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Termination Factor-Mediated DNA Loop between Termination and Initiation Sites Drives Mitochondrial rRNA Synthesis

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SUMMARY

The human mitochondrial transcription termination factor mTERF plays a central role in the control of heavy-strand rDNA transcription by promoting initiation, besides termination, of this transcription. However, until now, the mechanism underlying this stimulation of transcription by mTERF was not understood. In the present work, addition of mTERF to a HeLa cell mitochondrial lysate-based reaction mixture containing an artificial rDNA template did indeed specifically stimulate rDNA transcription. This stimulation required that mTERF be *simultaneously* bound to the rDNA transcription termination and initiation sites in the *same* molecule, thus forming a loop. Most significantly, a double binding of mTERF to the rDNA molecule, with resulting loop formation, was also shown in vivo. These results strongly suggest that, to satisfy the need for high rate of rDNA transcription, human mitochondrial rRNA synthesis involves mTERF-mediated rDNA looping that promotes recycling of the transcription machinery.

INTRODUCTION

According to evidence that we obtained about 25 years ago (Montoya et al., 1982, 1983), transcription of the HeLa cell mtDNA heavy (H) strand (rich in guanine [Vinograd et al., 1963]) starts from two initiation sites within the main control region (Figures 1A and 2C). One site (H1) is situated 16 bp upstream of the $tRNA^{Phe}$ gene and produces a transcript which terminates at the 3′ end of the 16S rRNA gene and is destined to be processed, yielding the two rRNAs, the $tRNA^{Phe}$ and the $tRNA^{Val}$. The second site (H2) is near the 5′ end of the 12S rRNA gene and produces a polycistronic molecule, which corresponds to almost the entire H strand and is processed to yield the mRNAs and most of the tRNAs encoded in the H strand. Subsequently, we showed that termination of transcription at the boundary between the 3′ end of the 16S rRNA gene and the $tRNA^{Leu(UUR)}$ genes is mediated by a termination factor, mTERF (Kruse et al., 1989; Fernández-Silva et al., 1997). This factor is a 342 residue DNA binding protein that carries three leucine zippers, of which one is bipartite, and two widely spaced basic domains. This factor was previously shown to bind to a 28 base pair region within the $tRNA^{Leu(UUR)}$ gene, at a position immediately adjacent to the 16S rRNA gene.

The rate of synthesis of rRNA is 15- to 60-fold higher relative to mRNA (Montoya et al., 1982, 1983). A striking observation in the analysis of mTERF (Kruse et al., 1989; Asin-Cayuela et al., 2004) was that, in an in vitro transcription system, mTERF stimulates transcription of mitochondrial rDNA, starting from its natural starting site (H1). However, the fact that this stimulation by mTERF was not observed when template DNAs containing a nonnatural starting site were used, even with a natural termination site (Hess et al., 1991; Prieto-Martín et al., 2004), suggested that mTERF interaction with both the natural termination and H1 initiation sites may be essential for this specific stimulation effect. In the present work, we demonstrate incontrovertibly the requirement, to achieve this stimulation, of a double DNA binding by mTERF, which causes a specific looping-out of the rDNA.

RESULTS

The Two Initiation Sites H1 and H2 for In Vivo mtDNA H Strand Transcription

First, we confirmed the precise identification and mapping of the two sites of human mtDNA H strand transcription initiation, H1 and H2, discovered earlier in HeLa cells (Montoya et al., 1982, 1983) by two approaches. One involved 5′ end labeling of the in vivo HeLa cell mtDNA nascent transcripts

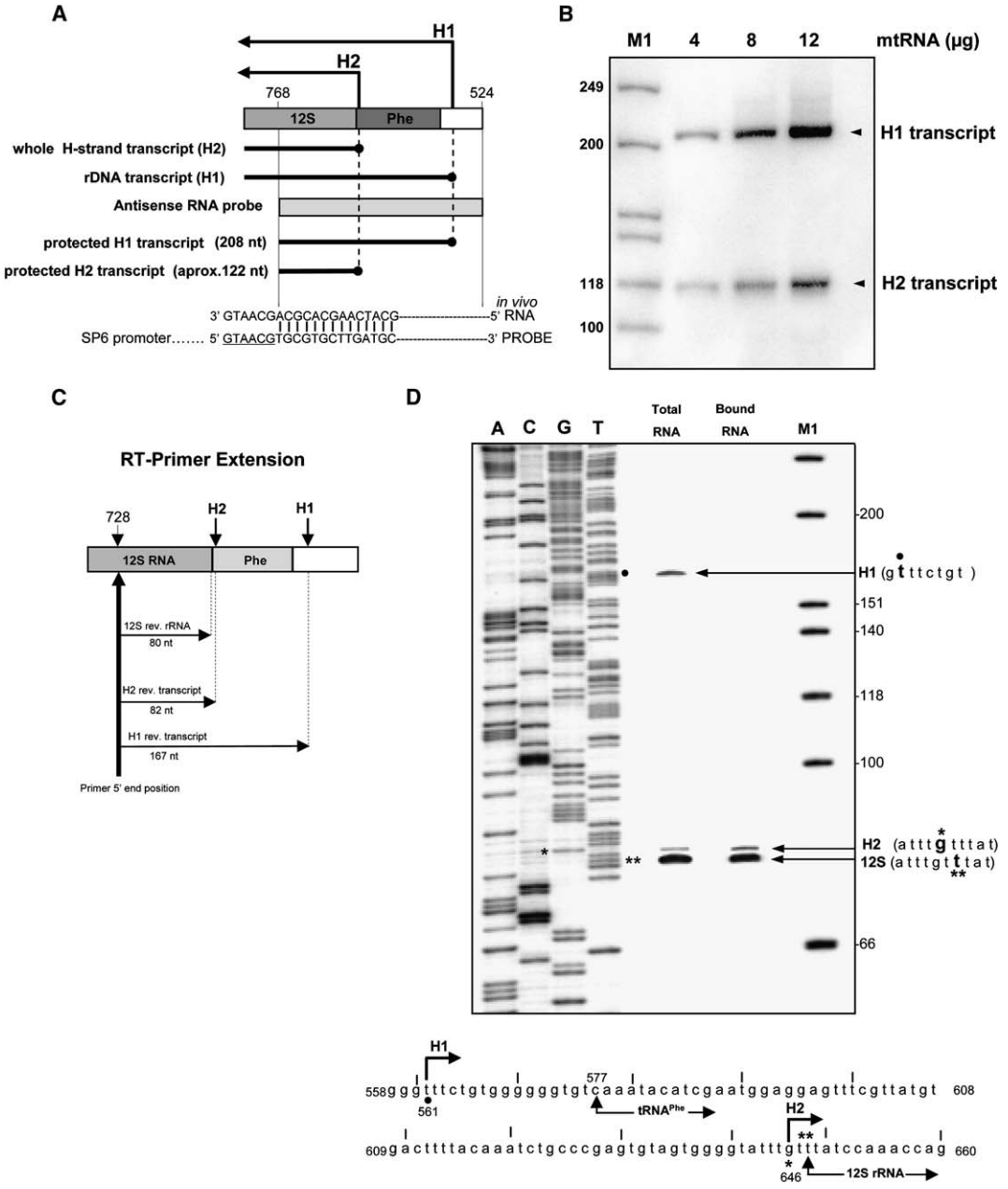


Figure 1. Detection and Precise Mapping of the Two In Vivo HeLa Cell H Strand Transcription Initiation Sites

(A and B) HeLa cell total mtRNA was in vitro labeled with [α-^{32}P]-GTP by guanylyltransferase at the 5′ end of the two initiation products of H strand transcription (H1 and H2 transcripts) and analyzed, as detailed in Experimental Procedures, by an S1 nuclease protection assay using an antisense RNA probe.

(A) Schematic representation of the S1 protection assay.

(B) The S1 protection products were analyzed by electrophoresis through an 8% polyacrylamide/7 M urea gel at 300V for 3 hr.

(C and D) A precise mapping, at the nucleotide level, of the 5′ ends of the H1 and H2 initiated transcripts in HeLa cell total and poly(A)$^+$ mtRNA samples was also made by reverse transcriptase (RT) primer extension, carried out in a 0.5% Tween 20 HeLa cell mitochondrial lysate.

(C) A 5′-end-labeled primer, complementary to the 12S RNA, with the 5′ end corresponding to mtDNA position 728, was used in the presence of AMV reverse transcriptase. In the schematic drawing, the three kinds of expected extended products are represented.

(D) Samples of total and poly(A)$^+$ mtRNA, the latter obtained as bound RNA by streptavidin-conjugate magnetic beads and biotinylated oligo(dT) primer, were run through an 8% polyacrylamide/7 M urea sequencing gel. In parallel were run the manual sequencing products obtained by using the *fmol*^{R} DNA Cycle Sequencing System (Promega) on the pTER mtDNA clone with the same 5′-end-labeled primer which was used for the RT-primer extension. The H1-initiated transcript has its 5′ end at the thymine (•) at position 561, while the H2 transcript has it at the guanine (*) at position 646, and the 12S RNA, at the thymine (**) at position 648. Both the H1 and H2 transcripts were of the expected size predicted by the S1 protection experiment (A). M1, ¢X174 DNA/HinfI markers (Promega) 5′ end labeled.

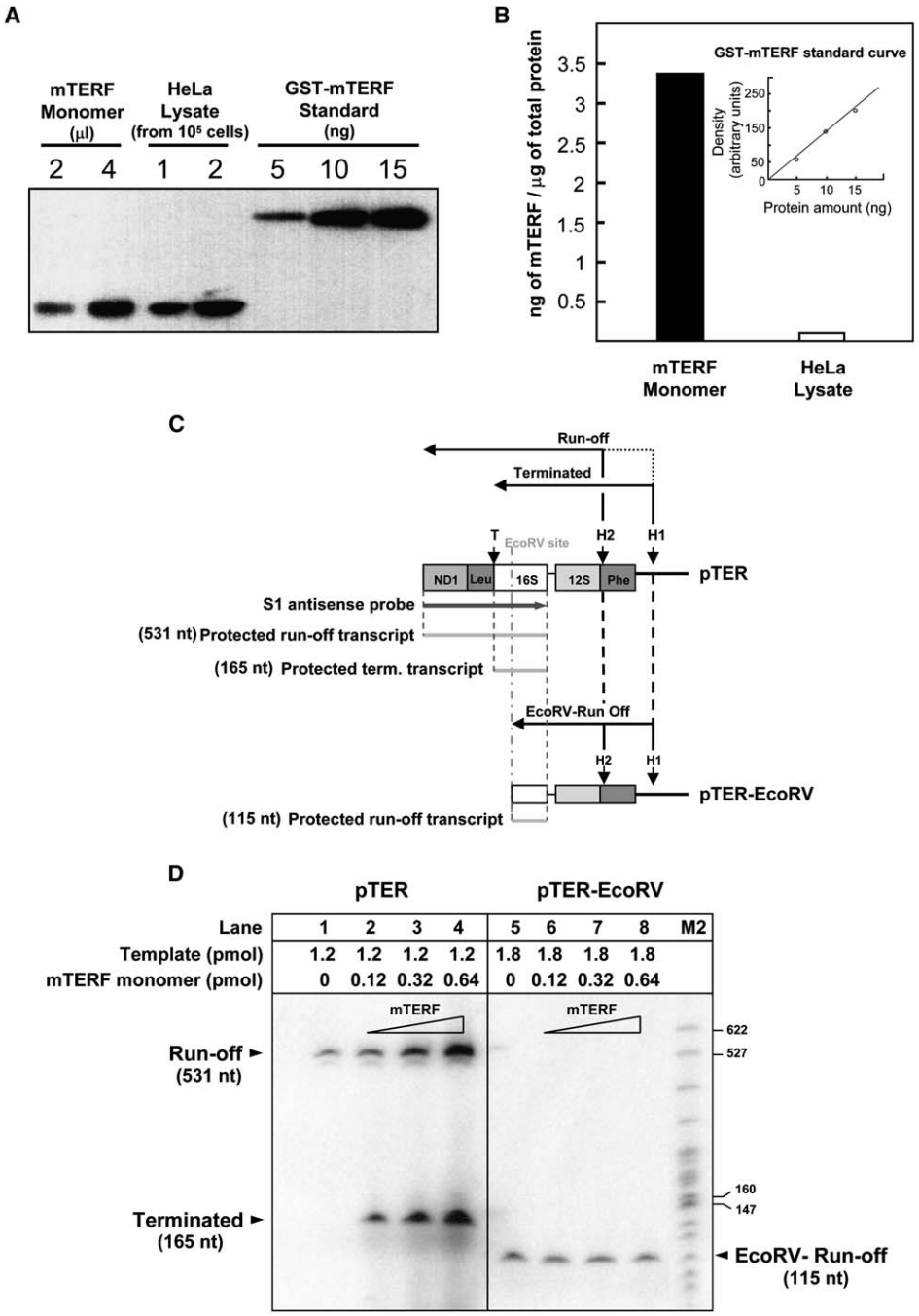


Figure 2. Stimulation of In Vitro Transcription at the H1 Site Requires Both mTERF and the Termination Site

In vitro labeled mtDNA transcription products, obtained in the presence of increasing amounts of mTERF monomer, were analyzed by S1 nuclease protection assays.

(A) Quantification by Western blot of the mTERF protein present in the mTERF-enriched fraction (mTERF monomer) and in a whole HeLa cell lysate. The purified GST-mTERF recombinant fusion protein was used as a standard for quantification purposes.

(B) Comparison of the mTERF amount present in the gel filtration mTERF monomer fraction and in a HeLa cell lysate.

(C) Schematic representation of the S1 protection experiments. Two kinds of templates were used for in vitro transcription, as detailed in Experimental Procedures: pTER contains the whole mtDNA H strand transcription initiation sequence and also the termination binding sequence for mTERF, while pTER-EcoRV is a truncated version of pTER, lacking the termination sequence. The run-off transcripts initiated at the H1 site were only observed in vitro.

(D) The products of in vitro transcription and of the S1 nuclease protection assay described in (C) were analyzed in an 8% polyacrylamide/7 M urea gel. The transcription experiments were carried out with 1 μg of pTER (1.2 pmol; lanes 1–4) or pTER-EcoRV template (1.8 pmol; lanes 5–8), with or without addition of increasing amounts of mTERF. M2, 5′-end-labeled pBR322-MspI DNA markers (New England Biolabs).

with [α-^{32}P]-GTP and guanylyltransferase (“capping” enzyme), followed by an S1 nuclease protection assay and analysis of the capped products by polyacrylamide/urea gel electrophoresis (Figures 1A and 1B). Figure 1B shows the two labeled S1-protected H1 and H2 transcripts. The second approach involved primer extension by reverse transcriptase (RT-primer extension) using a mitochondrial lysate (Figures 1C and 1D). In this approach, a 5′-end-labeled appropriate H strand oligodeoxynucleotide primer (see Experimental Procedures) was extended by the avian myeloblastosis virus (AMV) reverse transcriptase on the total and poly(A)$^+$ RNA samples up to the 5′ end of the three expected transcripts, i.e., the 12S RNA, the H2 nascent transcript, and the H1 nascent transcript (Figure 1C). The extended primers were run in a polyacrylamide/urea gel in parallel with the products of the sequencing reactions of the pTER DNA clone (Kruse et al., 1989). The two approaches agreed in confirming the H1 initiation site of rDNA transcription to be at position 561, 16 bp upstream of the tRNA^{Phe} gene, as previously established (Figure 1D; Chang and Clayton, 1984). Furthermore, the results determined for the first time the precise position of the H2 initiation site to be at nt 646 and confirmed the 5′ end of 12S rRNA to be at nt 648, i.e., two nucleotides downstream of H2 (Figure 1D).

Isolation and Quantification of mTERF

In the present work, the mTERF monomer-enriched fraction, isolated by gel filtration chromatography from the S-100 fraction of a HeLa cell mitochondrial lysate (Asin-Cayuela et al., 2004), was utilized (see Figure S1 in the Supplemental Data available with this article online) and quantified by Western blot assays using purified recombinant GST (glutathione S-transferase)-mTERF (see Supplemental Data) as a standard (Figures 2A and 2B). From the data in Figure 2A, it was calculated that each HeLa cell contains on the average $\sim 6 \times 10^5$ molecules of mTERF.

In Vitro Initiation of Transcription from the H1 Site Is Stimulated by the mTERF Monomer Only in Presence of the Termination Site

To understand the role of mTERF in mtDNA H strand transcription initiation at the H1 site, in vitro transcription experiments were carried out using the linearized pTER template and a truncated version of it lacking the termination binding site for mTERF, i.e., pTER-EcoRV, in a system utilizing the mitochondrial lysate (Figure 2C). The products were analyzed by S1 protection assays using an antisense RNA probe synthesized from the plasmid pKSM2 (Figure 2C). The S1 nuclease protection assays, in experiments using the pTER template, revealed that, in the presence of increasing amounts of mTERF monomer, not only was there termination of the transcripts at the boundary between the 16S RNA gene and the tRNA$^{Leu(UUR)}$ gene, but also an increase in the run-off transcripts (Figure 2D, left panel). This result, which agreed with previous observations (Kruse et al., 1989; Asin-Cayuela et al., 2004), strongly suggested that the protein not only acts as a transcription termination factor, but also stimulates transcription initiation. More surprisingly, experiments using the pTER-EcoRV template (Figure 2D, right panel) showed a total lack of stimulation of transcription by the same levels of mTERF monomer used with the pTER template, indicating that stimulation of mtDNA H strand transcription initiation by mTERF requires the presence of the H strand transcription termination site.

Quantitative Termination of Run-Off H1-Initiated Transcripts Requires a 2-Fold or Greater Molar Excess of mTERF Over Template

In the experiments of Figure 2D, the pTER template was in molar excess over mTERF monomer (1.2 pmol versus 0–0.64 pmol). In order to learn what is rate limiting for transcription initiation from the H1 site and for transcription termination, other experiments of in vitro transcription and S1 nuclease protection were carried out, this time using limiting amounts of pTER template, in the presence of increasing amounts of mTERF monomer (Figure 3). In these experiments, the level of mTERF monomer varied between 0- and 5-fold the molar amount of the template. As shown in Figure 3, the frequency of termination of the nascent transcripts increased with the amount of mTERF monomer, becoming nearly complete with a 2-fold molar excess and complete with a 5-fold excess. These experiments indicated that a significant molar excess of mTERF over the template was required for quantitative termination of the H1-initiated transcripts.

mTERF Stimulates Transcription from H1 on rDNA Template by Enhancing the Recycling of Transcription Machinery

To confirm that the stimulation of H strand transcription initiation by the mTERF monomer is specific for rDNA transcription from the H1 site, [α-^{32}P]-GTP-guanylyltransferase capping experiments were carried out on in vitro synthesized transcripts. As explained in Experimental Procedures, the capped in vitro nascent H1 and H2 transcripts shown in Figure 4 were 30 nucleotides shorter than the in vivo nascent transcripts shown in Figures 1A and 1B. As illustrated in the upper panel of Figure 4B (lanes 1–4), the analysis of the transcripts synthesized on the pTER template in the presence of increasing amounts of mTERF monomer confirmed that the termination factor promotes initiation from the H1 site without affecting the transcription from the H2 site. Furthermore, in transcription experiments using the pTER-EcoRV template, no stimulation of initiation from the H1 site by the mTERF monomer was observed (Figure 4B, upper panel, lanes 5–8), in agreement with the results shown in Figure 2D. Interestingly, the addition of the termination fragment (a 44-mer double-strand oligodeoxynucleotide corresponding to a mtDNA fragment containing the mTERF binding site) to transcription reactions using the pTER-EcoRV template restored partially, up to ~30% of the level observed with the pTER template, the deficient stimulating effect of mTERF monomer on transcription initiation at the H1 site observed with the latter template (Figure 4B, upper panel, lanes 9–11). This result strongly suggested that the molecular proximity between the H1 initiation and

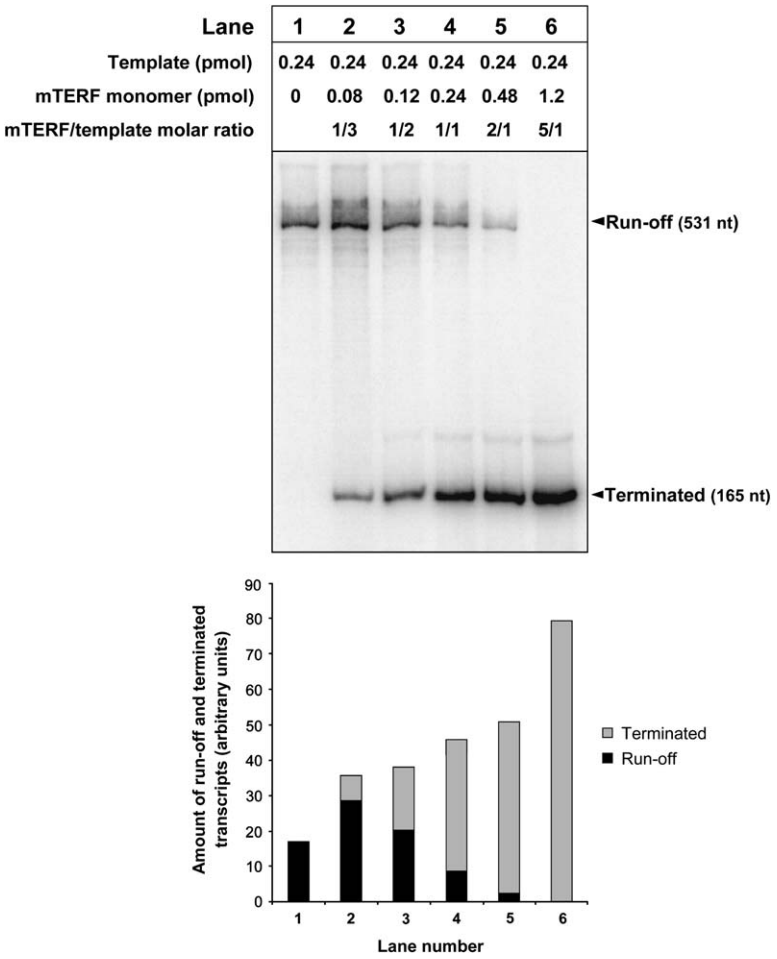


Figure 3. A 2-Fold or Greater Molar Excess of mTERF over Template Is Essential for Quantitative Termination of Run-Off Transcripts

In vitro transcription experiments and S1 nuclease protection assays were carried out, using a limiting amount of pTER template (0.24 pmol), in the presence of increasing amounts of mTERF monomer. The molar ratio of mTERF monomer to template in the in vitro transcription experiments varied between 1/3- and 5-fold (lanes 2–6). The samples where electrophoresed through an 8% polyacrylamide/7 M urea gel, and the gel was subsequently analyzed by a PhosporImager. At the bottom is represented the quantification of the amounts of run-off and terminated transcripts in each lane.

termination sites may be important to stimulate the H1-specific transcription by mTERF.

To analyze in detail how mTERF stimulates the H1-specific transcription, biotinylated transcription template plasmids immobilized on streptavidin magnetic beads (see Experimental Procedures) were used. This allowed a reduction of the available amount of transcription machinery, after preincubation of the templates with mitochondrial extract, by washing out the excess not interacting with the template. Under the conditions of low concentration of transcription machinery (including mitochondrial RNA polymerase) thus achieved, we found that the pTER template still exhibits the transcription stimulation effect of mTERF (Figure 4B, lower panel, lanes 1–4). By contrast, the pTER-EcoRV template did not show any transcription activation by mTERF, even after the addition of increasing amounts of mTERF and termination fragment under the same conditions (Figure 4B, lower panel, lanes 5–11). More importantly, no transcription from the H2 site was observed on the pTER template under conditions of low concentration of transcription machinery (Figure 4B, lower panel, lanes 1–4). This was in contrast to the results obtained with the same template not washed out of the excess amount of transcription machinery (Figure 4B, upper panel, lanes 1–4). These results suggest that the presence of mTERF and the relative proximity between H1 initiation site and termination site on the same artificial template stimulate the reinitiation of H1-specific transcription via repositioning of the transcription machinery without diffusion from template DNA.

To investigate the molecular basis of the H1 site-specific stimulation of transcription described above, the mtDNA binding capacity of the mTERF protein was tested. Very interestingly, in electrophoretic mobility shift assays (EMSA), the interaction of purified recombinant GST-mTERF protein with the H1 site (contained in a 5′-labeled H1 probe [see Supplemental Data]) was observed (Figure 4C, left panel). By contrast, the H2 site (contained in a 5′-labeled H2 probe) did not bind to GST-mTERF under the same experimental conditions (Figure 4C, right panel). This result provided the important clue that the previously unknown interaction of mTERF with the H1 site presumably plays a crucial role in the phenomenon investigated here.

H Strand Transcription Initiation and Termination Probes Are Band Coshifted with mTERF in EMSA Experiments

To study the molecular mechanism by which mTERF could control the rDNA transcription initiation and termination

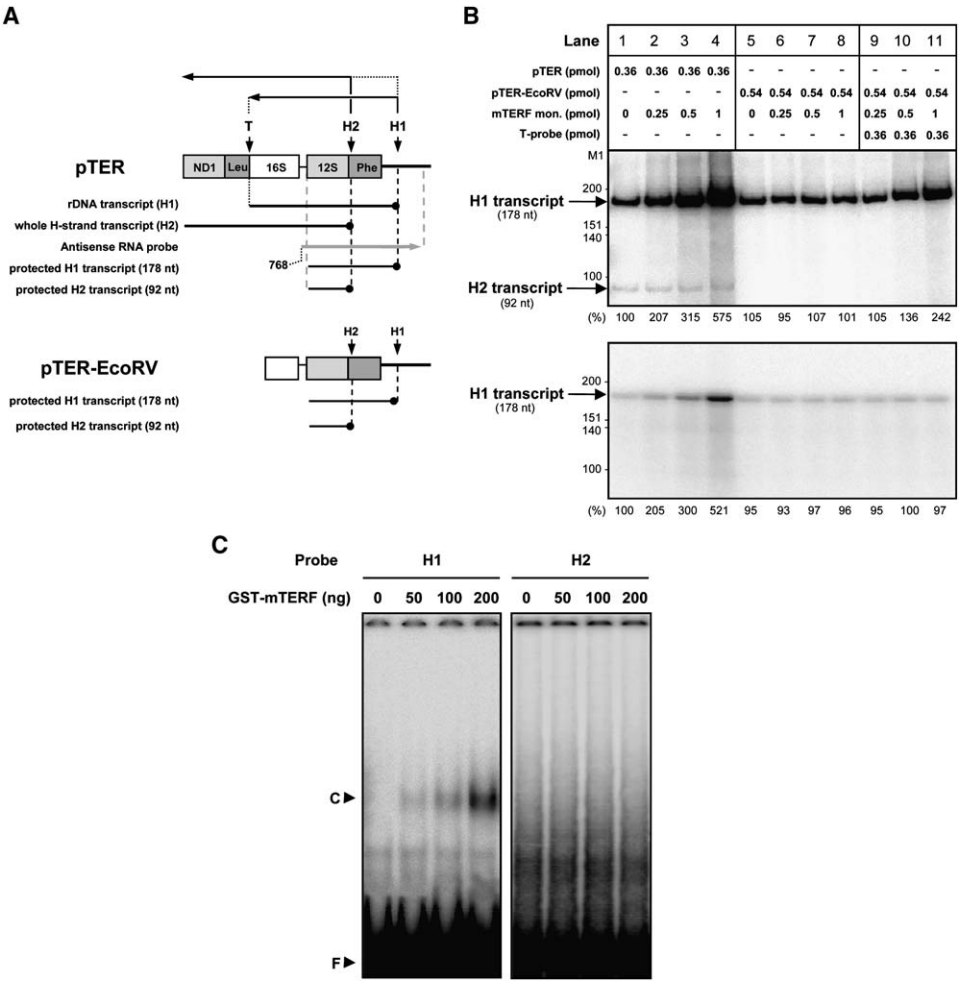


Figure 4. mTERF Stimulates Transcription from H1 on rDNA Template by Enhancing the Recycling of Transcription Machinery

(A) As shown in this schematic diagram, transcripts in vitro initiation at the H1 or H2 site were labeled with [α-^{32}P]-GTP and guanylyltransferase and analyzed by S1 nuclease protection assays (see Experimental Procedures). The DNA templates utilized were pTER and its derivative pTER-EcoRV. The run-off transcripts initiated at the H1 site were only observed in vitro.

(B) mTERF-dependent stimulation of H1 transcription was tested under two different conditions, that is, high (upper panel) and low (lower panel) amount of mitochondrial transcription machinery (see Experimental Procedures). The experiments with the pTER-EcoRV template were done without (lanes 5–8) or with the addition of a 44 bp transcription termination fragment (T probe; lanes 9–11). The products of the S1 protection assay were 30 nucleotides shorter than the products obtained by using in vivo synthesized RNA (Figure 1B), as explained in Experimental Procedures and illustrated in (A). The relative band intensities of H1 transcripts were quantified by densitometry using the ImageQuant program (Molecular Dynamics). The H1 transcription level of pTER in the absence of mTERF monomer was set as 100%.

(C) mTERF protein specifically binds to the H1 site. In these DNA mobility shift assays, an increasing amount of recombinant GST-mTERF protein (from 50 to 200 ng) was mixed with ^{32}P-end-labeled H1 or H2 probe (20 fmol each). The sequences of the H1 and H2 probes correspond to the human mtDNA L-strand nt 524–576 and nt 601–660, respectively. The purified recombinant GST-mTERF showed interaction with the H1 site (left panel), whereas the H2 site was not bound under these conditions (right panel). C, protein-DNA complex; F, free probe.

activities, EMSA experiments were performed using an infrared laser scanning system (Odyssey, LiCor), as previously described (see Experimental Procedures). Figure 5 shows the results of EMSA experiments with the mTERF monomer-enriched fraction and the T probe (green fluorescence), encompassing the termination binding site, and the I probe (red fluorescence), which contains the H strand transcription initiation sites (see Experimental Procedures). The mTERF monomer fraction showed a specific binding activity for the I probe (Figure 5A, lanes 2–4) and T probe (Figure 5A, lanes 6–8). The shifted bands were in each case specifically super-shifted (Figure 5A, lanes 3 and 7) by the addition of mTERF antiserum to the binding reaction mixture, whereas no super-shift was observed in the controls with preimmune serum (Figure 5A, lanes 4 and 8). The most important result was the presence of a band coshifted with the two DNA probes (Figure 5A, lanes 10–12), which also was specifically super-shifted by mTERF antiserum (Figure 5A, lane 11). This result points to a simultaneous interaction of mTERF (or mTERF plus some auxiliary protein[s] present in the

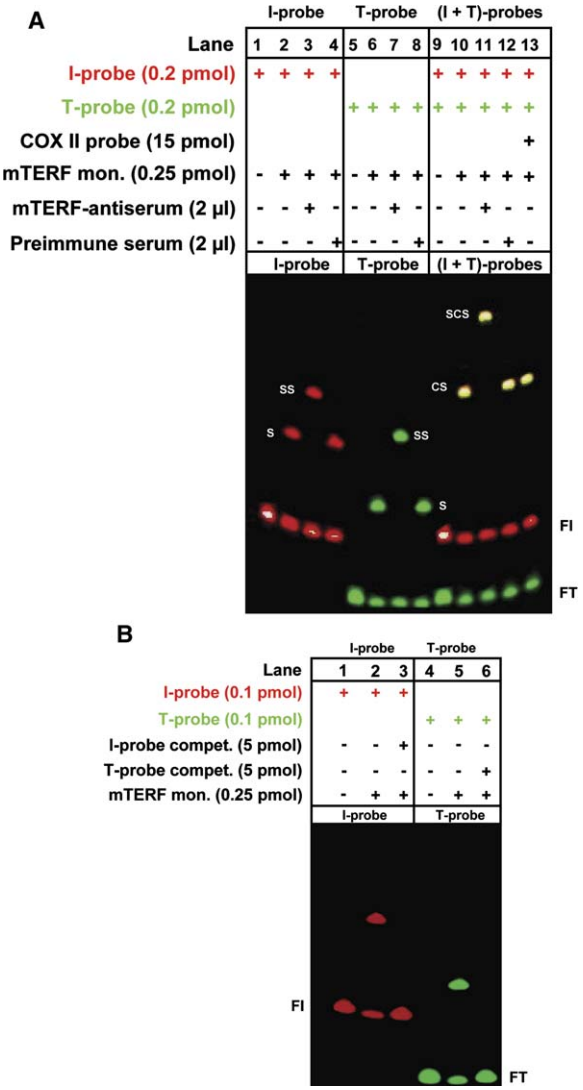


Figure 5. Transcription Initiation and Termination Probes Are Band Coshifted with mTERF

EMSA experiments with two different fluorescent DNA probes to test the mTERF binding sites for transcription initiation (I probe, red fluorescence) and for transcription termination (T probe, green fluorescence) were performed as described in Experimental Procedures.

(A) The specific binding activity of mTERF for the I probe (lanes 1–4) and the T probe (lanes 5–8) and its simultaneous binding interaction with the two DNA probes (lanes 9–12), as indicated by the yellow merged coshifted band. Lanes 3, 7, and 11 represent super-shift experiments after the addition of mTERF-antiserum, and lanes 4, 8, and 12 the corresponding controls with a preimmune serum. FI, free I probe, and FT, free T probe; S, shifted bands of the single probe binding test (lanes 2, 4, 6, and 8); SS, super-shifted bands of the single-probe bindings; CS, coshifted bands with the two probes; SCS, super-shift band of the CS. A control for nonspecific binding activity for other mtDNA sequences was done by using a 75-fold molar excess of a 100 bp unlabeled probe representing a fragment of the COXII gene (lane 13).

(B) Competition experiments for the I probe (lanes 1–3) and the T probe (lanes 4–6) binding activities using a 50-molar excess of the corresponding unlabeled probes.

monomer fraction) with the initiation and termination sites. A 75-fold molar excess of a fragment of the cytochrome c oxidase subunit II gene (COXII) was used to exclude the occurrence of nonspecific interactions of the mTERF fraction with mtDNA sequences (Figure 5A, lane 13). Figure 5B shows that the binding of mTERF to each of the two DNA fluorescent probes is competed for completely by a 50-fold molar excess of the same unlabeled probe.

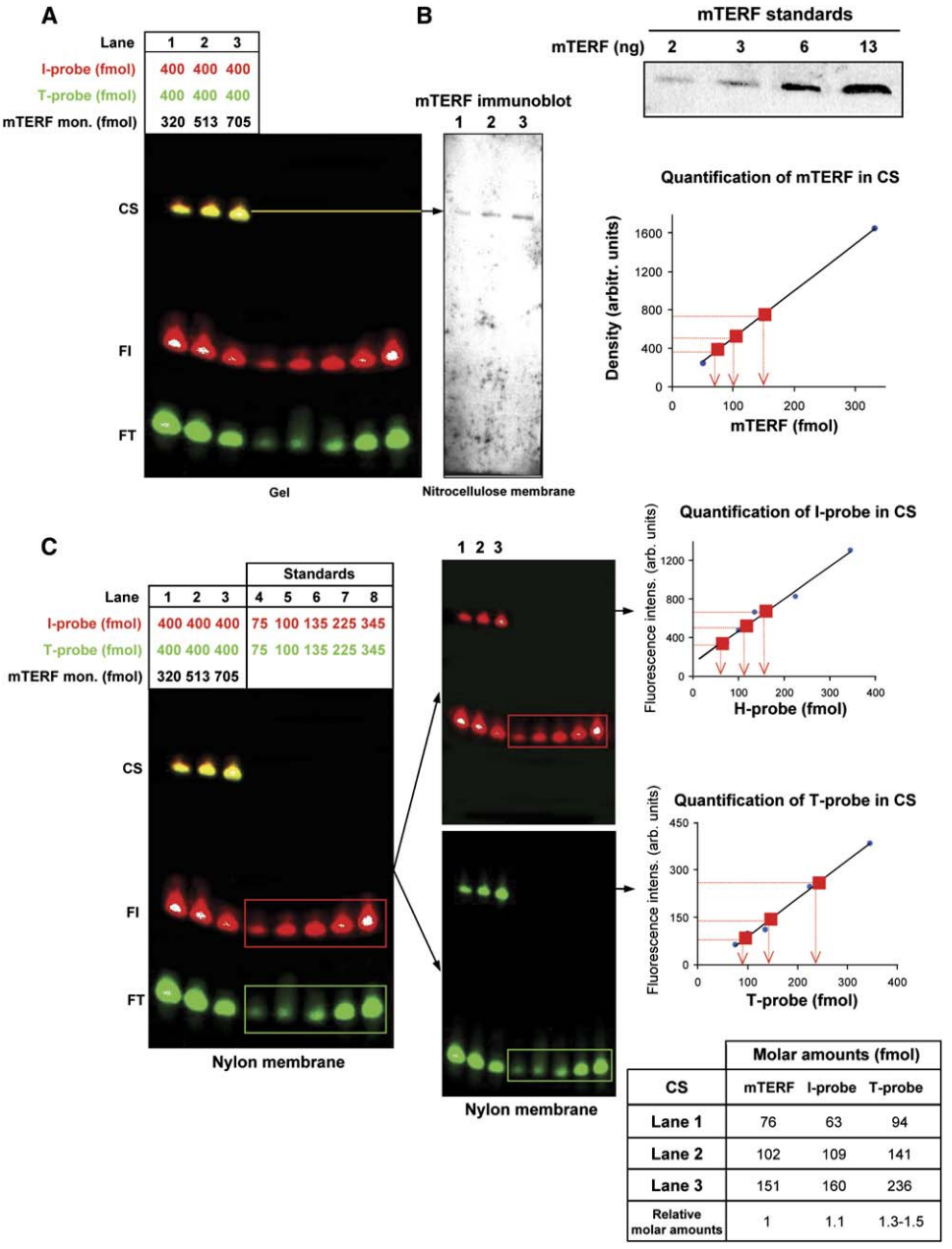
Interestingly, the mTERF comigration with the initiation and termination sites shown above was not detected with the recombinant mTERF protein. In fact, with the latter protein, a high concentration (100-fold molar excess) of unlabeled initiation fragment (H1, not H2) only partially competed with the ^{32}P-labeled termination fragment (TERM) and did not show the comigration (Figure S2, left panel). In contrast, with the recombinant mTERF protein, the unlabeled termination probe (TERM) competed effectively with the ^{32}P-labeled TERM (Figure S2, right panel). These results clearly show that the capacities of natural mTERF and recombinant mTERF for binding to both sites are different.

Molecular Analysis of the Band-Coshifted Complexes

To analyze the molecular nature of the coshifted complexes, a differential membrane transfer system (Demczuk et al., 1993) was used after the EMSA experiments. In these, the band-coshifted complexes were obtained using the same amounts of I probe and T probe and increasing amounts of mTERF (Figure 6A). In the same gel, different amounts of the two free DNA probes were loaded to construct standard curves (see Experimental Procedures). After electrophoresis, the protein and the two DNA probes were blotted onto two different membranes simultaneously. The nitrocellulose membrane used for the mTERF immunoblot (Figure 6B, left panel) shows the mTERF present in the coshifted complexes. By using the mTERF standard curve (Figure 6B, right panels), the amount of mTERF in these complexes could be estimated. Figure 6C (left panel) shows the two-wavelength infrared scanning of the nylon membrane used to transfer the DNA from the EMSA gel (Figure 6A), which gives a pattern identical to that of the gel. Figure 6C (central panels) shows the single wavelength scan image of the nylon membrane for each of the two DNA probes. The two probes are coshifted to give the yellow coshifted complex band in the merging image of the two-wavelength scans. Within a red or green contour are shown the samples for the standard curve of I-probe or T-probe, respectively. The DNA standard curves were in a very good linear range (Figure 6C, right panels). In the three coshifted complexes obtained with increasing amounts of mTERF monomer, the molar amounts of mTERF and the I probe were very similar, whereas the amounts of the T probe were 30%–50% higher (table in Figure 6C).

In Vivo Association of mTERF with the H1 Initiation and Termination Sites of H Strand Transcription Revealed by Chromatin Immunoprecipitation

To test the prediction that the mTERF protein should be associated with the H1 initiation sequence in vivo, the H1



	Molar amounts (fmol)		
CS	mTERF	I-probe	T-probe
Lane 1	76	63	94
Lane 2	102	109	141
Lane 3	151	160	236
Relative molar amounts	1	1.1	1.3-1.5

Figure 6. Molecular Analysis of the Band-Coshifted Complexes

Band-coshifted complexes were electroblotted onto a selective two-membrane-transfer system to analyze the relative molar amounts of I probe, T probe, and protein in the ternary complexes, as detailed in Experimental Procedures.

(A) Infrared fluorescence image of the EMSA gel prior to the electrotransfer, showing the two overlaid fluorescent channel scans of the coshifted complexes produced by incubating the two DNA probes with increasing amounts of mTERF monomer (lanes 1–3). The right side of the panel shows increasing amounts of free I probe or T probe loaded on the gel to construct standard curves for the DNA quantification described below in (C). FI, free I probe; FT, free T probe; CS, band-coshifted complex.

(B) Quantification of the mTERF present in the coshifted complex. The protein was transferred to a nitrocellulose membrane and analyzed by Western blotting using mTERF antiserum. The left subpanel shows the immunoblots of the band-coshifted complexes (CS) presented in (A) (lanes 1–3), which agree perfectly with the CS positions. For the quantification of mTERF in each lane, a standard curve with increasing amounts of the mTERF monomer fraction (filled blue circles) was constructed (right subpanels). The filled red squares represent the estimated amounts of mTERF monomer.

(C) Quantification and relative molar amount calculation of the coshifted DNA I and T probes. In the same two-membrane-transfer system described above, the nylon membrane, carrying the free DNA probes and the coshifted DNA probes, was analyzed by the infrared laser scanning system (Odyssey, LiCor). The left panel shows the two overlaid fluorescent channel scans of the nylon membrane, with the coshifted complexes produced in the presence of increasing amounts of mTERF monomer (lanes 1–3), as described in (A), and also the two fluorescence patterns obtained with increasing amounts of the free

initiation site was analyzed by the chromatin immunoprecipitation technique (ChIP; Orlando, 2000), using affinity-purified mTERF antibody (Figure 7A; Experimental Procedures). As a positive control, the in vivo interaction between mTERF and the transcription termination site was analyzed by using the same antibody and preimmune serum as a negative control. To detect mTERF-dependent immunoprecipitation of mtDNA fragments, specific primer sets (Table S1) were designed for amplification of several segments of mtDNA (Figure 7A). The amplification of every immunoprecipitated mtDNA segment was performed in the linear range, and the molecular abundances were quantified by densitometry of ethidium bromide-stained bands (Figures 7B and 7C). As expected, the transcription termination site and initiation site were immunoprecipitated and amplified comparably by the mTERF-specific antibody. By contrast, the amplification of other portions of the mitochondrial genome revealed a very weak or no signal above background (Figure 7B). Thus, the above described results showed that there is a good correlation between the in vivo ChIP results and results of the EMSA assays in which mTERF was tested against the H1 or H2 initiation site (Figure 4C).

Measurement of Affinities of mTERF for the H1 Initiation and Termination Sites

To measure the affinities of mTERF for the two mtDNA binding sites, the apparent equilibrium dissociation constants (K_D's) of mTERF for the two sites were determined by carrying out several independent EMSAs. Thus, the purified in vivo generated mTERF was shown to have very similar affinities to the termination and H1 sites (K_D values of 50 ± 2.5 nM and 62 ± 5.7 nM, respectively). This was in agreement with the finding that mTERF can bind simultaneously to the two sites, as shown in Figures 5 and 6. The latter data are perfectly consistent with the results of our ChIP experiments. By contrast, by using the recombinant human GST-mTERF protein expressed in a soluble form in *E. coli*, we ascertained the average K_D values (±SEM) for the termination and H1 sites to be 65 ± 1.2 nM and 750 ± 9.7 nM, respectively.

Mitochondrial rDNA Looping in the In Vitro Transcription Template Detected by Electron Microscopy (EM)

The results described above support a simultaneous interaction of mTERF with its two mtDNA binding sites. A plausible mechanism for this interaction may be a looping out of the rDNA. Figure 7D represents a schematic model of this mTERF-mediated rDNA loop. In this model, the binding of mTERF to its binding sites, presumably together with auxiliary transcription factors such as TFAM (Fisher and Clayton, 1988) and/or TFB1M or TFB2M (Falkenberg et al., 2002), specifically drives the RNA polymerase to the H1 initiation site. The loop structure might provide a mechanism to activate a new rDNA transcription event after the completion of the previous one.

To determine directly if protein-mediated rDNA looping occurs between the H1 and termination mTERF binding sites, the linearized 1.3 Kb pTER DNA template was incubated with a mitochondrial lysate from HeLa cells and the mTERF monomer protein (see Experimental Procedures). Following incubation, the protein was fixed with glutaraldehyde, free protein was removed by gel filtration chromatography, and the reaction products examined by EM. Examination of fields of molecules revealed four classes of DNA molecules: protein-free DNA, DNA with a single protein complex bound internally along its length, DNA with two distinct protein complexes bound internally (Figure 7E), and DNA with a large loop (representing roughly 1/3 of the total length) bound to a protein complex at its base (Figure 7F). The positions of the bound protein complexes were consistent with the location of the mTERF binding sites (Tables S2 and S3). There were also some DNA molecules with protein bound to one or both ends. The amounts of internal single binding or double binding protein complexes and the loop formation increased almost regularly with the amount of exogenous mTERF up to a certain saturating protein concentration (data not shown).

In Vivo Detection of Mitochondrial rDNA Looping by Chromosome Conformation Capturing Assay

We also demonstrated the mTERF-mediated rDNA loop formation in vivo by a chromosome conformation capturing (3C) assay, carried out as described (Dekker et al., 2002), with some modifications. A description of the detailed method used is available in Supplemental Data. The basic principle of this technique was that intact cells were treated with 1% formaldehyde to crosslink mTERF and portions of mtDNA which were in close proximity to each other (Figure 7G, upper panel). The crosslinked mtDNA-protein complex was digested with the restriction enzyme HpyCH4IV, and the mTERF-mediated DNA-protein complex was isolated by immunoprecipitation using affinity-purified mTERF antibody. The mTERF-crosslinked mtDNA fragments were then ligated at very low DNA concentrations that favor intramolecular interaction, rather than random intermolecular interactions. After ligation, the crosslinks were reversed by overnight heating and treatment with proteinase K. The ligation products were then analyzed by PCR using two different pairs of primers (a and b or a and c in Figure 7G). The expected sizes of PCR products obtained using the a + b primer set and the a + c primer set were 661 bp and 1043 bp, respectively. We also used other mtDNA samples prepared under different

probes. The two central panels show the single wavelength fluorescence channel images of the membrane observed for the I probe (700 nm channel) and T probe (800 nm channel). The three single-color bands in the upper portion of each panel are exactly at the same positions in the image, representing the coshifted complexes. The right panels show the points of the standard curve for each probe indicated by filled blue circles, and the estimated values for the band-coshifted DNA amounts indicated by filled red squares. The table represents the molar amounts of mTERF monomer, I and T probe in the band-coshifted complexes (CS), as well as the approximate estimated relative molar amounts of mTERF, I, and T probe in each of the three complexes.

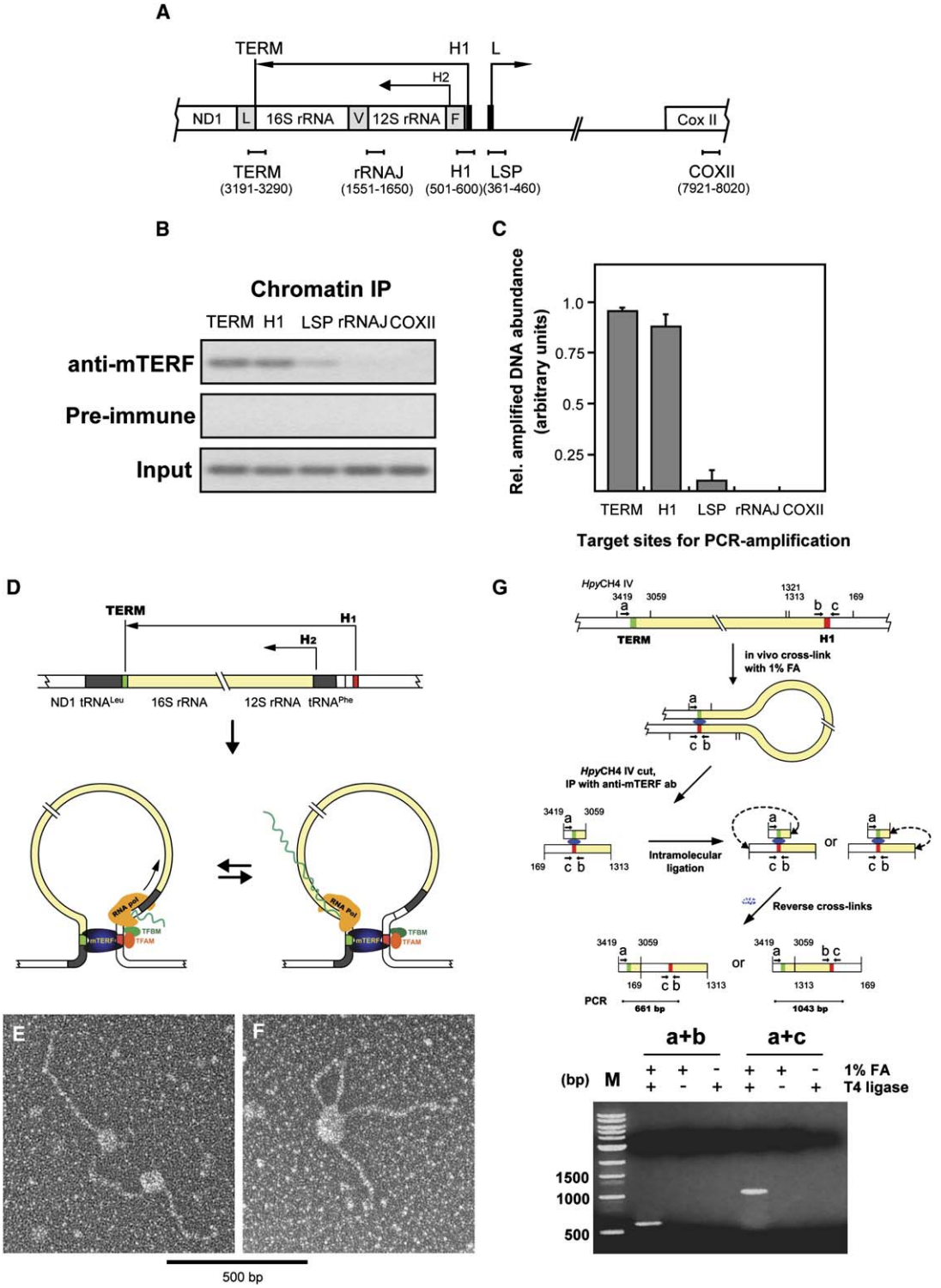


Figure 7. The mTERF Protein Interacts with the Initiation and Termination Sites of Mitochondrial rDNA Transcription and Mediates rDNA Looping In Vivo and In Vitro

(A) Map of target sites for PCR-mediated detection of the mtDNA fragments crosslinked to mTERF using the ChIP technique. The numbers in brackets indicate the positions of the PCR products in the Cambridge human mtDNA sequence (Anderson et al., 1981). TERM, mitochondrial rDNA transcription termination site; rRNAJ, junction between 12S rRNA and 16S rRNA genes; H1, mitochondrial rDNA transcription initiation site; LSP, light-strand promoter; COXII, cytochrome c oxidase subunit II gene; L, $tRNA^{Leu(UUR)}$; V, $tRNA^{Val}$; F, $tRNA^{Phe}$.

conditions, without the crosslinking or ligation step, as control templates. Representative agarose gel electrophoresis runs of PCR products obtained under the different conditions are shown in Figure 7G (lower panel). The production of PCR products of the right sizes was fully dependent on the crosslinking and ligation steps. Furthermore, the ligation-dependent generation of a new HpyCH4IV enzyme cut at the expected position in each PCR product was fully confirmed by redigestion with the same enzyme (Figure S3).

DISCUSSION

This work has provided strong evidence indicating that the mechanism underlying the regulation of human mitochondrial rDNA transcription by mTERF involves the simultaneous interaction of this factor with the initiation and termination sites and the resulting looping-out of the rDNA. In fact, the observation that, in in vitro transcription experiments, the stimulation of rDNA H strand transcription from the H1 site by the mTERF monomer did not occur if the artificial template lacked the mTERF termination binding site suggested that the transcription stimulating activity requires the mTERF monomers to be bound to the termination site. In the case of the truncated template (pTER-EcoRV), the stimulation of rDNA transcription by mTERF could be restored up to ~30% of the level observed with the full-length template (pTER) after addition of a 44-mer termination fragment to the reaction mixture, however, only in the presence of a high concentration of the mitochondrial transcription machinery. It is possible that mTERF has another stimulatory role, maybe by bending the DNA to an easy-to-initiate conformation or working as an enhancer effector that requires that mTERF DNA binding domains are bound to both DNA sites to have an active conformation. It should also be mentioned that, in previous reports, the stimulation effect by mTERF was not observed in an in vitro transcription system using template DNA containing a nonnatural promoter (such as LSP, SP6, or T7 promoter) and a nonnatural starting site (Hess et al., 1991; Prieto-Martín et al., 2004).

In the present work, a *simultaneous* binding of the natural mTERF monomer to the I probe and T probe was shown clearly in a series of EMSA experiments using infrared fluorescence measurements and following Western blot analysis. The I probe used in the present work included both the H1 and the H2 sites. However, other evidence indicated that mTERF did bind to and stimulated transcription from a mtDNA fragment encompassing only the H1 site, but not from a fragment carrying only the H2 site (Figure 4C). A quantification of the infrared fluorescence measurement and Western blot data showed the presence in the coshifted bands, produced from a nearly equimolar mixture of the three components, of average relative molar amounts of ~1, ~1, and 1.3–1.5 for mTERF monomer, I probe, and T probe, respectively.

That the capacity of mTERF monomer to interact in vitro with the two mtDNA fragments carrying the H1 initiation site or the termination site reflected an in vivo phenomenon was shown by in vivo ChIP assays, in which the simultaneous binding of mTERF to the two mtDNA sites occurred with two separated mtDNA segments in vivo. A very likely mechanism for this process, strongly supported by direct electron microscopy (EM) would involve a looping-out of the rDNA. The in vivo occurrence of an mTERF-mediated rDNA loop formation was fully confirmed by a chromosome conformation capturing assay (Figure 7G). The looping mechanism could explain the 15- to 60-fold higher rate of rRNA synthesis over mRNA synthesis in vivo (Montoya et al., 1983) as resulting from the enhancement of the reinitiation rate of the H1-specific transcription event. The 2-fold or greater molar excess of mTERF monomer over the artificial mtDNA template, which was required, in the in vitro transcription experiments, for quantitative termination of H1-initiated transcripts, may, at least in part, account for the large excess (>70 fold) of mTERF molecules per cell determined in this work (~6×10^5) over the amount of mtDNA per cell (7–8 $\times$ 10^3 [King and Attardi, 1989]) in HeLa cells. This high concentration of mTERF maintained in vivo may ensure a stable rDNA loop structure between the H1 initiation and the termination sites for each ''coupled'' rDNA transcription event.

It is possible that a single mTERF molecule binds to the termination site and to the H1 site of the *same* mtDNA molecule

(B) The relative amounts of the PCR products were compared after electrophoresis in a 1% agarose gel and EtBr staining. The PCR reactions of the products immunoprecipitated by preimmune serum and of the mitochondrial lysate input were used as negative and positive control, respectively. Only the mtDNA fragments containing the termination site (TERM) or initiation site (H1) of rDNA transcription could be amplified by their specific PCR primer sets. The input was 10% of the total mtDNA fragment pool used for immunoprecipitation.

(C) DNA fragments detected by PCR with primer sets for TERM, rRNAJ, H1, LSP, and COXII in HeLa cell mtDNA crosslinked and immunoprecipitated with purified anti-mTERF antibody. The mtDNA fragments containing the transcription termination site (TERM) or initiation site (H1) were immunoprecipitated and amplified comparably by the mTERF-specific antibody. Each bar represents the average of three independent experiments, and the SEM is indicated.

(D) Schematic representation of a proposed model for an mTERF-mediated rDNA loop structure. H1 (red fragment), rDNA transcription initiation and mTERF binding site; H2, total H strand transcription initiation site; TERM (green fragment), rDNA transcription termination and mTERF binding site. RNA Pol, human mtRNA polymerase; TFAM, human mitochondrial transcription factor A; TFBM, human mitochondrial transcription factors B1 and/or B2.

(E) EM image of a pTER DNA molecule with two bound protein complexes situated at the two mTERF binding sites, according to measurements of DNA lengths and positions of the bound protein complexes.

(F) EM image of a looped pTER DNA molecule. The sizes of the loop, long arm, and short arm in (E) and (F) are consistent with the protein-mediated looping occurring via the mTERF binding sites.

(G) In vivo interaction between the H1 site and the termination site, as detected by 3C assays. The upper panel shows the experimental scheme, the orientation of the primers (small arrows), and the location of the HpyCH4IV sites near the H1 (red) site and the termination (green) site. The mTERF or mTERF-mediated structure was represented as a blue oval. The lower panel shows the PCR products derived from primer combinations after ligation between the HpyCH4IV fragments harboring the H1 site and the termination site. The primer combinations are shown above the gel.

at the *same* time. Another possibility is that one mTERF molecule binds to each site of the *same* mtDNA molecule and that the two mTERF molecules then interact with each other, either directly or with the intermediary of one or more mTERF molecules, possibly with the participation of auxiliary proteins. However, the available results, and in particular the competition EMSA data, do not allow yet a decision about which of the models mentioned above operates in vivo.

Very recently, evidence has been reported that a recombinant rat mitochondrial transcription termination factor exhibited a binding activity for the mitochondrial promoter region (Prieto-Martín et al., 2004). In the above cited work, no evidence was found of the simultaneous formation of a complex of this rat mTERF with the termination probe and the promoter probe. However, the estimated K_D values for the termination and H1 sites of a rat His-mTERF were ~210 nM and ~1300 nM, respectively, which are reasonably close to those estimated in the present work for the recombinant human GST-mTERF protein expressed in *E. coli* (65 nM and 750 nM, respectively). This observation points to the possible role of the recombinant nature of mTERF in producing the difference in its affinity for the two sites, possibly as a result of differences in secondary modifications. In the present work, we have in fact demonstrated that there is a clear discrepancy between purified in vivo and in vitro generated human mTERF in their DNA binding properties, with the H1 binding affinity of purified in vivo generated mTERF being quite higher than that of recombinant mTERF. It is a reasonable idea that the natural mTERF protein may have a post-translational modification(s) or a binding cofactor(s) which increases its affinity for the H1 site.

In the present work, no analysis of the mTERF domains(s) interacting with the T probe and the I probe was carried out. However, the observation that the stoichiometry of binding in the ternary complex approximated 1:1:1 favors the conclusion that mTERF has two binding sites, one specific for the H1 promoter and the other for the terminator. This conclusion strongly supports the interpretation that regulation of human mitochondrial rRNA synthesis involves an mTERF-mediated rDNA looping. A role of DNA loops in control of gene expression has also been described in yeast. Thus, in a recent paper (O'Sullivan et al., 2004), findings are reported that illustrate the role of gene loops in juxtaposed promoters and terminators in *S. cerevisiae*.

Our work has revealed a quite novel mechanism for activating mitochondrial rRNA synthesis in mammalian cells. This mechanism involves, first, the specific selection of the mitochondrial rDNA transcription initiation site H1 between the two well-documented H strand initiation sites and then an mTERF-mediated molecular process that facilitates the recycling of mitochondrial RNA polymerase on the *same* rDNA loop-forming template mtDNA molecule.

EXPERIMENTAL PROCEDURES

HeLa Cell Mitochondrial RNA Extraction and Fractionation

The mitochondrial fraction was prepared by differential centrifugation (Kruse et al., 1995) from 3–4 × 10^9 cells, purified by Percoll/Metrizamide gradient centrifugation (Storrie and Madden, 1990; Helm and Attardi, 2004), and then incubated with micrococcal nuclease (Sigma) and, subsequently, with 100 μg/ml of proteinase K (Roche) for 30 min at 37ºC (Attardi and Montoya, 1983). RNA was finally extracted using the RNAbee system (Tel-Test, Inc.). The separation of poly(A)$^+$RNA (bound RNA) and non-poly(A)$^+$RNA from 0.5 mg of total mitochondrial RNA was obtained using a PolyATract mRNA isolation system (Promega).

In Vitro Transcription of mtDNA with Different Templates

In vitro transcription, using [α-^{32}P]-UTP in the 13,000 × g_{av} supernatant of a 0.5% Tween 20 mitochondrial lysate, was performed essentially as described (Kruse et al., 1995; Fernández-Silva et al., 1996; Asin-Cayuela et al., 2004). Two mtDNA templates were used (Figure 2C). One template was the human mtDNA clone pTER (Kruse et al., 1989) and the other, pTER-EcoRV (Figure 2C), was a derivative of pTER lacking the termination site, which was obtained by cutting it with EcoRV (New England Biolabs).

Partial Purification and Quantification of mTERF

The mTERF monomer-enriched fraction used in the present work was obtained by gel filtration chromatography of the S-100 fraction from a HeLa cell mitochondrial lysate, as described (Asin-Cayuela et al., 2004; Figure S1). The amount of mTERF in a single HeLa S3 cell or mTERF monomer fraction was determined by Western blot analysis, using a standard curve from various known amounts of the recombinant GST-mTERF, as described in Supplemental Data.

S1 Nuclease Protection Assay

The guanylyltransferase + [α-^{32}P]-GTP capping reaction specifically labels nascent transcripts which carry a di- or triphosphate nucleotide at their 5′ end. In vivo or in vitro synthesized mtRNA was labeled with guanylyltransferase and [α-^{32}P]-GTP following the procedure described by Kruse et al. (1995). After the capping reaction, the samples were analyzed by the S1 nuclease protection assay. For this purpose, [α-^{32}P]-GTP-labeled RNA samples were hybridized with the antisense RNA probe described in Supplemental Data (synthesized from the plasmid pGEHP23 with SP6 RNA polymerase), digested with S1 nuclease, and the capped products were analyzed by 8% polyacrylamide/7M urea gel electrophoresis (Figures 1A and 1B), as detailed elsewhere (Fernández-Silva et al., 1996).

For the in vitro-transcribed RNA, the bands resolved by urea-polyacrylamide gel electrophoresis in the S1 protection assays were 30 nucleotides shorter (Figure 4) than those obtained with in vivo synthesized RNA (Figures 1A and 1B). This was expected, since the 12S RNA gene fragment inserted into the artificial DNA templates pTER and pTER-EcoRV, utilized for in vitro transcription, starts at nucleotide 648 and ends at nucleotide position 739, and the antisense RNA probe utilized in the S1 nuclease protection assays encompasses a fragment of mtDNA from position 524 to position 768.

For a transcription reinitiation assay at a low concentration of transcription machinery, including the mitochondrial RNA polymerase, template plasmids immobilized on magnetic beads were used. The pTER plasmids were cut with HindIII, and biotin-14-dATP was then incorporated into the HindIII site using the Klenow enzyme. The biotinylated plasmids were cut with EcoRI or EcoRV and then purified by 1% agarose gel electrophoresis. Biotinylated templates (10 μg) were bound to 500 μl of streptavidin magnetic beads (Dynal) and incubated with 2% bovine serum albumin (BSA) to block nonspecific binding sites. The immobilized template plasmids were incubated with 2.5 μl of the S100 fraction of a mitochondrial lysate diluted with transcription buffer (10 mM Tris-HCl [pH 8.0; 25ºC], 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 10% glycerol) without NTPs at 30ºC for 15 min and washed once with the same buffer. Transcription was started by addition of 25 μl of transcription buffer containing 0.6 mM of each NTP and different amounts of purified natural mTERF and T probe and incubation at 30ºC for 30 min. The nascent transcripts were detected by in vitro capping and the S1 nuclease protection assay described above.

In the in vitro transcription experiments in which the transcribed mtRNA was labeled with [α-^{32}P]-UTP (Figures 2D and 3), the samples were also

analyzed by the S1 protection assay (Fernández-Silva et al., 1996), utilizing the other unlabeled antisense RNA probe described in Supplemental Data (synthesized from the pKSM2 plasmid with T3 RNA polymerase).

5′ End Mapping of In Vivo-Initiated Transcripts

In order to obtain a precise mapping of the 5′ ends of the mtDNA H strand nascent transcripts (H1 and H2 transcripts), purified total mtRNA and polyadenylated mtRNA from HeLa cells synthesized in vivo were analyzed by reverse transcription-primer extension (RT-primer extension; Calzone et al., 1987; Boorstein and Craig, 1989). For this purpose, a 5′-end-labeled oligodeoxynucleotide primer, complementary to a portion of the 12S RNA sequence (corresponding to the mtDNA segment between nucleotides 708 and 728; Figure 1C), and AMV reverse transcriptase (Promega) were utilized. The L strand primer sequence was: 5′-$_{(728)}$ gag ggtgaactcactggaacg $_{(708)}$-3′. After the RT-primer extension, 20 μl of urea loading dye was added to each sample, and the samples were heated at 90ºC for 10 min, and either loaded directly onto an 8% polyacrylamide/7 M urea gel and run in parallel with the pTER manual sequencing products described below or stored at −20ºC.

The manual sequencing analysis of the pTER clone was carried out by the fmol^R DNA Cycle Sequencing System (Promega), according to the manufacturer's instructions, using the same 5′-end-labeled primer described above for the RT-primer extension.

EMSA by a Two-Fluorescence Infrared Imaging System

A two-fluorescence infrared laser scanning system (Odyssey, LiCor) was used in the EMSA assays aimed at analyzing the double interaction of mTERF with the termination and initiation binding sites. The termination probe (T probe) and initiation probe (I probe) were prepared as described in Supplemental Data. The Odyssey system enables the simultaneous visualization of two molecules differently labeled with the IRDye 800 and IRDye 700. The standard band-shift assay reaction mixture was as previously described (Asin-Cayuela et al., 2004). The incubation was carried out at 4ºC for 20 min, and immediately the samples were loaded onto a native 8% polyacrylamide gel in Tris/glycine/EDTA buffer in the cold room. Supershift experiments were carried out by including 2 μl of anti-mTERF antiserum in the reaction, and controls were performed with 2 μl of preimmune serum.

We measured the apparent equilibrium dissociation constants (K_D's ± SE) of human mTERF for the DNA binding sites by using three independent EMSAs to estimate the amounts of the bound and unbound probes. The protocols in detail are available in Supplemental Data.

Selective-Membrane Transfer of the DNA Probes and mTERF from Band-Coshifted Complexes

The molecular analysis of the protein-DNA coshifted complexes was made, after the EMSA gel run and subsequent scanning (Figure 6A), by using a system of double membrane electroblotting (Demczuk et al., 1993), with some modifications, as detailed in Supplemental Data. The electroblotted membranes were analyzed by Western blotting or Odyssey infrared fluorescence laser system (LiCor), as described in Supplemental Data.

Chromatin Immunoprecipitation Technique and Chromosome Conformation Capturing Assay

ChIP experiments were carried using a technique based on a combination and modifications of previously described protocols (Orlando, 2000 and references therein). Sequences of mtDNA subregion-specific primers and a description of the detailed method used are available in Supplemental Data. A description of the procedure followed for the chromatin conformation capturing assay can also be found in Supplemental Data.

Preparation of DNA-Protein Complexes for EM

A 25 μl reaction mixture containing 150 ng of the linearized 1.3 kb pTER DNA template was incubated for 30 min at 30ºC in transcription buffer (Fernández-Silva et al., 1996), supplemented with 1.3 μl of mitochondrial lysate and mTERF monomer protein (3–25 ng). The reaction mixture was fixed, fractionated by gel filtration chromatography, and the fractions containing the DNA-protein complexes were prepared for EM as described (Griffith and Christiansen 1978).

The samples were examined in a Philips/FEI Tecnai 12 TEM at 40 KV, and the images were recorded using a Gatan Ultrascan US4000P slow scan CCD camera (Gatan). DNA lengths were measured using GATAN Digital Micrograph 3.0 software.

Supplemental Data

Supplemental Data include three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/123/7/1227/DC1/.

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