

The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA

Anne K. Hyvärinen¹, Jaakko L. O. Pohjoismäki¹, Aurelio Reyes², Sjoerd Wanrooij¹, Takehiro Yasukawa², Pekka J. Karhunen³, Johannes N. Spelbrink¹, Ian J. Holt² and Howard T. Jacobs^{1,4,*}

¹Institute of Medical Technology and Tampere University Hospital, FI-33014, University of Tampere, Finland, ²MRC-Dunn Human Nutrition Unit, Cambridge, UK, ³Department of Forensic Medicine and Tampere University Hospital, FI-33014, University of Tampere, Finland and ⁴Institute of Biomedical and Life Sciences, University of Glasgow, Scotland, UK

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ABSTRACT

The mammalian mitochondrial transcription termination factor mTERF binds with high affinity to a site within the tRNA^{Leu(UUR)} gene and regulates the amount of read through transcription from the ribosomal DNA into the remaining genes of the major coding strand of mitochondrial DNA (mtDNA). Electrophoretic mobility shift assays (EMSA) and SELEX, using mitochondrial protein extracts from cells induced to overexpress mTERF, revealed novel, weaker mTERF-binding sites, clustered in several regions of mtDNA, notably in the major non-coding region (NCR). Such binding *in vivo* was supported by mtDNA immunoprecipitation. Two-dimensional neutral agarose gel electrophoresis (2DNAGE) and 5' end mapping by ligation-mediated PCR (LM-PCR) identified the region of the canonical mTERF-binding site as a replication pause site. The strength of pausing was modulated by the expression level of mTERF. mTERF overexpression also affected replication pausing in other regions of the genome in which mTERF binding was found. These results indicate a role for TERF in mtDNA replication, in addition to its role in transcription. We suggest that mTERF could provide a system for coordinating the passage of replication and transcription complexes, analogous with replication pause-region binding proteins in other systems, whose main role is to safeguard the integrity of the genome whilst facilitating its efficient expression.

INTRODUCTION

The mitochondrial genome of animals is organized in a highly compact manner, with virtually no non-coding information between or within its 37 genes. The circular genome is transcribed by a phage-type RNA polymerase into polycistronic transcripts which, in mammals, encompass the entire genome on both strands (1,2). Production of these transcripts depends upon a set of closely spaced promoters located in the major non-coding region (NCR). The primary transcripts are then processed to mature mRNAs, rRNAs and tRNAs via a series of enzymatic steps requiring the tRNA-processing endonucleases RNase P and tRNAse Z, as well as other enzymes. The major coding strand (informationally the L-strand, but for the purposes of transcription conventionally referred to by the name of the template, H-strand) is transcribed from two distinct initiation sites at the heavy-strand promoter (HSP), P_{H1} and P_{H2}, separated by ~100 bp. The P_{H2}-derived precursor transcript covers virtually the entire genome and can give rise to all of the transcription products of the heavy-strand except tRNA^{Phe}, whose coding sequence overlaps the P_{H2} initiation site. The P_{H1} initiation site gives rise to a truncated transcript encompassing just the rRNAs (plus two tRNAs) and thus defines a distinct mitochondrial rDNA transcription unit.

Termination at the 3' end of the rDNA is brought about by a transcription termination factor, mTERF (3–6), which has also been proposed to interact with the RNA polymerase in initiation site selection (2,7). Recent data suggest that this involves formation of a DNA loop in which RNA polymerase complexes are recycled around the rDNA segment of the genome after terminating (7). mTERF binds sequence specifically with high affinity to a

*To whom correspondence should be addressed. Tel: +358 3 3551 7731; Fax: +358 3 3551 7710; E-mail: howard.t.jacobs@uta.fi

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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sequence element within the coding sequence of tRNA^{Leu(UUR)}, located immediately downstream of the rDNA (4). Current evidence indicates that mTERF interacts with its asymmetric-binding site as a monomer (8), although the tertiary structure of the protein and the structural basis of its interaction with DNA are unknown.

mTERF belongs to a recently identified superfamily of proteins whose functions are largely unknown (9–11). Homologues in *Drosophila* and in sea urchins have variously been implicated in transcriptional termination (12–14), regulation of DNA replication (15) and even mitochondrial protein synthesis (11). The sea urchin mTERF homologue mtDBP (D-loop-binding protein) has recently been shown to terminate transcription in a polar manner (14,16), analogous with the activity of mTERF (5). However, mtDBP is also a contra-helicase (15), and has been proposed to play a role in regulating the expansion of the short D-loop of sea urchin mtDNA and thus the initiation of productive replication of the genome.

Transcription and replication of mtDNA have long been regarded as interlinked processes. The primer for initiation of DNA replication has been assumed to be a product of transcription by the mitochondrial RNA polymerase. However, there is no consensus concerning the mechanism by which 3' ends are generated for extension by DNA polymerase, variously proposed to be RNA processing by endonuclease MRP (17) or protein-independent termination at one of the conserved sequence blocks of the NCR (18). The exact site of replication initiation is also unclear, and may vary between cell-types. A prominent cluster of 5' ends in H-strand DNA, designated as O_H, is generally regarded as the major origin of (unidirectional) replication. However, there is no direct experimental evidence that it functions thus, and bidirectional initiation clearly occurs in some molecules at sites downstream of O_H, both in cultured cells (19), especially when recovering from drug-induced mtDNA depletion (20), and in solid tissues (21,22). In a minority of molecules (21) these initiation sites can encompass the entire genome (in birds) or almost the entire genome (in mammals).

Mitochondrial DNA (mtDNA) was for over 25 years assumed to replicate by a unique, strand-asynchronous mechanism (23). However, more recent analysis of mtDNA replication intermediates (RIs) by two-dimensional neutral agarose gel electrophoresis (2DNAGE) failed to detect the extensively single-stranded products of such a replication mechanism (19–22,24–26) and instead revealed two classes of double-stranded RIs. One class consists of the predicted products of conventional strand-coupled replication (19,21,24,25); the other contains extended RNA segments (26) encompassing the entire lagging strand (RITOLS, 'RNA incorporation throughout the lagging strand', 19). Maturation of the lagging strand to DNA appears to occur with different kinetics and distinct sites of initiation in different organisms (19), and some RIs of the first class could be interpreted as molecules in which lagging-strand DNA synthesis has effectively caught up with the advancing

fork, as a result of replication pausing. The mechanism by which the RNA lagging strand is created is unknown.

mtDNA replication also depends on the HMG-box protein TFAM, named for its essential role as a cofactor for efficient and specific transcriptional initiation. TFAM is required for mtDNA maintenance (27) and appears to have several distinct roles in mtDNA metabolism. It is a major structural protein of the mitochondrial chromosome, but also influences mtDNA replication in ways connected with transcription. Overexpression of TFAM leads to a drop in mitochondrial transcript levels and a pronounced shift toward conventional, strand-coupled RIs (28). This could represent either a general slowing or stuttering of fork advance, attributable to a decreased availability of RNA to form the lagging strand (i.e. in which lagging-strand maturation frequently catches up with fork advance) or else a programmed switch to standard DNA synthesis.

As a factor affecting the outcome of mitochondrial transcription, mTERF might be expected also to have some influence over mtDNA replication if, as suggested, replication is intimately connected with transcription. We therefore embarked on a series of experiments to document the effects on mtDNA replication of modulating the expression of mTERF in cultured human cells. To this end, we set out initially to characterize better the binding specificity of mTERF, especially given recent reports of possible additional binding sites for mTERF *in vivo* (6,29). Electrophoretic mobility shift assays (EMSA) using mitochondrial protein extracts from cells induced to overexpress mTERF revealed additional, though weaker mTERF-binding sites clustered in strategically important regions of the mitochondrial genome. 2DNAGE and lagging strand 5' end mapping by ligation-mediated PCR (LM-PCR) identified the canonical mTERF-binding site as a replication pause site, with the frequency of pausing subject to the expression level of mTERF. Replication pausing in other regions of the genome, notably the NCR, was also affected by mTERF overexpression. These results indicate a role for mTERF in mtDNA replication, in addition to its role in transcription.

MATERIALS AND METHODS

Cells and cell culture

Human embryonic kidney-derived HEK293T cells, Flp-InTM T-RexTM-293 cells (Invitrogen), 143B osteosarcoma, Jurkat and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 4.5 g/l of D-glucose, 10% foetal calf serum (Sigma), 50 µg/ml uridine (Sigma) and 2 mM L-glutamine (BioWhittaker/Cambrex) at 37°C in an incubator with 5% CO₂ in air. Flp-InTM T-RexTM-293-derived cell-lines were cultured under selection with blasticidin and hygromycin according to the manufacturer's protocol. Expression of mTERF or mTERF-MycHis (C-terminally tagged) was induced in transfected Flp-InTM T-RexTM-293 cells with 10 ng/ml doxycycline (Sigma-Aldrich), which was replenished every 48 h. Cells were passaged routinely

every 3–4 days at 1:10 or 1:20 dilution. Adherent cells were detached either by pipetting alone or, for HeLa cells, by treatment with Trypsin-EDTA (Bio-Whittaker/Cambrex). Suspension cells (Jurkat) were passaged by centrifugation and resuspension in fresh medium.

Oligonucleotides and plasmids

Oligonucleotides used to create EMSA or hybridization probes by PCR from purified human mtDNA or cloned segments thereof are listed in Supplementary Table 1. The mTERF coding region, including the mitochondrial targeting signal and 24 nt of the 5' untranslated region, a total of 1221 bp, was amplified from HeLa cell cDNA (30) using the following chimeric primer pairs (all sequences shown 5' to 3', restriction sites used for cloning underlined): BamHI-mTERF F1, CGCGGATCCCCTGTTCTC CAGCCTTTCTGG plus HindIII-mTERF R1, CCC AAGCTTGGCAAATCTGCTTAACTTTTT to create an in-frame C-terminal fusion to the Myc epitope tag; BamHI-mTERF F1 plus HindIII-mTERF R STOP, CCCAAGCTTTCAGGCAAATCTGCTTAACTTTTT to create an mTERF expression construct containing the stop codon at the natural position (shown in italics, underlined, complementary strand). After sequence verification PCR products were digested with BamHI and HindIII (Fermentas, manufacturer's recommended conditions) and ligated to similarly digested pcDNA3.1(-)/Myc-His A (Invitrogen) vector DNA to create the mTERF and mTERF-MycHis expression constructs. For induced expression using the Flp-In™ T-REx™-293 cell system these plasmids were digested with PmeI (New England Biolabs), which cuts on either side of the insert, ligated into PmeI-digested DNA of the vector pcDNA5/FRT/TO (Invitrogen), and stably transfected into the recipient cells as previously (31).

DNA and siRNA transfections

HEK293T cells were transfected either with 3 µg of plasmid DNA and 30 µl of LipofectAMINE (Invitrogen) or 10 µg of DNA and 40 µl of TransFectin™ Lipid Reagent (Bio-Rad), according to manufacturers' protocols. Transiently transfected cells were subsequently harvested for different assays, or placed under selection using 2 mg/ml G418 Sulfate (Calbiochem), in order to select clones of stably transfected cells expressing mTERF-MycHis. mTERF-specific siRNAs were synthesized by means of *in vitro* transcription using the Silencer™ siRNA construction kit (Ambion). Candidate target sites for specific mTERF silencing were chosen using a prediction programme provided by Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html). One out of five tested siRNAs was found to be efficient in mTERF silencing (see Results section), the relevant target site in mTERF mRNA being nt 585–605 (5'-AAGCGGGUGAAAGCUAACAUU-3'). To knock-down mTERF expression, HEK293T cells (with or without prior stable transfection with the mTERF-MycHis expression construct) were transfected with 10 nM (final concentration) of mTERF-specific siRNA molecules using Lipofectamine™ 2000 transfection reagent (Invitrogen),

as per manufacturer's recommendations. An siRNA reagent targeted on 5'-GGAGAAGGUACGAGGGGC AUU-3' (siRNA Control) was used as a negative control.

Immunocytochemistry

For immunocytochemistry cells were grown on coverslips, seeded at low density. Twenty-four hours after transfection or induction with doxycyclin, cells were washed with DMEM and then incubated in fresh medium containing 100 mM MitoTracker® Red CMXRos (Molecular Probes) at 37°C for 10 min, then washed twice with PBS. After incubation in fresh medium at 37°C for 2 h, cells were again washed twice with PBS and fixed in 4% formaldehyde/5% sucrose in PBS at 37°C for 15 min. After three further PBS washes cells were permeabilized by incubation in 0.5% Triton X-100 in PBS at 37°C for 15 min, washed twice with PBS, incubated in blocking solution (5% w/v non-fat milk powder in PBS) at room temperature for 45 min, then again washed three times with PBS. After incubation in primary antibody solution, mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals, stock 5 mg/ml) 1:1000 in PBS for 1 h at room temperature, cells were washed three times with PBS, then incubated for 1 h at room temperature in a 1:200 dilution of secondary antibody, fluorescein-conjugated horse anti-mouse IgG (Vector Technologies, stock 1.5 mg/ml). After three final PBS washes the coverslips were mounted on slides using Vectashield with DAPI (Vector Technologies). Cells were visualized and photographed using an Olympus IX70 inverted confocal microscope at 100× magnification, with excitation at 568 nm (emission 607/45) for Mitotracker Red and 488 nm (emission 525/50) for fluorescein, using an Andor iXon DV885 front-illuminated CCD camera.

Western blotting

SDS-PAGE used 12% polyacrylamide gels under standard conditions (32). Protein extraction and western blotting were carried out essentially as described previously (30). Primary antibodies used were mouse anti-Myc monoclonal 9E10 (as above, diluted 1:15000) and rabbit anti-human mTERF antibody, custom-supplied (Invitrogen) as an anti-peptide (KLH-conjugated CSNDYARRSYANIKE) antibody, 1 mg/ml, diluted 1:5000. Kodak Biomax™ ML X-ray film was exposed to the filter membrane for between 5 s and 5 min.

Preparation of mitochondrial lysates

Cells were harvested without trypsinization, resuspended in 1 ml (per 10 cm plate of cells) of resuspension buffer (0.133 M NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 25 mM Tris-HCl pH 7.5) and centrifuged at 1200g_{max} for 2 min at 4°C. The pellet was resuspended in 0.5 ml of swelling solution (10 mM NaCl, 1.5 mM CaCl₂, 10 mM Tris-HCl pH 7.5) and incubated on ice for 15 min. After swelling, the cells were dounce-homogenized (20–25 strokes, tight-fitting pestle) on ice and breakage of the cells was checked microscopically. An equal volume of sterile filtered sucrose/EDTA buffer (0.68 M sucrose, 2 mM EDTA, 20 mM Tris-HCl pH 7.5) was added immediately after

breaking the cells. Nuclei and debris were pelleted by centrifugation at $1200g_{\max}$ for 10 min at 4°C . The supernatant was transferred to a fresh tube and centrifugation was repeated. The supernatant was collected and recentrifuged at $16000g_{\max}$ for 30 min at 4°C . The mitochondrial pellet was washed once with $200\mu\text{l}$ of PBS and frozen at -80°C or lysed immediately. For processing large quantities of cells the volumes were scaled up. Mitochondrial lysates were prepared essentially as described by Fernandez-Silva *et al.* (33), except using 'Complete, Mini protease inhibitor cocktail' (Roche) instead of PMSF.

EMSA

DNA fragments for EMSA were PCR amplified using mtDNA as template and primer pairs shown in Supplementary Table 1, followed by sequence verification of the product. dsDNA oligonucleotide probes for EMSA (Supplementary Table 1) were prepared by mixing equal amounts of complementary oligonucleotide pairs in $500\mu\text{l}$ of H_2O to a final concentration of 2mM , followed by incubation for 5 min at 100°C and cooling to room temperature on the bench. Total of 300ng of each PCR fragment or 20pmol of each dsDNA oligonucleotide were labelled using 8 U of T4 polynucleotide kinase (Fermentas) and $15\mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]$ ATP (Amersham Pharmacia Biotech, $3000\text{Ci}/\text{mmol}$) in $15\mu\text{l}$ final volume of PNK buffer (MBI Fermentas). Reactions were stopped on ice and diluted to $100\mu\text{l}$ with H_2O . EMSA was carried out in $20\mu\text{l}$ binding reactions according to Fernandez-Silva *et al.* (33) with minor modifications. Reactions contained at least $10\mu\text{l}$ of the binding buffer (25mM HEPES-KOH, pH 7.5, 12.5mM MgCl_2 , 20% glycerol, 0.1% Tween-20, 1mM DTT), 0.2pmol of labelled dsDNA oligonucleotide or 3ng of labelled PCR product as probe, $5\mu\text{g}$ of mitochondrial lysate, 100mM KCl, $5\mu\text{g}$ BSA and $5\mu\text{g}$ of non-specific competitor DNA poly(dI-dC)-(dI-dC) (Amersham Pharmacia Biotech). Reactions were incubated at room temperature for 20 min and terminated on ice with addition of 0.25 volumes of 30% glycerol. Competition EMSA reactions contained also up to 100-fold excess of the non-labelled competing probe. Supershift EMSA reactions contained $0.5\mu\text{g}$ of anti-Myc antibody (as above), or $1\mu\text{g}$ of anti-FLAG[®] M2 antibody (Sigma), which was added 30 min prior to the labelled probe. Depending on the length of the fragment, reaction products were analysed on 5–10% non-denaturing polyacrylamide TBE gels, pre-run at 4°C in $2.2\times$ TBE at 100 V for 1 h at 4°C , then run at 100 V for 30 min and 175 V for 3–5 h depending on the size of the probe fragment. Gels were dried and autoradiographed using KODAK BioMax[™] MS film.

SELEX

Creation of a randomized DNA ligand library was carried out essentially as described by Blackwell (34). The 46 nt long oligonucleotide template contained 14 internal random nucleotides, flanked on either side by 16 nt fixed ends corresponding with standard primers, containing recognition sites for BamHI and EcoRI, respectively.

Second-strand synthesis was carried out in a reaction volume of $20\mu\text{l}$ containing $1.6\mu\text{g}$ of template, $500\mu\text{mol}$ of primer, 2mM dNTPs and 5 U of Klenow fragment (Fermentas) in Klenow fragment buffer at 46°C for 1 min, followed by 37°C for 7.5 min. The reaction was stopped by heating at 75°C for 10 min and the dsDNA ligand library was gel-purified from an EtBr-stained 14% native polyacrylamide gel using the QIAEX kit (QIAGEN) according to the manufacturer's protocol. Ligand selection was carried out in $25\mu\text{l}$ reactions under essentially the same conditions as EMSA, using $10\mu\text{g}$ of mitochondrial protein lysate from mTERF-MycHis expressing Flp-In[™] T-Rex[™]-293-cells, $0.8\mu\text{g}$ of the ligand DNA and $6.25\mu\text{g}$ of non-specific competitor DNA poly(dI-dC)-(dI-dC) incubated for 20 min at room temperature. Pre-swollen anti-myc-Sepharose beads (Amersham Biosciences) were suspended in EMSA buffer (25mM HEPES-KOH, pH 7.5, 12.5mM MgCl_2 , 20% glycerol, 0.1% Tween-20, 1mM DTT, 100mM KCl, $0.2\mu\text{g}/\mu\text{l}$ BSA), washed once in the same buffer and resuspended in 1.5 volumes of the same buffer containing $0.25\mu\text{g}/\mu\text{l}$ poly(dI-dC)-(dI-dC). To each binding reaction was added $100\mu\text{l}$ of the bead suspension, followed by gentle rotation for 2 h at 4°C . Beads were then washed in EMSA buffer containing $0.25\mu\text{g}/\mu\text{l}$ poly(dI-dC)-(dI-dC), followed by a further seven times in the buffer without poly(dI-dC)-(dI-dC) and gentle rotation overnight in $100\mu\text{l}$ of K buffer (10mM Tris-HCl, 0.5mM EDTA 50mM NaCl, pH 8.0) containing $100\mu\text{g}/\text{ml}$ of freshly dissolved proteinase K (Fermentas). DNA was recovered from the beads by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitation, washed once with 70% ethanol and resuspended in a minimal volume of H_2O ($\sim 7.5\mu\text{l}$). PCR was then carried out using $2\mu\text{l}$ of this template in a $50\mu\text{l}$ reaction volume containing $0.2\mu\text{M}$ of each SELEX primer (GGTGAATTCGCTCACG and GAACGGATCCCTTTCG, both shown 5' to 3', with restriction sites for cloning underlined) and 2.5 U of Pfu DNA polymerase (Promega). Thirty amplification cycles were carried out using a 15 s extension step, after which the enriched ligand DNA was gel-purified from an EtBr-stained 12% native polyacrylamide gel as above. After seven such enrichment cycles, the ligand DNA was cloned into pCR[®]4Blunt-TOPO[®] vector (Invitrogen) and individual clones were sequenced using standard primers on an ABI 3100 sequencer using the BigDye[®] Terminator v3.1 cycle sequencing kit (Applied Biosystems).

DNA extraction and mtDNA copy number estimation

For the preparation of mtDNA (mitochondrial nucleic acids) for analysis of RIs from cultured cells, mitochondria were isolated and processed as described by Pohjoismäki *et al.* (28). Total DNA for analysis of mtDNA copy number was extracted from cells by standard methods (35), and copy number was determined using quantitative PCR, as described previously (28), with amyloid precursor protein (APP) as a single-copy nuclear DNA standard. Human placental mtDNA was prepared as previously (24). Total DNA for analysis of mtDNA RIs

was extracted from frozen human tissue blocks (~7 × 7 × 7 mm³) obtained via forensic autopsies. The samples were taken as part of the Tampere Coronary Study, approved by the Ethics Committee of Tampere University Hospital (DNO 1239/32/200/01) and the National Authority for Medicolegal Affairs. Heart, brain, skeletal muscle and kidney tissue samples were cut into thin slices with a sterile blade and suspended in 2 ml DNA extraction buffer (28). One-tenth volume of 10% SDS and 0.5 mg proteinase K were added. The crude homogenate was passed several times through a 5 ml pipette tip with a sawn-off end, to disperse the larger tissue fragments. The homogenate was incubated overnight with gentle swirling at 37°C. After incubation, 2 volumes of phenol–chloroform–isoamylalcohol (25:24:1) were added, and the mixture was shaken gently for 1 h. The mixture was then transferred to Eppendorf 15 ml Phase Lock Gel™ Heavy tubes and centrifuged at 5000g_{max} for 15 min. The aqueous phase was recovered and the extraction step repeated. DNA was precipitated by the addition of 0.2 volumes of 10 M ammonium acetate and 2 volumes of ethanol. The mixture was incubated on ice for 10 min and DNA was spooled out using a glass rod, washed once with 70% ethanol, air dried gently and resuspended in 300–700 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), depending on the pellet size. 2DNAGE analysis used 10 µg aliquots of heart and brain DNA and 20 µg aliquots of kidney and skeletal muscle DNA.

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 treatment with S1 nuclease was used, DNA was first recovered by ethanol precipitation and resuspended in the appropriate reaction buffer, before treatment with 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 as described previously [(28), note different gel conditions for fragments in different size classes].

Radiolabelled probes and blot hybridization

For Southern hybridization, probes were created by *Pfu*-PCR, using cloned segments of human mtDNA as template (see Supplementary Table 1), and subsequently sequenced to confirm their identity. Probes were labelled using Rediprime™ II random prime labelling kit (Amersham) and [α -³²P] dCTP (Amersham; 3000 Ci/mmol).

LM-PCR

LM-PCR was carried out as described by Yasukawa *et al.* (20), using oligonucleotide primer sets as indicated in figure legends and as detailed in Supplementary Table 2.

Mitochondrial DNA immunoprecipitation

Cells were processed for mitochondrial DNA immunoprecipitation (mIP) essentially as described by Lu *et al.* (36). The mtDNA was sheared to fragments of average size 500–600 bp using a Sonics Vibra-Cell sonicator, 3 mm tip at 25% power for 3 × 20 s (1 s on, 1 s off) with incubation on ice for 30 s between. Complete, Mini protease inhibitor cocktail (Roche) was included in the lysis buffer. Lysates were pre-cleared with pre-swollen Protein A Sepharose (Amersham Biosciences) and immunoprecipitations were carried out with 5 µg mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals) or anti-FLAG® M2 antibody (Sigma) overnight at 4°C. Final PCR reactions used primers listed in Supplementary Table 1 and the minimum number of amplification cycles required to generate substantial product bands from the input DNAs (generally 25–26 cycles, depending on the fragment, based on preliminary tests), thus avoiding saturation.

RESULTS

mTERF has multiple binding sites in the human mitochondrial genome

In order to study the effects of mTERF on mtDNA replication, we established HEK293-derived cells expressing both natural mTERF and C-terminally Myc epitope-tagged mTERF. Mitochondrial targeting was verified by immunocytochemistry of transiently transfected HEK293T cells expressing mTERF-MycHis (Figure 1a). Induction of protein expression in Flp-In™ T-Rex™-293 cells stably transfected with the mTERF or mTERF-MycHis constructs was verified by western blotting (Figure 1b). Protein levels were the same after 24 or 48 h of induction. Prolonged overexpression of mTERF (6 days) had no significant effect on mtDNA copy number as estimated by Southern blotting (data not shown) or by quantitative PCR (Supplementary Figure 1a).

Since the main aim of the study was to determine the effects of altered mTERF expression on mtDNA replication *in vivo*, we first tested the effects of mTERF expression on protein binding to mitochondrial DNA, using EMSA with mitochondrial protein extracts from cells overexpressing mTERF. In contrast to earlier studies using purified, bacterially expressed mTERF, this tests the effects of altered mTERF expression level on protein–DNA interactions in the mitochondrial milieu, in which other mitochondrial proteins, including TFAM, are present and may influence binding.

Using EMSA with probes covering the previously identified, canonical mTERF-binding site in the tRNA^{Leu(UUR)} gene, we confirmed that overexpression of natural mTERF, whether by transient or stable transfection (data not shown), or under tetracycline induction (Figure 1c), leads to a large increase in sequence-specific binding activity. The protein complex formed from the Myc epitope-tagged protein migrated slightly slower than the complex formed by endogenous

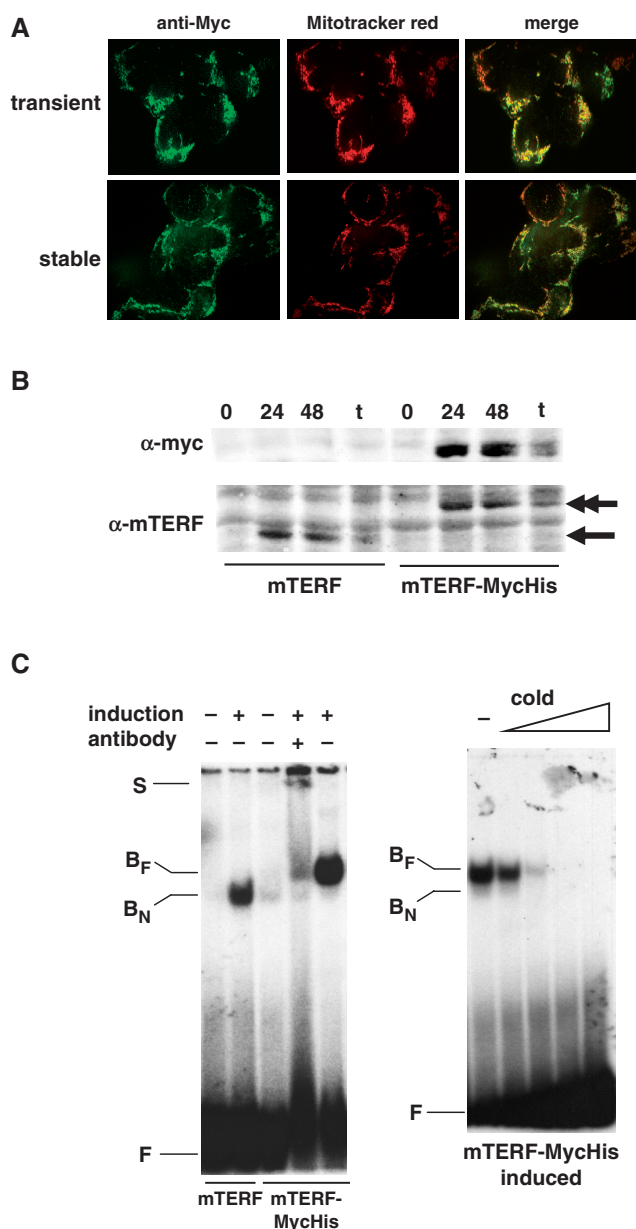


Figure 1. Overexpression of mTERF in cultured cells. (A) Immunofluorescence of HEK293T cells transiently or stably transfected with mTERF-MycHis, using anti-Myc monoclonal antibody, counterstained with Mitotracker Red. (B) Western blots of mitochondrial protein extracts from Flp-InTM T-RExTM293 cells transfected with the mTERF or mTERF-MycHis constructs and induced for expression as indicated (0, 24, 48 h) or from transiently transfected (t) HEK293T cells, probed with anti-Myc or anti-mTERF antibodies, as indicated. The endogenous mTERF protein detected by the anti-mTERF antibody is singly arrowed. The mTERF-MycHis fusion protein detected by the same antibody is indicated by a double arrow. (C) EMSA using Leu-short dsDNA oligonucleotide probe and mitochondrial protein extracts from Flp-InTM T-RExTM293 cells transfected with the mTERF or mTERF-MycHis constructs and induced for expression as indicated. EMSA was carried out with or without anti-Myc antibody as shown (left-hand panel), or (right-hand panel) in the presence of an increasing amount of cold Leu-short dsDNA oligonucleotide competitor (1-, 10-, 100- and 1000-fold mass excess) or without competitor (-). The free probe (F), complexes formed by natural mTERF (B_N) or the mTERF-MycHis fusion protein (B_F), and the antibody-supershifted complex (S) are indicated. See also Supplementary Figure 1.

or overexpressed natural mTERF, and was supershifted by an anti-Myc monoclonal antibody (Figure 1c), but not by other antibodies (e.g. anti-FLAG, Supplementary Figure 1b). The anti-Myc antibody did not supershift the complex formed by endogenous or overexpressed natural mTERF (Supplementary Figure 1b).

These properties next allowed us to test other regions of the mitochondrial genome for specific binding of mTERF to DNA, using EMSA. Using overlapping fragments of ~150 bp, we scanned the major NCR and its flanking sequences, the minor NCR (O_L), its surrounding tRNA gene cluster, the region extending from O_L to the canonical mTERF-binding site in the tRNA^{Leu(UUR)} gene, the ATPase 6 gene and its junction with the COIII gene and several other segments of the genome (Figure 2, Supplementary Figures 2 and 3). We estimated relative binding affinities using competition EMSA against the tRNA^{Leu(UUR)} gene fragment and vice versa. As shown in Figure 2 and Supplementary Figure 2, we identified a cluster of four moderately strong mTERF-binding sites within the ND1 coding sequence and the adjacent IQM tRNA gene cluster (see Figure 2e for summary). Competition EMSA indicated that the binding to fragment ND1.1 (Figure 2b) was between one and two orders of magnitude weaker than to the canonical binding site in tRNA^{Leu(UUR)}. Binding to the ND1.1 fragment was tested further, using shorter, overlapping fragments (Supplementary Figure 2d). The results suggest that fragment ND1.1 contains two distinct binding sites. We also identified a binding site adjacent to O_H (fragment OH1) at least two orders of magnitude weaker than the canonical binding site, based on competition EMSA data (data not shown), as well as four other binding sites in the D-loop portion of the NCR and one at O_L, plus a possible site at the HSP (fragment OH5, see Supplementary Figure 2e).

Alignment of the sequences of these binding sites suggested a consensus which was verified by SELEX (Table 1). Most of the SELEX output clones analysed (82/109) contained at least one match to the consensus TGGT or TYGGT, and 43 clones showed an identical or almost identical (8/9) match to the extended consensus TGGT(N₅)TYGGT (or its complement). Of 28 control clones analysed, subjected to the same number of amplification cycles but without antibody selection, none matched this consensus. Comparing the SELEX consensus with the canonical mTERF-binding site in the tRNA^{Leu(UUR)} gene, and with the findings of an earlier application of PCR-based selection on a smaller scale using only EMSA (37), the invariant features of the binding site would appear to be two pairs of G residues on the same strand, separated by eight nucleotides (see also Supplementary Table 3).

In order to verify that mTERF is able to bind to at least some of its non-canonical binding sites *in vivo*, we carried out semi-quantitative mIP, using a minor adaptation of the method recently published by Lu *et al.* (36). For this assay we used cells inducibly expressing mTERF-MycHis, and carried out immunoprecipitation using anti-Myc antibody, as well as a control antibody (anti-FLAG) or no antibody.

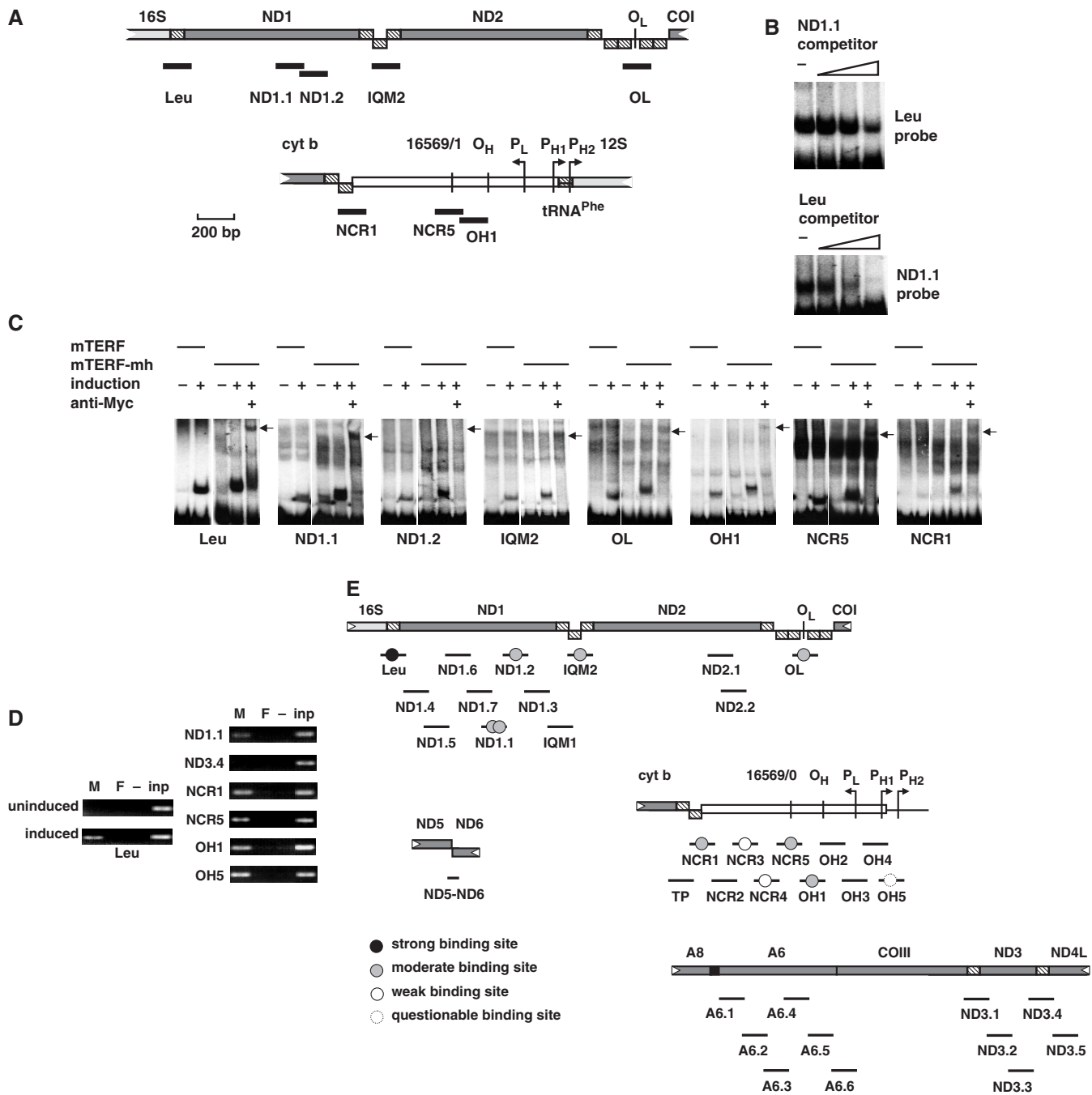


Figure 2. EMSA and mIP analysis of alternate mTERF-binding sites in human mtDNA. (A) Schematic diagram of regions of the mitochondrial genome in which binding was detected, showing NCR (white box), 16S and 12S rRNA genes (pale grey boxes), protein-coding genes ND1, ND2, COI and cyt b (darker grey boxes), tRNA genes (cross-hatched boxes), O_H, O_L and the promoters/transcriptional initiation sites of the two strands (P_L, P_{H1} and P_{H2}). Genes transcribed to the right shown above the centre line, genes transcribed to the left shown below. Nucleotide coordinates are as Ref. (82). Black bars indicate the positions of the 150 bp probe fragments which were found by EMSA to contain strong or moderate binding sites for mTERF, as shown in panels b and c. (B) Competition EMSA using the probes and competitors as shown, plus mitochondrial protein extract from cells induced to express mTERF-MycHis. The amounts of cold competitor represent 1-, 10- and 100-fold mass excess over the probe. Similar results were obtained using extracts from cells overexpressing natural mTERF (data not shown). (C) EMSA analyses of binding to 150 bp probe fragments as indicated, using mitochondrial protein extracts from Flp-InTM T-RExTM-293 cells transfected either with natural mTERF or with mTERF-MycHis (mTERF-mh) and induced for expression (or not) as indicated. Supershifted complexes are denoted by arrows. Although the supershifted complex is minor in some cases, the main complex is always efficiently removed by the antibody, confirming the presence of mTERF-MycHis. Other antibodies tested (e.g. anti-FLAG) gave no supershifting and did not inhibit the formation of these complexes. For further experiments confirming specificity of binding and negative/weak findings using other fragments, see Supplementary Figures 1 and 2. (D) mIP analysis of mTERF-MycHis binding *in vivo*. Immunoprecipitation used anti-Myc (M), anti-FLAG (F) or no antibody (-). Amplification of immunoprecipitates alongside corresponding input DNAs used the same primer pairs as were employed to generate the corresponding fragments for EMSA (see Supplementary Table 1), Samples were from Flp-InTM T-RExTM-293 cells induced for mTERF-MycHis expression, except for fragment Leu, where extracts from uninduced cells were also tested.

Induction of mTERF-MycHis expression enabled immunoprecipitation of several key fragments of the mitochondrial genome in which binding was found *in vitro* (Figure 2d). The fragment containing the canonical mTERF-binding site in the tRNA^{Leu(UUR)} gene was routinely detected in the anti-Myc immunoprecipitate from induced cells, but was not immunoprecipitated by control antibody (anti-FLAG) or no antibody. Immunoprecipitates from uninduced cells were negative under comparable conditions, but using excess anti-Myc antibody we sometimes observed weak amplification of this fragment (data not shown), consistent with a low level of leaky expression of the mTERF-MycHis transgene and the high affinity of the protein for the canonical binding site. Consistent positive signals were also seen in the anti-Myc immunoprecipitate from induced cells, but not control immunoprecipitates, for the HSP-containing fragment OH5 and for the three D-loop fragments (NCR1, NCR5 and OH1) which gave the strongest EMSA signals *in vitro* (Figure 2c). The ND1.1 fragment internal to the ND1 coding sequence was also weakly amplified from anti-Myc immunoprecipitates from induced cells (Figure 2d). Fragments from the ND3 gene (e.g. ND3.4), or others which were negative for binding *in vitro* using EMSA, gave either very faint signals or no signal at all after immunoprecipitation. Overall, these findings are consistent with the proposition that mTERF, when overexpressed, can bind *in vivo* to specific, non-canonical binding sites, which correspond with binding sites detected *in vitro*.

Replication pause sites map close to sites of mTERF binding in human mtDNA

In previous studies using 2DNAGE we noted the occurrence of a number of stereotypic pause sites in mitochondrial DNA of both sea urchins (38) and vertebrates (21,24). In sea urchins, pause sites correspond with sites of specific protein binding (39–41). We therefore considered the hypothesis that some of the replication pause sites in human mtDNA may map to locations of mTERF binding.

We initially analysed the region of the genome in which the canonical mTERF-binding site in the tRNA^{Leu(UUR)} gene is located. 2DNAGE analysis of the PvuII–AccI fragment covering this site, extending from O_L into the rDNA, in several different cell lines and tissues (Figure 3), revealed a number of pause sites of varying prominence. To visualize their positions more clearly we treated parallel samples with S1 nuclease, thus digesting partially degraded RITOLS intermediates, including any attached RNA tails. The two epithelia-derived cell-lines, HEK293T

and HeLa, gave very similar patterns, with a clear, though relatively weak pause site signal in the region of the tRNA^{Leu(UUR)} gene (designated ‘a’ in Figure 3b), a second, more prominent pause located in the 3’ part of the ND1 gene or in the adjacent IQM tRNA cluster (designated ‘b’), a third, near O_L (designated ‘d’), and accumulated material in a broad region of ND2 (designated ‘c’).

In 143B (osteosarcoma) and Jurkat (T-cell leukaemia) cells the steady-state abundance of all mtDNA RIs was quantitatively less, though the patterns were qualitatively similar to those seen in HEK293T or HeLa cells. In S1-untreated material the pause sites were poorly resolved, and the descending segment of the Y-arc was very weak. Region ‘c’ was not seen as a discrete species, even after S1 treatment. Following S1 treatment, the ratio of the other pauses differed between cell-types: for example, pause ‘b’ was much more prominent than pause ‘d’ in Jurkat cells, whereas in 143B cells they were at similar abundance. The tRNA^{Leu(UUR)} gene pause ‘a’ was seen clearly in all cell-lines tested.

In tissue samples (Figure 3c), pause ‘a’, near to the canonical mTERF-binding site, was most prominent in the brain, but weak in other tissues tested. Pause ‘d’ was more prominent than pause ‘b’ in heart and brain, but weaker than pause ‘b’ in skeletal muscle and in kidney. Pause ‘c’, was seen only in kidney, where both it (and pause ‘b’) appeared to be even more diffuse than in other tissues or cell-lines. An additional pause site was seen in brain, between ‘c’ and ‘d’ (denoted ‘d’). Pause ‘d’, near O_L, was also detected as an extended pause region in human placenta [(24), Supplementary Figure 3].

Pausing near two other sites at which mTERF binding to DNA was seen both *in vitro* and *in vivo* (Figure 2), namely O_H and the TAS region, is already well documented from previous studies, and further examples are seen in Figure 4 (see also Supplementary Figures 3 and 4). Although originally proposed as a unique unidirectional origin, recent data indicate that O_H also functions as a site of fork arrest when bidirectional replication initiates elsewhere, and may thus also be considered as the terminus of replication [(20–22,24), see also Supplementary Figure 3]. The TAS region is, by definition, adjacent to the termination site for the synthesis of D-loop 7S DNA.

mTERF overexpression enhances replication pausing in human mtDNA

In order to test whether the level of mTERF expression influences replication pausing we carried out 2DNAGE

(E) Summary of EMSA results combining the data from this figure, Supplementary Figure 2, and other (negative) data not shown. The regions of the genome which were probed are reproduced from part (a) of the two figures, plus the ND5-ND6 gene junction which was probed using a dsDNA oligonucleotide. Binding is denoted as strong (filled circles), moderate (grey circles), weak (open circles), questionable (dotted circle, fragment OH5, as discussed in the text and legend to Supplementary Figure 2) or absent (no circles). Our inference of binding is based on the fact that EMSA signals were enhanced by induction of expression of both mTERF and mTERF-MycHis, that the complexes migrated at slightly different positions consistent with the presence of the epitope tag in the latter case, and that the complexes formed by mTERF-MycHis were supershifted by the anti-Myc antibody. The assertion that binding is strong, moderate or weak is based either on actual competition experiments (OH1 and ND1.1), or simply on the strength of the EMSA signal. Where the above criteria were not fulfilled, binding was scored as negative. In summary, the ND1 coding region and following IQM tRNA cluster contain at least four weak binding sites for mTERF. The NCR contains three weak binding sites, as well as three additional sites which showed very weak or questionable mTERF binding, as shown. A weak binding site was also found in the vicinity of O_L.

Table 1. SELEX analysis of the mTERF-binding site

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Occurrences ^a														
A	0	0	0	3	30	17	23	21	1	0	0	0	0	2
C	0	0	0	7	4	6	1	3	0	0	15	0	0	0
G	8	43	43	2	4	16	6	16	31	0	0	43	43	0
T	35	0	0	31	5	4	13	3	11	43	28	0	0	41
Consensus ^b	t	G	G	t	a	r	a	r	g	T	Y	G	G	t

^aOut of 43 clones analysed which matched a clear consensus (see text).

^bNucleotides found in 43/43 clones shown in upper case, others in lower case, Y = pyrimidine, R = purine.

analysis of RIs in mtDNA extracted from cells induced to overexpress mTERF, compared with uninduced cells (Figure 4, Supplementary Figure 4). Within the 3.6 kb PvuII–AccI ND2-containing fragment (Figure 4b), pause site ‘a’ (tRNA^{Leu(UUR)}, mapping near the canonical site of mTERF binding) was strongly enhanced by mTERF overexpression, compared with the unit-length restriction fragment (denoted 1n in Figure 3d). Pause sites ‘b’ (ND1/IQM tRNA cluster) and ‘d’ (O_L) were also enhanced, as was the more diffuse pause region ‘c’. In mTERF-overexpressing cells we also detected a more prominent X-form intermediate (designated ‘x’ in Figure 4b, Supplementary Figure 4a) in restriction fragments (e.g. HincII or AccI) containing the tRNA^{Leu(UUR)} gene at a central location.

Within the NCR mTERF overexpression enhanced the abundance of a paused intermediate migrating near or beneath the bubble arc (designated ‘f’ in the HincII fragment and ‘n’ in the AccI fragment, O_H probe), as well as the arc leading to it from the unit-length fragment (Figure 4c, Supplementary Figure 4b). These forms were sensitive to S1 nuclease (Supplementary Figure 4b) and are probably equivalent to the classical D-loop. mTERF overexpression also appeared to increase the relative amount of 7S DNA as well as introducing subtle alterations to the various forms of mtDNA resolved on 1D gels (Supplementary Figure 4f). mTERF overexpression also diminished the relative abundance of termination intermediates (designated ‘t’ in Figure 4c and d) and increased that of Y-form intermediates in which a single fork appears to have paused when approaching O_H (designated ‘g’). The distribution of material on the termination arc also appeared to be subtly different from that of uninduced cells.

In other regions, a prominent pause site (‘h’, Figure 4c), located near to the ND5/ND6 gene boundary, was unaffected by mTERF overexpression, whereas a novel pause was induced in the coding region of ND3 (Supplementary Figure 4c). Note, however, that strong mTERF binding was not found *in vitro* in either region (Supplementary Figure 2 and other data not shown).

Digestion of mtDNA from mTERF-overexpressing cells with restriction enzymes having only a single recognition sequence in the genome generated 2DNAGE patterns consistent with enhanced pausing in the region of ND1/tRNA^{Leu(UUR)} and consequent delayed resolution in the NCR (Figure 4d, Supplementary Figure 4d and e). Note that mTERF overexpression produced subtle, site-specific effects, rather than a

general slowing of replication e.g. as would be attributable due to non-specific stalling.

mTERF knockdown diminishes replication pausing in the ND1/tRNA^{Leu(UUR)} region

To test whether the modulation of replication pausing resulting from mTERF overexpression represents the signature of a finely tuned physiological process rather than just an overexpression artefact, we downregulated the expression of mTERF by RNA interference. This produced a reciprocal effect on mtDNA replication pausing at the canonical mTERF-binding site. We first tested several different mTERF-directed siRNAs in transient transfection assays, using cells stably transfected with the mTERF-MycHis expression construct, enabling us to evaluate knockdown at the protein level by western blotting (Figure 5a). One particular siRNA (mTERF.1) gave consistently strong knockdown, as judged also by immunocytochemistry on mTERF-MycHis-expressing cells (Figure 5b) and EMSA (Figure 5c). Based crudely on the autoradiographic EMSA signals, functional knockdown of >90% was routinely achieved 48 h after transfection with siRNA mTERF.1. The effects of mTERF knockdown on replication pausing in the ND1/tRNA^{Leu(UUR)} region were then studied using 2DNAGE (Figure 5d). Pause site ‘a’ (tRNA^{Leu(UUR)}) was no longer detectable, even on long autoradiographic exposure, and the prominence of X-forms was also diminished by mTERF knockdown. The abundance of other pauses was altered less substantially, though the relative amount of species ‘b’ compared with ‘c’ appeared to be decreased.

mTERF overexpression enhances lagging strand 5' ends near to specific replication pause sites

One signature of increased replication pausing during strand-coupled DNA replication should be the enhancement of persistent, lagging strand 5' ends adjacent to pause sites (Supplementary Figure 5). We used LM-PCR to map such ends in the vicinity of the major pauses regulated by mTERF, and to determine the effects upon them of mTERF overexpression. Comparing mtDNA from cells overexpressing mTERF with that from uninduced cells, we analysed 5' ends on the L-strand near the canonical tRNA^{Leu(UUR)} binding site, as well as in the whole of ND1 and the adjacent tRNA genes, and also on the H-strand in the NCR. A cluster of L-strand 5' ends in or adjacent to the tRNA^{Leu(UUR)} gene, notably at np 3211,

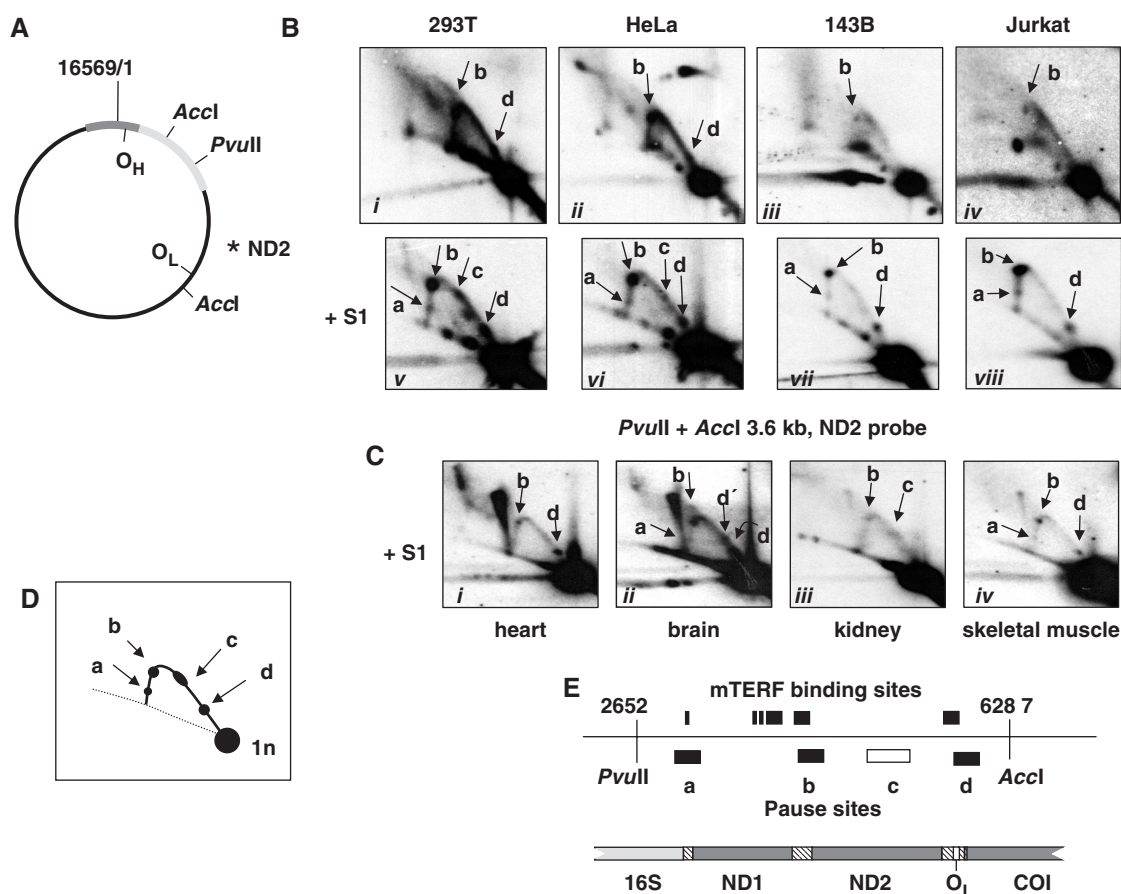


Figure 3. 2D-NAGE analysis of replication pausing in human mtDNA (region spanning from 16S rDNA through O_L). (A) Schematic map of human mtDNA showing relevant restriction sites, O_H , O_L , the approximate locations of the probe used (ND2, see Supplementary Table 1), denoted by an asterisk, the NCR (bold, dark grey) and rDNA (bold, pale grey). (B) 2D-NAGE analysis of ND2-containing (3.6 kb) PvuII–Accl fragment from four human cell-lines as indicated, with and without treatment, after digestion, with S1 nuclease. Prominent pause sites ‘a’–‘d’, arrowed, schematized in part (D). A prominent intermediate, lying below the Y-arc between ‘c’ and ‘d’, is a product of S1 nuclease digestion, and probably represents a paused species containing a single-stranded gap. (C) 2D-NAGE analysis of the 3.6 kb PvuII–Accl fragment from four human autopsy tissues as indicated, treated with S1 nuclease after digestion. Prominent pause sites ‘a’–‘d’, arrowed, schematized in part (D). (E) Map of the 3.6 kb PvuII–Accl fragment showing the approximate locations of the pause sites and mTERF-binding sites, with the gene locations in the region (16S rRNA in light grey, ND1, ND2 and COI protein-coding genes in dark grey, tRNAs cross-hatched, non-coding DNA at O_L in white) shown below. Limits of the mTERF-binding sites are based on the EMSA data of Figure 2 and Supplementary Figure 2. Approximate locations of pause sites extrapolated from first-dimension migration, calibrated by positions of 1n, 2n, linear partials and apex of Y-arc. Depending on compression artefacts, actual pause sites may be located up to 150 bp further from the ends of the segments as shown. Pause ‘c’ (open box), when present, appears to extend over a wider segment than the other pauses.

3234 and 3310, were strongly enhanced during 72 h of induction of mTERF overexpression (Figure 6a, Supplementary Figure 5b). In the vicinity of the IQM tRNA gene cluster, LM-PCR revealed L-strand 5' ends enhanced by mTERF overexpression at np 4476 (close to the 5' end of ND2 mRNA) and np 4434 (within the tRNA^{Met} gene), against a background of heterogeneous 5' ends that were generally unaffected by mTERF overexpression (Figure 6b and c). The significance of this heterogeneous background of abundant 5' ends is unclear, although the two sites enhanced by mTERF overexpression lie in the vicinity of pause ‘b’. Within the remainder of the ND1 and ND2 coding regions (Supplementary Figure 5c–h) we detected only weak LM-PCR signals which were not affected by mTERF overexpression. The prominent 5' ends in the tRNA^{Cys} gene adjacent to O_L were also unaffected by mTERF overexpression.

In the NCR, H-strand 5' ends at O_H , as well as those clustered in the distal region of the D-loop (np 16311, 16337, 16370, 16411 and more weakly at np 16197) were strongly induced by mTERF overexpression. This is consistent with delayed resolution, arising from more frequent pausing at the canonical mTERF-binding site. H-strand 5' ends in the NCR are on the lagging strand for initiation events outside of the NCR, and thus may also be enhanced by pausing of replication forks entering the NCR from the ‘cytochrome *b* side’.

DISCUSSION

mTERF is a modulator of replication as well as transcription

In this study, we showed that mTERF binding at its canonical binding site in the tRNA^{Leu(UUR)} gene

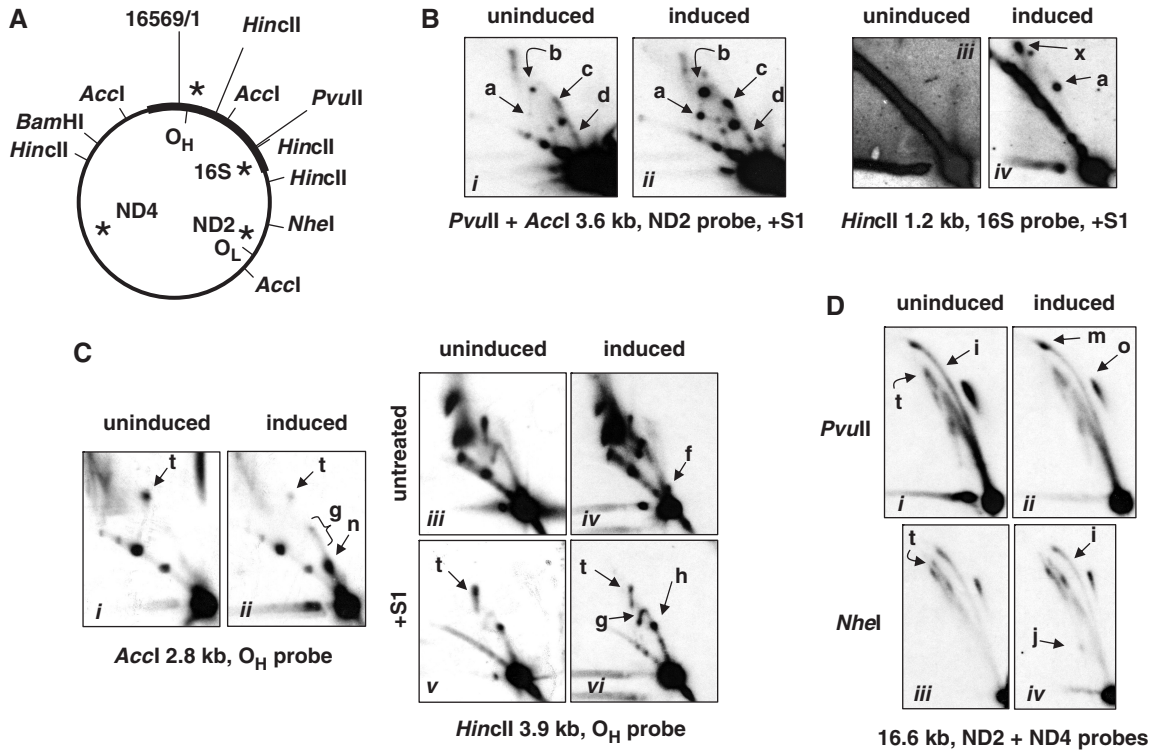


Figure 4. 2DNAGE analysis of mtDNA replication pausing in mTERF-overexpressing cells. (A) Schematic map of human mtDNA using same nomenclature as Figure 3a, showing relevant restriction sites and approximate locations of probes. (B–D) 2DNAGE analysis of mtDNA from cells induced to overexpress mTERF, compared with uninduced cells. To facilitate visualization of pause sites, samples were treated with S1 nuclease following restriction digestion, where indicated. Pause sites 'a'–'d' arrowed, plus other species as discussed in text. The exposures of the uninduced and induced panels *i* and *ii* of (b) were adjusted for comparability, based on loading, after measuring phosphorimager signals from the unit-length fragment. Pause site 'h', as visualized in part (c), appears to be unrelated to mTERF expression. In part (d), the paused bubble, 'm' (PvuII digest), and the highly asymmetric double-Y species 'j' (NheI digest), both enhanced relative to other species by mTERF overexpression, are predicted products of pausing in the ND1/tRNA^{Leu(UUR)} region. Subtle modifications are also seen in the region in which X, double-Y and broken theta molecules migrate, notably a decrease in the abundance of putative termination intermediates 't' (see also Supplementary Figure 4). 'o'—uncut circles and gapped circles.

influences replication pausing near to this site. Overexpression of mTERF enhanced this pausing and increased the steady-state abundance of lagging strand 5' ends adjacent to the binding site, whereas mTERF knockdown by RNA interferences decreased pausing in the tRNA^{Leu(UUR)} gene region. In addition, based on studies *in vitro* (EMSA, SELEX) and supported by findings *in vivo* (mIP), we identified novel sites of mTERF binding, elsewhere in the genome. Binding at these sites was weaker than in the tRNA^{Leu(UUR)} gene, but replication pausing in these regions was nevertheless influenced by mTERF overexpression. The data support a role for mTERF as a modulator of replication, especially at its canonical binding site. Close parallels with the properties of replication pause-region binding proteins in bacteria and eukaryotic nuclei provide some intriguing hypotheses which we now discuss.

mTERF binding to additional sites in human mtDNA

The present study revealed novel sites of mTERF binding in the NCR and ND1 gene. mIP assays were consistent with binding at least at some of these sites by overexpressed mTERF *in vivo*. mTERF homologues in invertebrates, such as DmTTF in *Drosophila* (12) or

DBP in sea urchins (39,41), also have diverse and multiple binding sites, typically demarcating the 3' ends of oppositely transcribed gene clusters. Although mTERF binding to the novel binding sites appeared weaker than at the canonical binding site, many of them are clustered, suggesting that cooperative binding might promote site occupancy *in vivo*, consistent with the results of mIP. The binding we observed *in vivo* might also depend on other mitochondrial nucleoid proteins, including TFAM, as well as possible post-translational modifications.

The effects of mTERF overexpression on mtDNA RIs from the NCR (Figure 4c and d), suggest that mTERF may interact with this region *in vivo* to promote fork arrest at the replication terminus. The termination zone for mtDNA replication appears not to be a single point (O_H), but an extended region of the NCR (Figure 4c). mTERF overexpression resulted in increased stalling of replication forks as they approach O_H from the 'cytochrome *b* side'. It also appeared to elevate the abundance of 7S DNA (Supplementary Figure 4f) and of S1 nuclease-sensitive species probably equivalent to the classical D-loop (Figure 4c), consistent with increased pausing in the TAS region. Protein-binding sites within the TAS region were previously mapped by *in vivo* footprinting (42) and by EMSA (43), and mTERF might be one of the

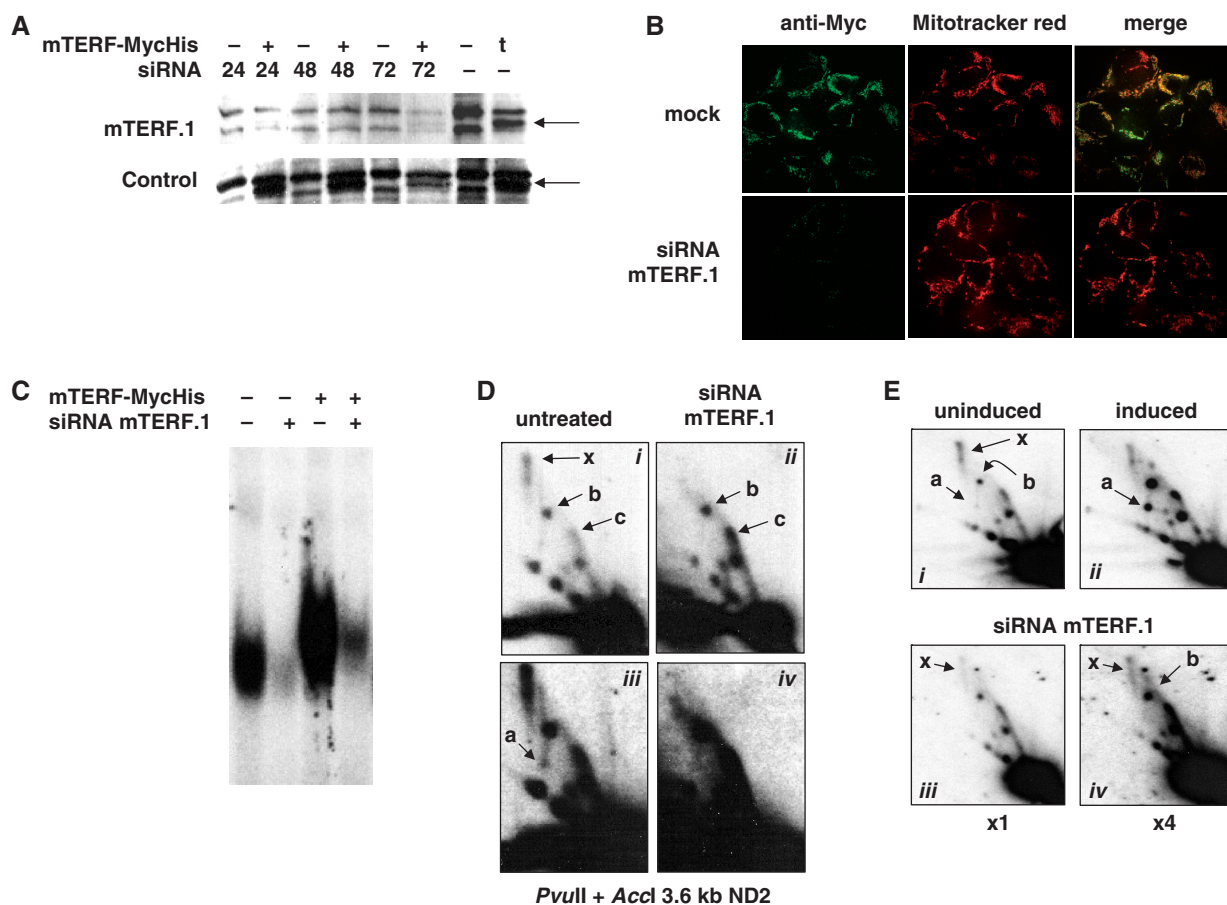


Figure 5. Downregulation of mTERF expression by RNA interference. (A) Western blot assay of mTERF knockdown by siRNA mTERF.1 (directed against mTERF mRNA) and siRNA Control. HEK293T cells were either untransfected (–), transiently transfected (t) or stably (+) transfected with an mTERF-MycHis expression construct. Cells were then assayed 24, 48 and 72 h following siRNA transfection or else without such transfection (–). The arrowed band is the mTERF-MycHis fusion protein, migrating between two background bands which appear in all westerns and thus provide an internal loading control. Note that the sample from untransfected, non-siRNA-treated cells in the upper panel (penultimate lane) is approximately 3-fold overloaded. (B) Immunocytochemistry of HEK293T cells stably transfected with mTERF-MycHis expression construct and then either mock transfected or transiently transfected with siRNA mTERF.1. Immunocytochemistry used the anti-Myc monoclonal antibody, and counterstaining with Mitotracker Red. (C) EMSA using Leu-short dsDNA oligonucleotide probe and mitochondrial protein extracts from HEK293T cells with or without stable transfection of mTERF-MycHis expression construct, followed by transient transfection for 48 h with or without siRNA mTERF.1. Despite the apparent difference in signal, the experimental conditions are the same as in Figure 1c: only the exposure time is different, and the amount of background signal in the gel. (D) 2DNAGE of mtDNA from untreated HEK293T cells or cells transfected with siRNA mTERF.1 for 48 h. PvuII + AccI digest (S1 treated) probed for the 3.6 kb fragment using ND2 probe. Panels *iii* and *iv* are longer exposures of panels *i* and *ii*, respectively. Note the down-regulation of the X-spike (*x) and pause site 'a', as well as of pause site 'b' relative to pause region 'c' (see Figure 3). (E) Phosphorimager-calibrated exposures of 2DNAGE blots from siRNA-treated cells (panel *iii*) alongside the corresponding images (panels *i* and *ii*) from uninduced and induced mTERF over-expressing cells, reproduced from Figure 4b. A longer exposure (panel *iv*) confirms the absence of pause 'a'.

proteins involved. The D-loop remains enigmatic. DNA synthesis arrest at TAS might be a switching mechanism relevant to copy number control, or may have other purposes, such as mitochondrial nucleoid organization (44).

The *Escherichia coli* Tus protein, which regulates the termination of chromosomal DNA replication, may represent a useful paradigm for mTERF. Tus binds multiple copies of the *Ter* sequence flanking the terminator region, and acts directionally as a contrahelicase (45) to trap replication forks in this region (46). The sea urchin mTERF homologue DBP has also been shown to function as a contrahelicase *in vitro* (15). Like Tus, mTERF binding to sites on both sides of the replication terminus region might regulate the entry of oppositely moving replication forks into the region, facilitating their

orderly synopsis. Increased mTERF expression resulted in elevated levels of persistent H-strand 5' ends in the NCR (Figure 6d), an expected signature of delayed resolution if fork passage through rDNA is more restricted. The orientation of potential mTERF-binding sites in the genome appears highly non-random. Taking the simplified sequence GG(N₈)GG as the minimal binding site, its 12 occurrences in the NCR all bear the same orientation. The same applies to the cluster of seven such sites in the 3' portion of ND1 and the adjacent tRNA gene cluster. In contrast, the canonical binding site shows the opposite orientation, although is flanked on each side by two oppositely oriented copies of the minimal binding site (Supplementary Figure 6).

Although we detected only ambiguous mTERF binding *in vitro* in the H-strand transcriptional initiator region,

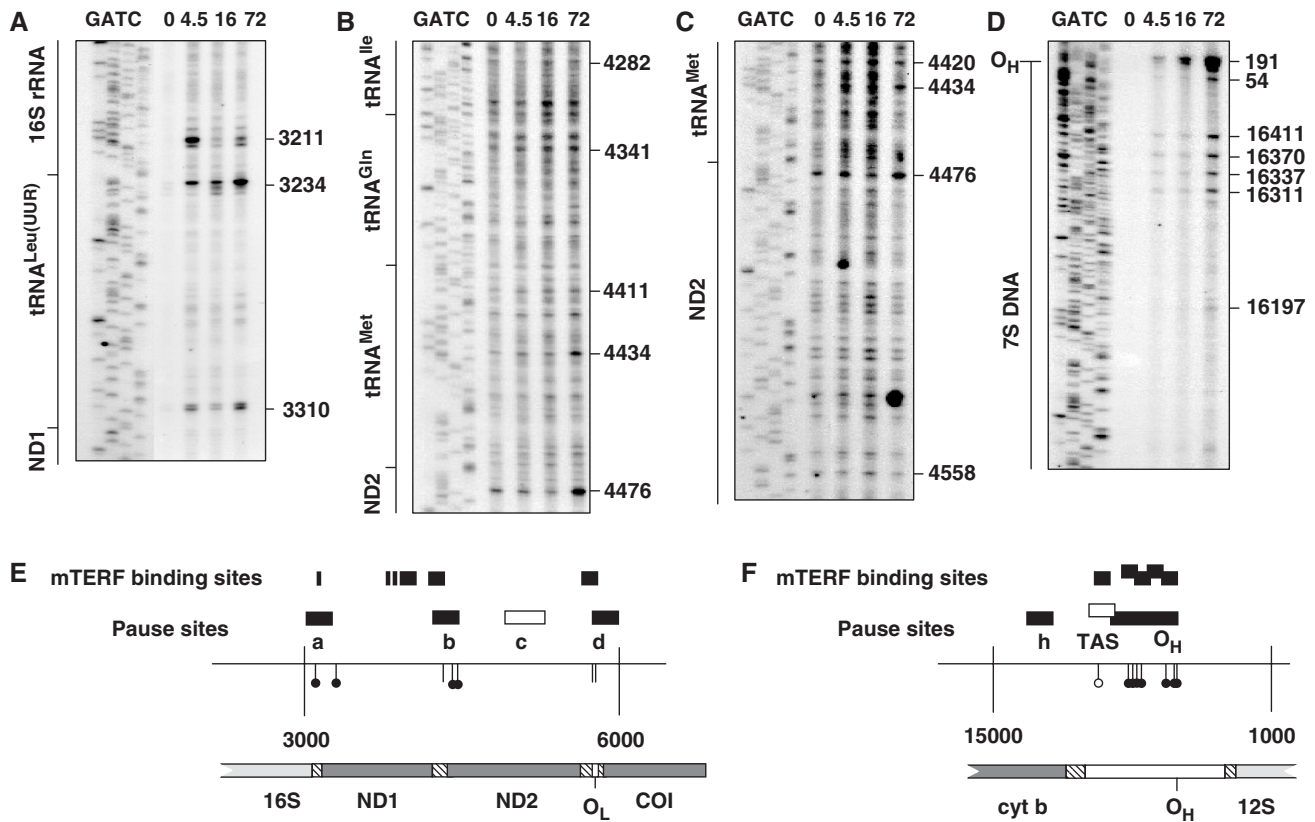


Figure 6. LM-PCR analysis of DNA 5' ends. (A–D) Analysis of L-strand 5' ends in the ND1–ND2 region, using primer sets TL1/TL2/TL7, TL8/TL9/L11 and TL8/TL9/TL11, respectively, shown alongside sequencing ladders for the corresponding segments. Samples analysed represent a time-course of induction of mTERF overexpression from 0–72 h (d). Analysis of H-strand 5' ends in the O_H region using primer set H1/H2/H5. (E) and (F) Schematic summary diagrams of the LM-PCR findings in the 16S rRNA–O_L and O_H regions, respectively. Shown below the scale lines are the positions (vertical lines) of the major 5' ends detected, with those exhibiting clearly increased abundance in mTERF-overexpressing cells also indicated by filled circles. Gene locations shown below (12S and 16S rRNA in light grey, cyt b, ND1, ND2 and COI protein-coding genes in dark grey, tRNAs cross-hatched, non-coding DNA in NCR and at O_L in white). LM-PCR data are compiled from parts (a–d) of this figure, plus parts (b–i) of Supplementary Figure 5. Above the scale lines are indicated the positions of mTERF-binding sites and replication pauses inferred from other experiments: in (e) reproduced from Figure 3d, and in (f) compiled from data of Figures 2 and 4, plus Supplementary Figure 2. Based on the data of Figure 4, the O_H pause region enhanced by mTERF overexpression extends across most of the NCR. The white box in (f) indicates the assumed position of the pause site giving rise to species 'n' in Figure 4c, i.e. assuming initiation close to O_H. The open circle denotes the minor lagging strand 5' end mapping in this region (np 16197), which was enhanced by mTERF overexpression. The positions of the various mTERF-binding sites in the NCR were inferred from EMSA using overlapping 150 bp fragments, as shown.

(Supplementary Figure 2e, EMSA probe OH5), mIP findings suggest that the site is efficiently bound *in vivo* by overexpressed mTERF. The latter is consistent with previous findings that recombinant mTERF (7,29) binds only weakly to this site, whereas partially purified, endogenous mTERF binds more strongly and establishes a DNA loop required for efficient rDNA transcription (7). This may require a post-translational modification or limiting accessory factor found only *in vivo*. mTERF exists in multiple isoforms with distinct properties (4,47), and has been reported to convert to an inactive trimeric form *in vitro* (48). The initiator fragment also contains binding sites for TFAM, which may promote binding but may also interfere with the interpretation of the EMSA assay (see Supplementary Figure 2e).

Regulated passage of replication and transcription complexes

The role of mTERF as a transcriptional terminator is well established from *in vitro* studies, and DmTTF

also functions thus *in vivo* in *Drosophila* (13). However, there is no compelling evidence that mTERF regulates mitochondrial RNA levels physiologically. The disparity in relative abundance between mRNAs and rRNAs in mammalian mitochondria can largely be accounted for by post-transcriptional regulation, notably differences in half-life (49) and RNA processing efficiency (50). Despite causing reduced mTERF-binding affinity and terminator activity *in vitro*, the 3243A>G MELAS mutation has almost no effect on mitochondrial RNA levels *in vivo* (5). Moreover, manipulation of mTERF levels *in vivo* by overexpression or RNA interference has remarkably little effect on steady-state mitochondrial RNA levels (Hyvärinen *et al.*, manuscript in preparation). The observation that mTERF also modulates mtDNA replication pausing suggests a different physiological meaning for its action as a transcriptional terminator, i.e. it coregulates replication and transcription.

The unregulated collision of oppositely moving transcription and replication complexes drastically inhibits DNA replication and provokes genomic instability in both bacteria (51,52) and yeast (53,54). In *E. coli*, head-on collision of the transcription and replication machineries severely impedes the progress of the replication fork (51) whereas codirectional transcription has no effect. Within the *E. coli* chromosome almost all essential genes are oriented such that transcription and replication are codirectional (55), which is proposed to minimize the mutagenic effect of repeated replication stalling and recombinational restart, following head-on collisions (51,56). In bacterial or yeast plasmids, or yeast rDNA, such head-on collisions can trigger genomic instability (53,57,58), e.g. due to knotting of daughter duplexes (59). In mammalian nuclei, head-on collisions can trigger the formation of HSRs (homogeneously staining regions of chromosomes), the signature of massive gene amplification events (60).

Proteins with dual roles in replication and transcriptional arrest are well documented. The *E. coli* Tus protein, described in the preceding section, preferentially blocks transcription with a similar polarity as DNA replication (61,62). Passage of a transcription complex from the permissive direction relieves the block on DNA synthesis (61) by provoking the dissociation of bound Tus (63). The mouse TTF-I protein binds at the 3' end of the rDNA transcription unit, where it terminates transcription by RNA polymerase I (64) and arrests replication forks arriving from the other direction (65,66), via its polar conrahelicase activity (67). This organization of the rDNA locus is relatively conserved throughout eukaryotes, although in some species the TTF-I homologue co-operates with or depends upon other proteins to maintain the replication fork barrier (RFB), including Sap1p and Reb1p in *Schizosaccharomyces pombe* (68–70), with involvement of Swi1p and Swi3p to stabilize the stalled forks (71), or Fob1p in *Saccharomyces cerevisiae* (72,73), with Sir2p regulating recombination at the stalled forks (74).

The entry of replication forks into the mtDNA termination zone around O_H requires traversal of the heavily transcribed rDNA region in the antisense direction, with potentially catastrophic consequences if a transcription complex is encountered. By binding at the rDNA boundary, mTERF may thus serve a function related to those of both Tus and TTF-I, facilitating the regulated passage of oppositely moving transcription and replication machineries, and regulating fork access to the termination zone.

The passage of a transcriptional complex in the permissive direction may also serve a regulatory role, such as hypothesized for mtDBP in relieving the block on D-loop expansion in sea urchins (15).

mTERF overexpression enhanced X-like species in fragments where the canonical mTERF-binding site was centrally located (Figure 4b, Supplementary Figure 4a), whereas mTERF knockdown by RNA interference depleted such species (Figure 5d). Although these may be recombination intermediates (see below), they might also comprise termination complexes centred on the

mTERF-binding site. If increased mTERF activity enhances rDNA transcription (6), it may also restrict the entry of replication forks into rDNA in the antisense direction and perhaps even shift the resolution site in some molecules from the NCR to the tRNA^{Leu(UUR)} gene. However, this must be a very minor fraction of molecules, since we did not see a complete Y-arc in O_H-containing fragments.

Transcription termination and the bootlace model

Previous studies of vertebrate mtDNA replication indicated that, in the majority of molecules, the lagging strand is initially laid down in the form of extended RNA segments which are subsequently converted to DNA via a maturation step (19). We hypothesized that the RNA lagging-strand may arise via either of two highly unorthodox mechanisms: either via a primase capable of synthesizing extended RNA primers, or by the hybridization of preformed L-strand RNA with the displaced H-strand in a 3' to 5' direction as the replication fork advances, the so-called bootlace model.

mTERF-dependent replication pausing may be construed as circumstantial evidence supporting the bootlace model. Transcriptional termination by mTERF adjacent to a paused replication complex would provide a 3' end capable of priming lagging-strand DNA synthesis, at the same time as delivering a fresh RNA bootlace to enable the replication fork to proceed in the forward direction. The mTERF-dependent enhancement of lagging strand 5' DNA ends near the canonical (and some other) mTERF-binding sites, (Figure 6), supports this idea. In sea urchin mtDNA, the major replication pause-region, which interacts with at least two DNA-binding proteins (38,40,41), also appears to be a major lagging-strand origin, as well as a site of transcriptional termination and/or RNA processing.

The effect of overexpression of mTERF is much more site-specific than that produced by overexpression of TFAM (28), by treatment with mtDNA replication inhibitors such as dideoxycytidine (28), or by expression of dominant-negative versions of the mtDNA helicase Twinkle (31). Unlike these treatments, mTERF overexpression did not cause a general slowing of replication, and did not alter globally the ratio of strand-coupled and RITOLS type RIs. By facilitating lagging-strand maturation, mTERF may serve merely to minimize the extent of the region of mtDNA maintained in the more vulnerable RNA-DNA hybrid form, thus contributing to genome stability.

Recombination at the mTERF-binding site

An alternate interpretation of the X-like molecules centred on the tRNA^{Leu(UUR)} gene region, which were enhanced by mTERF overexpression and depleted by mTERF knockdown, is that they represent true recombination intermediates. Such forms would be expected to arise if persistent mTERF binding and consequent prolonged pausing entrain fork collapse, requiring either a double-strand break or fork regression to generate a recombinogenic end for restart of replication. We previously

noted that the canonical mTERF-binding site was a frequent break-point in rearranged mtDNA molecules ('sublimons') detectable at a low level in all cell-types, but especially prominent in human heart (75), and also in mice expressing a disease-equivalent version of the Twinkle helicase (76) associated with autosomal dominant external ophthalmoplegia (PEO). It is tempting to ascribe such molecules to aberrant recombination following pausing and fork collapse at the tRNA^{Leu(UUR)} gene.

In yeast, double-strand breaks at the site of the RFB in rDNA, giving rise to low-level genomic rearrangements implicated in ageing, are evident even in wild-type strains (54). However, in strains defective for the DNA helicase Rrm3p, which is required for the processing of paused replication forks at sites of protein binding (77,78), the frequency of such events is greatly increased. If the balance between the pause-inducing and pause-processing machineries is disturbed, recombinational mechanisms must be employed to restart replication, with the concomitant risk of genomic instability. Thus, even though replication pausing systems such as mTERF may have evolved to limit genomic instability by preventing collisions of the replication and transcription machineries, their dysregulation, including by overexpression, could itself lead to instability. It follows that mTERF is a candidate gene for involvement in those cases of genetic disorders mediated by mtDNA rearrangements (e.g. PEO), whose genetic basis has not yet been elucidated.

In some cases replication pausing is merely a signature of defective replication (79,80). In others it is clearly a programmed event which facilitates other processes and preserves genome stability (81). As indicated by our findings (Figure 3), the phenomenon of pausing in human mtDNA is not confined to just one cell-type, nor is it an *in vitro* artefact seen only in cultured cells. The fact that it exhibits differences between cell-types and tissues strengthens the proposition that it is of physiological significance. The involvement of mTERF in modulating mtDNA replication pausing in human mtDNA, and the analogies with programmed replication pausing in other systems, support the idea that mTERF represents a system for safeguarding the integrity of the mitochondrial genome, whilst facilitating its efficient expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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