGE gels. One possibility is that they are held together by hemicatenation after ligation of at least one strand, although such forms should be S1 nuclease-sensitive (53). Another is that they are converted to true Holliday junctions. A third option is that they contain a single or even double chicken-foot structure.

Whatever their precise nature, a complex enzymatic machinery should be required both to form and to resolve them, in which TFAM is a plausible player, both because of its DNA-binding preferences and bending properties and by its putative ability to recruit other proteins to the DNA. DNA-bending is required for the protein-based partition systems of low copy-number bacterial plasmids such as P1 (54) and binding sites for the DNA-bending proteins H-NS and FIS appear to be clustered around the replication terminus in *Escherichia coli* (55). HMG and related proteins are widely implicated as cofactors in recombination. For example, mammalian HMGB1 facilitates V(D)J recombination by the RAG proteins (56). In bacteria, HU is essential for efficient homologous recombination (57) and IHF is required for the action of lambda integrase (58). HMG proteins are able to facilitate recombination even in heterologous systems [see Ref. (59)]. In yeast mitochondria, overexpression of Abf2p promotes the formation of recombination intermediates (60). DNA-bending by TFAM may facilitate analogous processes in human mtDNA.

A rather different interpretation would also be consistent with the 2DNAGE data, namely that TFAM overexpression leads to a high frequency of strand breakage at O_H . This would account both for the relative paucity of bubble arcs in O_H -containing fragments, (Figure 2b and c), for the corresponding increase in simple Y arcs and for the loss of termination intermediates (Figures 2c, and 3b). Such strand-breakage is very unlikely to be an extraction artefact, since these differences from control cells were seen in all of the

many DNA preparations analysed. If this interpretation is correct, it may imply a need for true recombination reaction to resolve daughter molecules, which could be much slower than the usual termination/resolution step. Copy number depletion might therefore be due to interference with termination when TFAM is either overexpressed or downregulated.

In addition, TFAM overexpression resulted in apparent decatenation of mtDNA, whereas TFAM knockdown increased the level of linear molecules and of one decatenated species that could also be generated in control cell mtDNA by treatment with T7 gp3 endonuclease, a promiscuous junctional resolvase. It is tempting to suggest that these species may be aberrant breakdown products resulting from failure of the normal termination step under conditions of TFAM deficiency. The loss of catenated species in favour of monomeric supercoils under conditions of TFAM overexpression may again reflect the altered kinetics of mtDNA replication, since it too was brought about by ddC treatment. Alternatively an overcompacted state of mtDNA resulting from excess TFAM may directly prevent catenation or stimulate decatenation, as is known for the *E. coli* HU protein acting on circular plasmid molecules (61). Decatenation might also result from frequent strand-breakage at O_H. Under conditions of decreased copy number, decatenation should be important for dispersal of newly replicated mtDNAs into nucleoids, to restrain mitotic segregation of possibly deleterious mtDNA sequence variants. In yeast, the TFAM homologue Abf2p is required for efficient parsing of mtDNA into nucleoids (62), although this process appears to involve recombination rather than decatenation.

Physiological role of TFAM as a mtDNA copy number regulator

Our findings indicate that, at least in this particular proliferating cell background, TFAM expression is finely poised. Any marked deviations from what appears to be an optimal expression level provoke alterations to mtDNA replication that result in decreased mtDNA levels.

Previous studies of the transcriptional regulation of the TFAM gene indicate that it is sensitive to signals connected with metabolite supply (63) and redox stress, via the phosphorylation of NRF-1 (64) and to proliferative, differentiation-linked and environmental signals via the co-activators PGC1 and PRC (65–67) and the transcription factor Myc (68). This raises the issue of whether there is an additional homeostatic, mechanism to fine-tune TFAM expression to the physical state of mtDNA, which can be circumvented by the manipulations we carried out. A retrograde signalling pathway of this type might serve to ensure that disturbances in mtDNA replication or segregation do not lead to a copy number catastrophe. Alternatively, the level of TFAM expression and possibly also its post-translational modification (69), may function *in vivo* to accelerate or decelerate mtDNA replication, thus constituting a key determinant of copy number.

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