# Supporting Information

## Lodeiro et al. 10.1073/pnas.1118710109

### SI Materials and Methods

**Recombinant Mitochondrial Transcription Proteins.** The proteins used to perform this study can be obtained from Enzymax, LLC. Expression and purification of human mtRNA polymerase (POLRMT), mitochondrial transcription factor B2 (TFB2M), and mitochondrial transcription factor A (TFAM) were performed as described previously (1, 2). Expression and purification of ssDNA binding protein (mtSSB) and termination factor (mTERF) were performed as described previously (3, 4).

**Purification and 5′-$^{32}$P Labeling of DNA Oligonucleotides.** DNA oligonucleotides were purified by denaturing PAGE and end-labeled by using [γ-$^{32}$P]ATP (7,000 Ci/mmol) and T4 polynucleotide kinase as described previously (1).

**Annealing of DNA Oligonucleotides.** For each pair of oligonucleotides, the nontemplating and templating strands (top and bottom strands) were annealed at 10 μM in 10 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, and 50 mM NaCl using a Progene Thermocycler (Techne). Annealing reactions were heated to 90 °C for 1 min and slowly cooled to 10 °C at a rate of 5 °C/min. Annealing was confirmed by native 6% (wt/vol) PAGE. dsDNA was further purified by native 6% (wt/vol) PAGE. The concentration of dsDNA was determined by absorbance at 260 nm using a NanoDrop spectrophotometer and an extinction coefficient corrected for hypochromism (http://biophysics.idtdna.com/UVSpectrum.html) (5).

**Description of Plasmids and Linearization.** Both the heavy-strand promoter 1 (HSP1) -LONG and HSP2-SHORT templates were cloned in the pGEM-T EZ backbone. The HSP1-LONG template consisted of joined mtDNA segments from nucleotides 468–708 and 3,224–3,325. The HSP2-SHORT template consisted of joined segments from nucleotides 572–708 and 3,224–3,325. Both segments were cloned into restriction sites *SacII* and *SpeI*. Linearization reactions contained 5–10 μg pGEM plasmid DNA, and digestions were performed as specified by New England Biolabs using restriction enzymes *SphI* and *SacI* outside the original cloning sites to ensure different length runoff transcripts. The digested DNA templates were gel-purified using a Qiagen kit according to the manufacturer's specifications, and the final concentration was determined using a NanoDrop spectrophotometer.

**In Vitro Transcription Reactions Using Plasmids as Templates.** Reactions contained 10 mM Tris·HCl, pH 8.0, 20 mM NaCl, 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 μg/μL BSA, 400 μM ATP, 150 μM CTP, 150 μM GTP, 10 μM UTP, 0.2 μCi/μL [α-$^{32}$P]UTP (3,000 Ci/mmol), 3.4 nM linearized pGEM plasmid DNA template, 10 nM h-TFAM, 16 nM h-TFB2M, and 16 nM h-POLRMT (final concentrations). Reactions were performed by incubating linearized plasmid DNA in the reaction buffer and then adding the recombinant purified proteins. The reactions were carried out at 32 °C for 30 min, quenched by the addition of 22.5 μg Proteinase K in 10 mM Tris·HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 0.5% SDS, and 100 ng/μL yeast tRNA, and incubated at 42 °C for 1 h. Transcription products were ethanol-precipitated, dried, resuspended in 25 μL formamide gel loading buffer, resolved on denaturing 5% (wt/vol) (37:3 acrylamide:bis-acrylamide ratio) PAGE, and visualized by phosphorimaging. An end-labeled 10-bp ladder (Invitrogen) was used to estimate transcript sizes.

**In Vitro Transcription Using Oligonucleotides as Templates.** Reactions were performed essentially as described previously (1). They contained 10 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 μg/μL BSA, 1 μM DNA oligonucleotide duplex, 500 μM ATP, 500 μM CTP, 500 μM GTP, 10 μM UTP, 0.2 μCi/μL [α-$^{32}$P]UTP, 1 μM h-TFAM, 1 μM h-TFB2M, and 1 μM h-POLRMT (final concentrations). Reactions were assembled and initiated as described above and quenched at various times by the addition of stop buffer [35% (vol/vol) formamide, 0.0125% bromophenol blue, 0.0125% xylene cyanol, and 50 mM EDTA final]. Products were resolved by denaturing 15% (wt/vol) (37:3 acrylamide:bis-acrylamide ratio) PAGE and visualized by phosphorimaging. Proteins were diluted immediately before use in 10 mM Hepes, pH 7.5, 1 mM DTT, and 20% (vol/vol) glycerol. The volume of protein added to any reaction was always less than or equal to one-tenth of the total volume. Any deviations are indicated.

**EMSA.** Binding reactions contained 10 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 μg/μL BSA, 1 nM $^{32}$P-labeled DNA oligonucleotide duplex, and various concentrations of TFAM. The $^{32}$P-labeled DNA duplex contained only one of the DNA strands labeled in a given reaction (template or nontemplate strand). The $^{32}$P-labeled DNA duplex was incubated with increasing concentrations of proteins for 5 min at 25 °C. The reactions were loaded in a 6% (wt/vol) native polyacrylamide gel containing 0.33× Tris Borate EDTA (TBE). The complexes were resolved by electrophoresis in 0.33× TBE for 2 h at 150 V. Gels were visualized by phosphorimaging and quantitated by using ImageQuant Software (GE).

**DNase I Footprinting.** Reactions were assembled as described above for transcription reactions using oligonucleotide templates but using 1 nM $^{32}$P-labeled DNA oligonucleotide duplex. DNase I footprinting reactions were performed essentially as described previously (1). Reactions were assembled and then initiated by the addition of RQ1 DNase (0.002 units/μL) and $CaCl_2$ (1 mM). The reaction was allowed to proceed for 2 min and then quenched by stop/trap buffer [35% (vol/vol) formamide, 0.0125% bromophenol blue, 0.0125% xylene cyanol, 50 mM EDTA final] and 50 nM trap strand final. The trap strand is an unlabeled DNA oligonucleotide that has the same sequence as the $^{32}$P-labeled DNA oligonucleotide in the reaction, which is necessary for improved resolution of the DNA fragments during PAGE (6). Quenched reactions were heated to 90 °C for 1 min and slowly cooled to 10 °C at a rate of 5 °C/min using a Progene Thermocycler before PAGE. Products were resolved by denaturing 8% (wt/vol) PAGE (37:3 ratio acrylamide:bis-acrylamide) and visualized by phosphorimaging.


1. Lodeiro MF, et al. (2010) Identification of multiple rate-limiting steps during the human mitochondrial transcription cycle in vitro. *J Biol Chem* 285:16387–16402.
2. Smidansky ED, Arnold JJ, Reynolds SL, Cameron CE (2011) Human mitochondrial RNA polymerase: Evaluation of the single-nucleotide-addition cycle on synthetic RNA/DNA scaffolds. *Biochemistry* 50:5016–5032.
3. Longley MJ, Smith LA, Copeland WC (2009) Preparation of human mitochondrial single-stranded DNA-binding protein. *Methods Mol Biol* 554:73–85.
4. Yakubovskaya E, Mejia E, Byrnes J, Hambardjieva E, Garcia-Diaz M (2010) Helix unwinding and base flipping enable human MTERF1 to terminate mitochondrial transcription. *Cell* 141:982–993.
5. Tataurov AV, You Y, Owczarzy R (2008) Predicting ultraviolet spectrum of single stranded and double stranded deoxyribonucleic acids. *Biophys Chem* 133:66–70.
6. Arnold JJ, Ghosh SK, Bevilacqua PC, Cameron CE (1999) Single-nucleotide resolution of RNA strands in the presence of their RNA complements. *Biotechniques* 27:450–456.

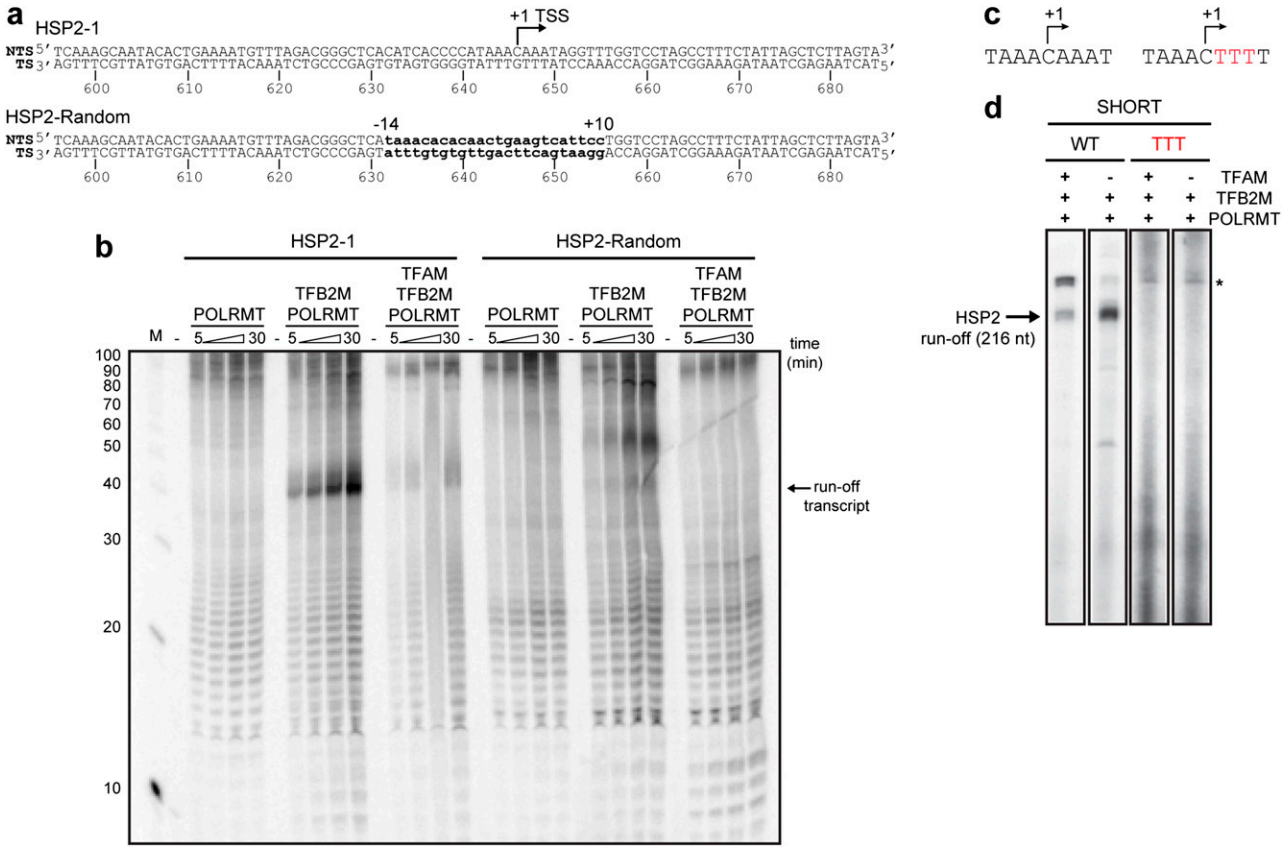


**Fig. S1.** Reconstitution of HSP2 transcription in vitro using DNA oligonucleotides and plasmid-based templates is specific to the predicted transcription start sites. (*A*) Schematic of the HSP2-1 and HSP2-Random DNA oligonucleotides used for in vitro transcription. For the HSP2-Random DNA oligo, the central 24-bp portion (−14 to +10) surrounding the proposed transcription start site (1) was randomized (shown as bold lowercase). (*B*) In vitro transcription assays using HSP2-Random DNA oligonucleotide as template. Reactions were performed by combining double-stranded HSP2 DNA oligonucleotide template with POLRMT alone or in the presence of TFAM and/or TFB2M as indicated. Transcription from HSP2-Random failed to produce the ∼40-nt RNA transcript as observed with HSP2. (*C*) HSP2 transcription in vitro using plasmid-based templates is abolished when mutations are incorporated upstream of the transcription start site. The sequence upstream of the proposed HSP2 transcription start site was changed from TAAACAAAT to TAAACTTTT (HSP2-TTT SHORT template). (*D*) Runoff transcription products from use of HSP2-WT or HSP2-TTT SHORT templates in the presence and absence of TFAM. RNA transcription was not observed when HSP2-TTT was used as template.

1. Martin M, Cho J, Cesare AJ, Griffith JD, Attardi G (2005) Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. *Cell* 123: 1227–1240.

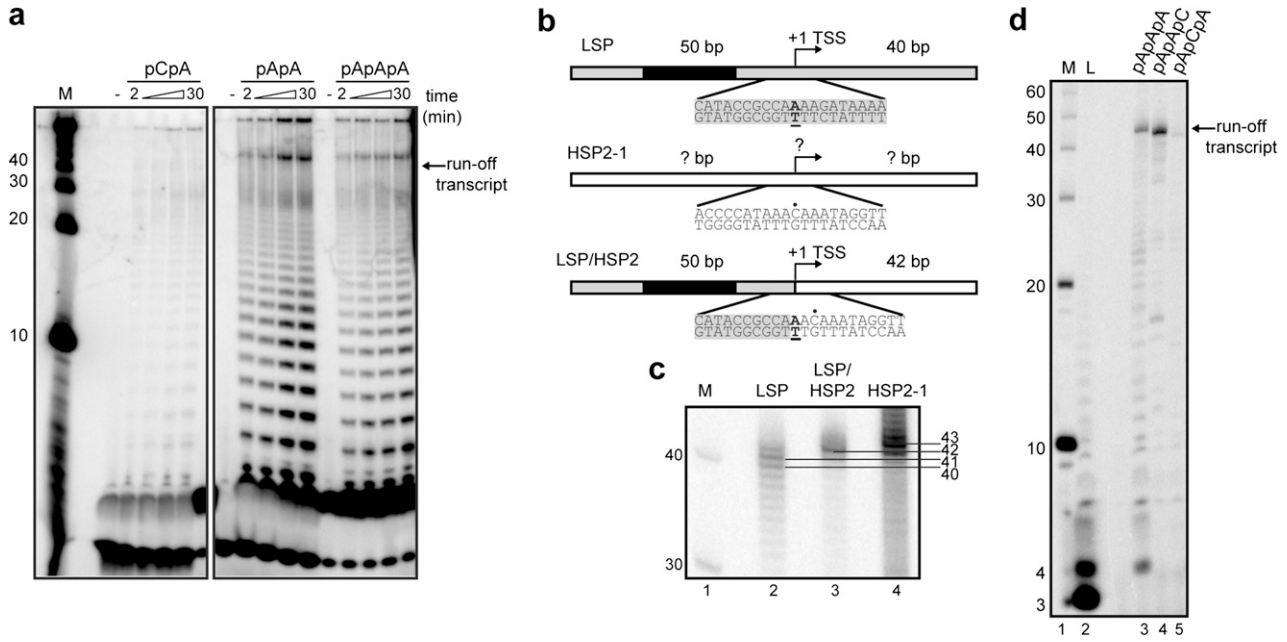


**Fig. S2.** Evaluation of the specificity of HSP2 transcription in vitro. (*A*) Design of a light-strand promoter (LSP) /HSP2 chimeric promoter to map the site of initiation from HSP2. The chimera should produce a 42-nt RNA that migrates with the major product from HSP2 if the transcription start site is at position 644 of mtDNA. (*B*) Runoff transcription from the templates shown in *A* by POLRMT-TFB2M in the absence of TFAM. (*C*) Evaluation of HSP2 transcription in the presence of pCpA, pApA, and pApApA di- or trinucleotide primers. Reactions were performed by combining HSP2-1 DNA oligonucleotide template with POLRMT and TFB2M in the presence of $^{32}$P-labeled di- and trinucleotide primers as indicated. HSP2 RNA transcription was not observed in the presence of pCpA; however, efficient transcription was observed with pApA or pApApA. (*D*) Runoff transcription from the HSP2-1 template in the presence of pApApA, pApApC, or pApCpA trinucleotide primer. Reactions were assembled by combining 100 nM HSP2-1 DNA oligonucleotide template with 100 nM POLRMT and 100 nM TFB2M in the presence of 50 μM trinucleotide primer as indicated. Reactions were initiated by the addition of NTPs (5 μM each) containing 0.2 μCi/μL [α-$^{32}$P]ATP, incubated at 32 °C for 30 min, and then quenched.

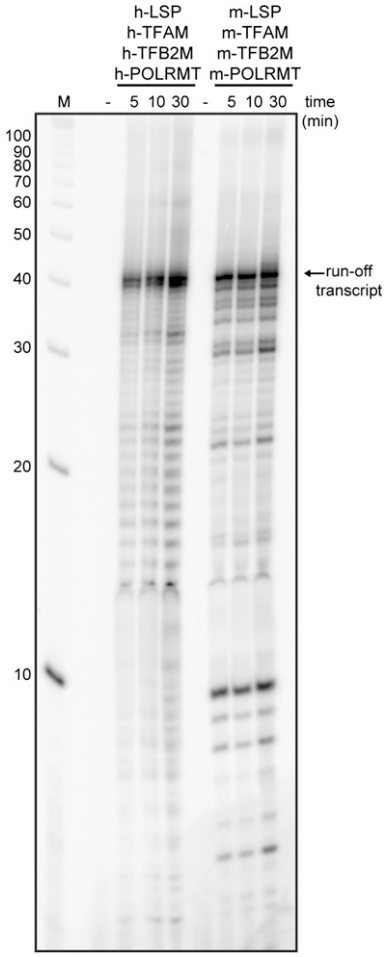


**Fig. S3.** Reconstitution of mouse mitochondrial transcription in vitro. In vitro transcription assays using human- and mouse-LSP DNA oligonucleotides and the corresponding human and mouse transcription components (POLRMT, TFB2M, and TFAM). Reactions were performed by combining the double-stranded LSP DNA oligonucleotide template with POLRMT, TFB2M, and TFAM as indicated. The mouse mitochondrial components were productive for promoter-specific transcription similar to the human components.

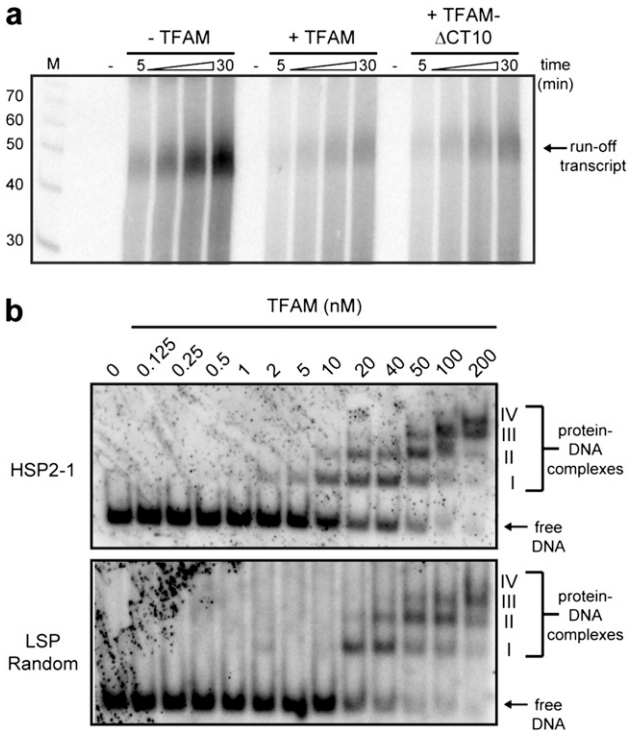


**Fig. S4.** TFAM requirements for HSP2 transcription inhibition. (*A*) Evaluation of HSP2 transcription in the presence of TFAM-ΔCT10. Reactions were performed by combining HSP2-1 DNA oligonucleotide template with POLRMT and TFB2M in the absence or presence of TFAM and/or TFAM-ΔCT10 as indicated. TFAM-ΔCT10 inhibited transcription from HSP2 similar to full-length TFAM. (*B*) Comparison of the DNA binding activities of TFAM with HSP2 and a randomized LSP DNA oligonucleotide. Titration of TFAM with HSP2-1 and an LSP-Random DNA oligonucleotide and evaluation of the protein–DNA complexes by EMSA. Increasing concentrations of TFAM readily produced four protein–DNA (I–IV) complexes with HSP2, whereas higher concentrations of TFAM were required to produce similar complexes with LSP-Random; additionally, these complexes were less abundant.

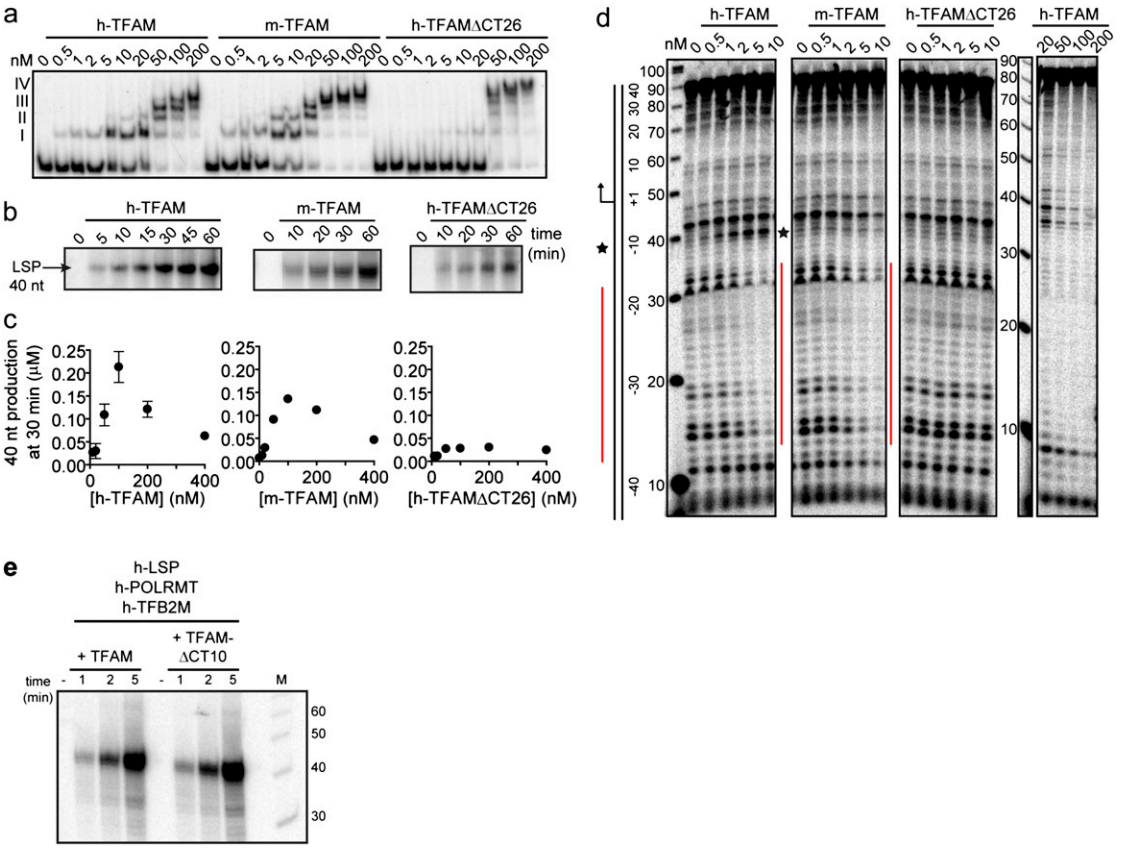


**Fig. S5.** Functional analysis of TFAM-LSP TFAM-responsive element (TRE) interactions. (*A*) Comparison of the DNA binding activities of h-TFAM, m-TFAM, and h-TFAM-ΔCT26 with LSP DNA oligonucleotide. TFAM was assembled on LSP DNA oligo (1 nM) as indicated, and the resulting protein–DNA complexes were evaluated by EMSA. Several TFAM-LSP species were observed. Increasing concentrations of both h-TFAM or m-TFAM readily produced four protein–DNA (I–IV) complexes. h-TFAM-ΔCT26 failed to produce protein–DNA complex I equivalent to complexes observed with h-TFAM or m-TFAM. Species I is consistent with the form that gives rise to LSP TRE protection, hyperactivity, and productive LSP transcription as observed in subsequent panels. (*B*) In vitro transcription assays using h-LSP DNA oligonucleotide as a template. Reactions were performed by combining a double-stranded LSP DNA oligonucleotide template with h-POLRMT and h-TFB2M in the presence of h-TFAM, m-TFAM, or h-TFAM-ΔCT26 as indicated. The reaction components of POLRMT, TFB2M, TFAM, and LSP DNA oligonucleotide template were assembled at a stoichiometry of 1:1:1:1. Both h-TFAM and m-TFAM were capable of activating LSP transcription, whereas activation by h-TFAM-ΔCT26 was not as robust. (*C*) LSP TFAM concentration-dependent transcription. Amount of 40-nt product produced in 30 min as a function of TFAM concentration. h-TFAM and m-TFAM maximally activate LSP transcription at concentrations that produce complex I. Higher concentrations inhibit transcription. h-TFAMΔCT26 failed to maximally activate LSP transcription consistent with the inability to form complex I. (*D*) DNase I footprinting provides evidence for binding of TFAM to LSP. TFAM was assembled on $^{32}$P-labeled LSP oligo (1 nM) as indicated. DNA cleavage was initiated by the addition of DNase I; a 10-bp ladder is indicated. The red line indicates the region protected by TFAM up to 10 nM, and the star (★) indicates the hyperactive site that is only observed in the presence of h-TFAM. High concentrations of TFAM (≥100 nM) protect the entire oligo from cleavage by DNase I. Similar results were obtained with m-TFAM. h-TFAMΔCT26 failed to provide protection similar to h-TFAM. (*E*) Evaluation of LSP transcription in the presence of h-TFAM-ΔCT10. Reactions were performed by combining LSP DNA oligonucleotide template with h-POLRMT and h-TFB2M in the presence of h-TFAM or h-TFAM-ΔCT10 as indicated. h-TFAM-ΔCT10 was capable of activating transcription from LSP.

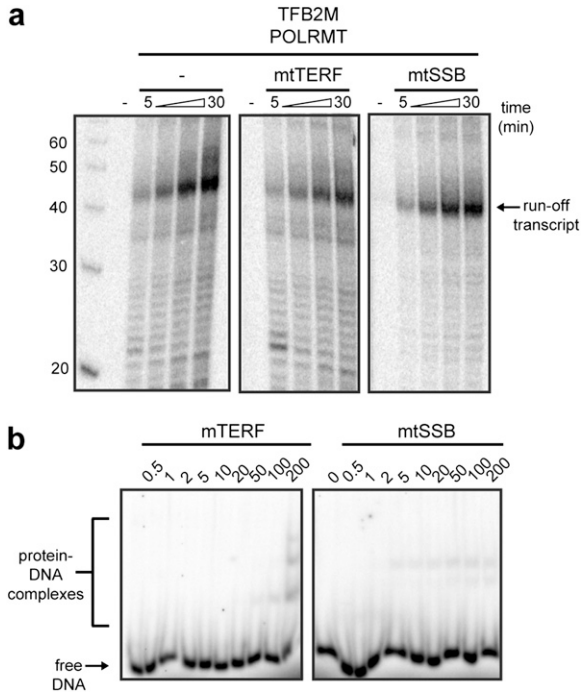


**Fig. S6.** Other mtDNA binding proteins do not inhibit HSP2 in vitro transcription or bind HSP2 DNA. (*A*) In vitro transcription assays in the presence of mTERF and mtSSB. Reactions were performed by combining double-stranded LSP DNA oligonucleotide template with POLRMT and TFB2M in the presence of mTERF and mtSSB as indicated. Both mTERF and mtSSB failed to inhibit HSP2 transcription. (*B*) Evaluation of the ability of mTERF and mtSSB to bind HSP2 DNA. mTERF and mtSSB were combined with HSP2-1 DNA oligo (1 nM) as indicated, and the resulting protein–DNA complexes were evaluated by EMSA. Both mTERF and mtSSB failed to produce substantial protein–DNA complexes equivalent to those complexes observed with TFAM.

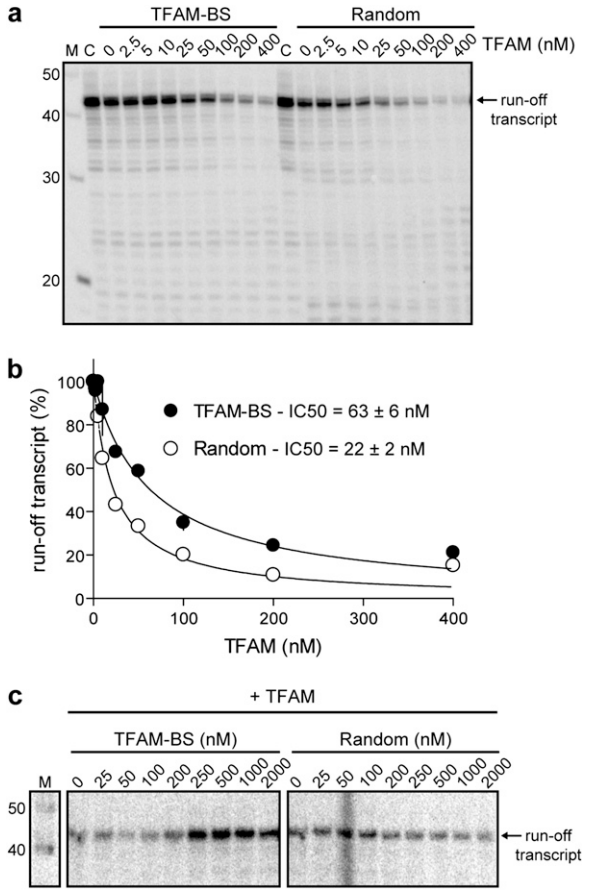


**Fig. S7.** TFAM inhibition of HSP2 transcription is antagonized by dsDNA containing the TFAM LSP TRE. (*A*) Evaluation of HSP2 transcription inhibition by TFAM in the presence of a competitor dsDNA oligonucleotide (28 bp) containing either the LSP TRE (TFAM-BS) or a randomized sequence (random). Reactions were assembled by combining 100 nM HSP2-1 DNA oligonucleotide template with 100 nM POLRMT and 100 nM TFB2M in the presence of 500 nM competitor dsDNA oligo and a range of concentrations of TFAM. Reactions were initiated by the addition of NTPs (5 μM each) containing 0.2 μCi/μL [$\alpha$-$^{32}$P]UTP, incubated at 32 °C for 30 min, and then quenched. (*B*) Determination of the $IC_{50}$ value for TFAM in the presence of competitor dsDNA. The phosphorimage in *A* was quantitated, and the amount of RNA transcript normalized to the amount with zero-added TFAM was plotted as a function of TFAM concentration. The data were fit to a hyperbola yielding an $IC_{50}$ value for TFAM of 63 ± 6 and 22 ± 2 nM in the presence of TFAM-BS or random dsDNA oligo, respectively. (*C*) Evaluation of HSP2 transcription in the presence of TFAM and a range of concentrations of competitor dsDNA oligo. Reactions were performed by combining 100 nM HSP2-1 DNA oligonucleotide template with 100 nM POLRMT and 100 nM TFB2M in the presence of TFAM and competitor dsDNA. Reactions were initiated by the addition of NTPs (5 μM each) containing 0.2 μCi/μL [$\alpha$-$^{32}$P]UTP, incubated at 32 °C for 30 min, and then quenched. TFAM inhibition of HSP2 transcription was antagonized by dsDNA containing the LSP TRE (TFAM-BS).

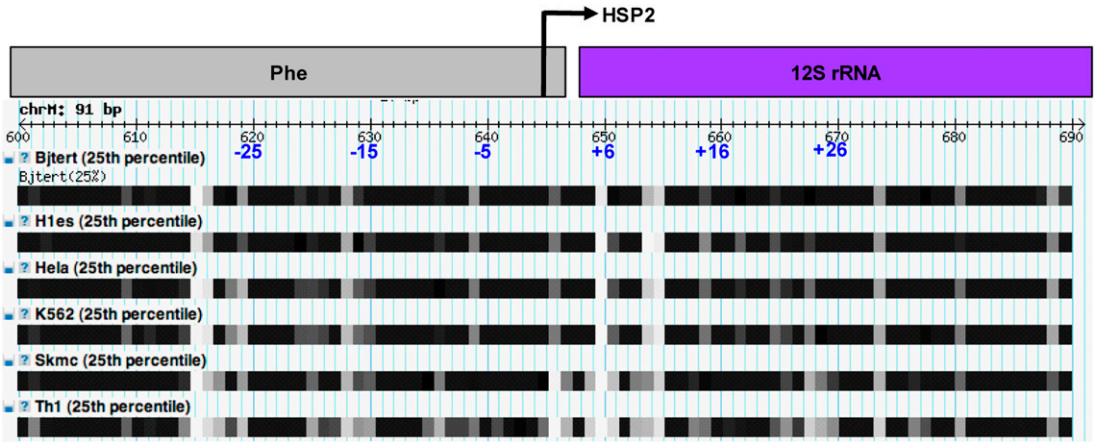


**Fig. S8.** DNA binding protein occupancy surrounding the HSP2 transcription start site as determined using high-throughput in vivo DNase I footprinting (1). The lowest 25th percentile range of DNase I cleavage frequency for each nucleotide (region 600–690 of mtDNA) within Bjtert, H1es, HeLa, K562 Skmc, and Th1 cells was obtained from http://mitochondria.matticklab.com/. The sensitivity of nucleotides to DNase I cleavage is shown. Dark regions indicate a high susceptibility to DNase I cleavage. Lighter regions are less susceptible to DNase I cleavage, indicating sites of protection by proteins. Sites of protection are observed surrounding the transcription start site of HSP2 consistent with in vitro data.

1. Mercer TR, et al. (2011) The human mitochondrial transcriptome. *Cell* 146:645–658.

**Table S1. DNA oligonucleotides used in this study**

| DNA oligo | Sequence |
|---|---|
| HSP2-1-NTS | 5′-TCAAAGCAATACACTGAAAATGTTTAGACGGGCTCACATCACCCCATAAACAA ATAGGTTTGGTCCTAGCCTTTCTATTAGCTCTTAGTA-3′ |
| HSP2-1-TS | 5′-TACTAAGAGCTAATAGAAAGGCTAGGACCAAACCTATTTGTTTATGGGGTGATGTGAGCCCGTCTAAACATT TTCAGTGTATTGCTTTGA-3′ |
| HSP2-2-NTS | 5′-ACACTGAAAATGTTTAGACGGGCTCACATCACCCCATAAACAAATAGGTTTGGTCCTAGCC TTTCTATTAGCTCTTAGTA-3′ |
| HSP2-2-TS | 5′-TACTAAGAGCTAATAGAAAGGCTAGGACCAAACCTATTTGTTTATGGGGTGATGTGA GCCCGTCTAAACATTTTCAGTGT-3′ |
| HSP2-3-NTS | 5′-GCTTACCTCCTCAAAGCAATACACTGAAAATGTTTAGACGGGCTCACATCACCCCATAAACAAATAGGTTTGGT CCTAGCCTTTCTATTAGCTCTTAGTA-3′ |
| HSP2-3-TS | 5′-TACTAAGAGCTAATAGAAAGGCTAGGACCAAACCTATTTGTTTATGGGGTGATGTGAGCCCGTCTAAA CATTTTCAGTGTATTGCTTTGAGGAGGTAAGC-3′ |
| HSP2-4-NTS | 5′-ACACTGAAAATGTTTAGACGGGCTCACATCACCCCATAAACAAATAGGTTTGGTCCTAGCCTTT CTATTAGCTCTTAGTAAGATTACACA-3′ |
| HSP2-4-TS | 5′-TGTGTAATCTTACTAAGAGCTAATAGAAAGGCTAGGACCAAACCTATTTGTTTATGGGGTGATGTG AGCCCGTCTAAACATTTTCAGTGT-3′ |
| HSP2-Random-NTS | 5′-TCAAAGCAATACACTGAAAATGTTTAGACGGGCTCATAAACACACAACTGAAGTCATTCCTGGTCC TAGCCTTTCTATTAGCTCTTAGTA-3′ |
| HSP2-Random-TS | 5′-TACTAAGAGCTAATAGAAAGGCTAGGACCAGGAATGACTTCAGTTGTGTGTTTATGAGCCCG TCTAAACATTTTCAGTGTATTGCTTTGA-3′ |
| h-LSP-NTS | 5′-GGAAAATAATGTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAAGATAAAATTT GAAATCTGGTTAGGCTGGTGTTAGGGT-3′ |
| h-LSP-TS | 5′-ACCCTAACACCAGCCTAACCAGATTTCAAATTTTATCTTTTGGCGGTATGCACTTTTAACAG TCACCCCCCAACTAACACATTATTTTCC-3′ |
| LSP-HSP2-NTS | 5′-GGAAAATAATGTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAACAAATAGGTTT GGTCCTAGCCTTTCTATTAGCTCTTAGTA-3′ |
| LSP-HSP2-TS | 5′-TACTAAGAGCTAATAGAAAGGCTAGGACCAAACCTATTTGTTTGGCGGTATGCACTTTTAACAGT CACCCCCCAACTAACACATTATTTTCC-3′ |
| m-LSP-NTS | 5′-TGTAAAATTTTTACAAGTACTAAAATATAAGTCATATTTTGGGAACTACTAGAATTGATCAGGAC ATAGGGTTTGATAGTTAATATTATA-3′ |
| m-LSP-TS | 5′-TATAATATTAACTATCAAACCCTATGTCCTGATCAATTCTAGTAGTTCCCAAAATATGACTTA TATTTTAGTACTTGTAAAAATTTTACA-3′ |
| LSP-Random-NTS | 5′-CGACAGAGTCCGTGCACCTACCAAACCTCTTTAGTCTAAGTTCAGACTAGTTGGAAGTTTGTCT AGATCTCAGATTTTGTCACTAGAGGA-3′ |
| LSP-Random-TS | 5′-TCCTCTAGTGACAAAATCTGAGATCTAGACAAACTTCCAACTAGTCTGAACTTAGACTAAA GAGGTTTGGTAGGTGCACGGACTCTGTCG |
| TFAM-BS-top | 5′-ATGTGTTAGTTGGGGGGTGACTGTTAAA-3′ |
| TFAM-BS-bot | 5′-TTTAACAGTCACCCCCCAACTAACACAT-3′ |
| Random-Top | 5′-GATGTTAGCTGTTGGTGGAGAGGTTATA-3′ |
| Random-Bot | 5′-TATAACCTCTCCACCAACAGCTAACATC-3′ |