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Phosphorylation of rat mitochondrial transcription termination factor (mTERF) is required for transcription termination but not for binding to DNA

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ABSTRACT

Despite the crucial importance of mitochondrial transcription, knowledge of its regulation is poor. Therefore, characterization of mammalian mitochondrial transcription termination factor (mTERF) functionality and regulation is of fundamental biological interest in order to understand the regulation of mitochondrial transcription. Here we report that mTERF is the first protein having a role in mammalian mitochondrial gene expression that appears to be controlled by phosphorylation. Recombinant mature rat mTERF protein has specific DNA-binding capacity for the sequence required for transcription termination. Furthermore, unlike recombinant human mTERF, the rat protein bound to its mitochondrial DNA binding site promotes the termination of transcription initiated with heterologous RNA polymerase. Interestingly, mTERF is a phosphoprotein with four phosphate groups, and while the DNA-binding activity of mTERF is unaffected by the phosphorylation/dephosphorylation state, only the phosphorylated form of the protein is active for termination activity. Moreover, natural human mTERF is also a phosphoprotein and its termination activity is inhibited by dephosphorylation. These data suggest that mTERF functioning *in vivo* is regulated by phosphorylation.

INTRODUCTION

Mitochondria are involved in many essential metabolic pathways, including the production of most of the cellular ATP through oxidative phosphorylation (OXPHOS system). In mammalian cells, each organelle contains several copies of the mitochondrial genome, which is represented as a closed circular DNA of ~16.6 kb. The mitochondrial DNA (mtDNA) encodes 37 genes: 22 tRNA, 2 rRNA and 13 polypeptides that are components of the OXPHOS system (1). However, most of the subunit components of the aforementioned system, and all the proteins necessary for mitochondrial replication, transcription and translation, are encoded by nuclear genes. These proteins are synthesized by cytoplasmic ribosomes as precursors and then imported into mitochondria (2).

Expression of mammalian mtDNA is initiated from a regulatory site in the molecule called the D-loop region that contains an origin of replication (O_H) and the transcription promoters. In this mtDNA there are two major transcription initiation sites (I_{H1} and I_L); I_{H1} is located in the H-strand promoter (HSP) and I_L in the L-strand promoter (LSP) (3). A second initiation site for H-strand transcription (I_{H2}) is located in the $tRNA^{Phe}$ gene, immediately adjacent to the gene for 12S rRNA (4,5). According to this dual H-strand transcription model, transcription starting at I_{H2} is less frequent and results in polycistronic molecules corresponding to almost the entire H-strand, yielding 12 mRNAs and 12 tRNAs. However, transcription starts relatively frequently at I_{H1} and then terminates at the downstream end of the 16S rRNA gene, directing the synthesis of the 2 rRNAs (16S and 12S) and 2 tRNAs (4–6). The difference, then, in the synthesis rate of rRNA and mRNA, besides being regulated at the level of transcription initiation, is explained by an attenuation event at the border of the 16S rRNA and $tRNA^{Leu(UUR)}$ genes (7,8). The factor mediating termination of transcription is the mitochondrial transcription termination factor (mTERF), a DNA-binding protein that protects a 28 bp region within the $tRNA^{Leu(UUR)}$ gene at a position immediately adjacent to the downstream end of the 16S rRNA gene (8,9). Cloning of human mTERF has provided evidence for a novel DNA-binding motif, in which three leucine zippers form an intramolecular three-stranded coiled-coil that brings two widely separated basic domains into close register with the mTERF target DNA sequence (10).

In the present work, rat mTERF cDNA has been cloned, sequenced and expressed *in vitro* and in bacteria. The recombinant protein has been imported into isolated mitochondria and processed to the mature form. The mature recombinant protein exhibits the expected specific DNA-binding capacity to the corresponding termination site in rat mtDNA and it promotes *in vitro* transcription termination. Furthermore, the capability of rat mTERF to terminate transcription is modulated by multiple phosphorylations.

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MATERIALS AND METHODS

Cloning and sequence of rat mTERF cDNA

Using the sequence of human mTERF (10), primers were designed to perform the RACE protocols, in order to obtain the entire cDNA sequence from rat liver poly(A)$^+$ RNA.

Plasmid constructs

The r-mTERF plasmid used to produce rat mTERF precursor by *in vitro* translation was constructed by inserting a EcoRI–HindIII fragment that contains the entire open reading frame (ORF) of rat mTERF into the pSPUTK vector (Stratagene). The sequence encoding the mature protein was PCR amplified using primers that produce a substitution of amino acids FSVE(+4) with MGAQ (R1, GGG CCC AAT GTA ACA GTA AAG ACG G-385; R3, CTG CAG CTA CCC ACT TAA TCT CTT C-1352). The PCR fragment vector was digested with ApaI and PstI and ligated to pSPUTK to give the r-mTERFm plasmid. The pLEX-His vector [which contains the sequence that encodes the histidine tag and the polylinker from pBlueBacHis2B vector (Invitrogen)] was used to express the mature protein in bacteria with a 14 amino acid histidine tag (MPRGSHHHHHHGMA) at the N-terminus. The pLEX-His-mTERF plasmid was constructed by PCR amplification of an appropriate fragment using primers R2 (GCT AGC GTG GAA TGT AAC AGT AAA G-381) and R3. The PCR product was digested with NheI and PstI, cloned into pLEX-His and transformed into *Escherichia coli* GI724 (Invitrogen). The correct nucleotide sequence of all constructs was verified.

For construction of the pGTER plasmid used in the transcription termination experiment, the rat mtDNA region between nucleotide positions 2542 and 2761 (11) was PCR amplified and cloned into pGEM-Teasy vector (Promega). The mTERF-binding sequence was placed in direct polarity with respect to the SP6 promoter and in reverse polarity with respect to the T7 promoter (Fig. 4A). The pGHLT plasmid was obtained by inserting an AflII–SalI human mtDNA fragment into pGTER. The chimeric fragment of 171 bp comprising nucleotides 360–520 of human mtDNA (12) was PCR amplified using one primer with an AflII site (360-A ACA GTC GAC CCT AAC ACC AGC CTA AC) and another with a SalI site (520-G GAG ACA TGT GTG TGT GCT GGG TAG). This fragment contains the human L-strand promoter with the initiation site (I_L). In this way, the human L-strand promoter replaced the SP6 promoter of pGTER.

Expression of rat mTERF

In vitro expression. The proteins (rat mTERF and rat mTERFm) were expressed in the TNT-coupled transcription–translation system (Promega), which uses a rabbit reticulocyte lysate, following the manufacturer's protocols. [^{35}S]methionine (1000 Ci/mmol) (Amersham) was used to label proteins and the incorporation of radioactive label and the amount of protein synthesized were calculated. When necessary, the encoding proteins were expressed in an uncoupled way as described previously (13).

In bacteria expression. The protein rat his-mTERF (in pLEX vector) was expressed in bacteria using the P_L Expression System (Invitrogen) following the manufacturer's protocols. *Escherichia coli* transformed with pLEX-His-mTERF were grown in induction medium to an OD_{550} of 0.5 at 30°C. The expression was induced at 37°C for 4 h by the addition of 100 μg/ml tryptophan. The purification of rat his-mTERF was carried out as described previously (14). Following induction of expression, bacterial pellets were isolated, dissolved in 6 M guanidinium chloride, 20 mM Tris–HCl (pH 7.9) and 500 mM NaCl and bound to Ni^{2+}-loaded HiTrap affinity columns (Pharmacia-Biotech). The columns were washed with 6 M urea, 20 mM Tris–HCl (pH 7.9), 500 mM NaCl and 20 mM imidazole and the urea removed with 20 mM Tris–HCl (pH 7.9) and 150 mM NaCl. The soluble protein (his-mTERF) was eluted from the column with 20 mM Tris–HCl (pH 7.9), 150 mM NaCl and 50 mM EDTA and analyzed by 10% (w/v) SDS–PAGE and Coomassie blue staining. The protein content was determined by comparison with marker proteins using densitometry.

Purification of natural human mTERF

Purification of human mTERF was carried out using a human S-100 mitochondrial lysate (h-mtS100) prepared as previously described (15) from HeLa cells, by sequential chromatography through a heparin–agarose column and an oligodeoxynucleotide affinity column.

Mitochondrial import/processing assay

The rat liver mitochondrial fraction was isolated as described (16), washed twice in incubation medium (10 mM Tris–HCl, pH 7.4, 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH_2PO_4, 0.05 mM EDTA and 5 mM $MgCl_2$), resuspended in the same medium at ~10 mg/ml and immediately used for the import/processing assays at a final concentration of ~2.5 mg/ml. The rat mTERF precursor was synthesized *in vitro* in the presence of [^{35}S]methionine and reticulocyte lysate was then added to 12.5% (v/v) to samples of the mitochondrial suspension. Induction medium (1 mg/ml BSA, 2 mM sodium succinate, 1 mM ATP and 1 mM methionine, included to avoid labeling of newly synthesized proteins) was added to the reaction mixtures and these were then incubated at 37°C for 1 h. The mitochondria were washed twice with ice-chilled incubation medium and the final pellets were lysed with 1 vol of twice concentrated gel sample buffer. In some experiments, trypsin was added to the incubation mixture at 50 μg/ml after the import/processing reaction and the samples were incubated at room temperature for 10 min before washing and lysis of the mitochondria. In a control experiment in which, beside trypsin (50 μg/ml), Triton X-100 was added to the reaction mixture to 1% (v/v), the washing of mitochondria was omitted. In other assays, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was included in the reaction mixture at 1.7 μg/ml and incubated at 37°C for 30 min. The mitochondrial lysates were analyzed by SDS–PAGE and the gels were fixed, treated with Amplify (Amersham), dried and exposed.

DNA binding assays

DNA-binding activity to the rat termination site was determined by mobility shift assays using a double-stranded 40mer oligodeoxynucleotide probe (L-strand, GG AAG TTA TTA GGG TGG CAG AGC CAA GTA ATT GCG TAA GA-2686; H-strand, GG TCT TAC GCA ATT ACT TGG CTC TGC CAC CCT AAT AAC TT-2649), which was labeled by

filling in the 3′-recessed ends with Klenow enzyme and [α-^{32}P]dCTP. Various amounts of protein were incubated at 25°C for 15 min in 20 µl of reaction mixture containing 10 nM probe, 25 mM HEPES–KOH (pH 7.8), 50 mM KCl, 12.5 mM $MgCl_2$, 0.05% (v/v) Tween-20, 1 mM dithiothreitol (DTT), 20% (v/v) glycerol, 0.1 µg/µl poly(dI·dC) (added as non-specific competitor DNA) and 0.25 µg/µl BSA. After incubation, the reaction mixtures were loaded on a native 5% (w/v) polyacrylamide gel in Tris–glycine buffer and run at 4°C.

Transcription termination assays

The transcription termination reactions were carried out for 30 min at 30°C in a 25 µl volume using 500 fmol (20 nM) appropriate digested plasmid, its corresponding RNA polymerase and various amounts of rat mTERF proteins in transcription buffer (10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, 10% v/v glycerol, 1 mM ATP, 0.1 mM GTP and CTP, 0.01 mM UTP and 10 µCi [α-^{32}P]UTP). Termination of transcription by human mitochondrial RNA (mtRNA) polymerase was tested in the presence of 10% (v/v) h-mtS100 of a Tween-20 mitochondrial lysate (15). A transcription reaction using 5 U SP6 or T7 RNA polymerase was carried out in the presence of NcoI- or SalI-digested pGTER plasmid, respectively. When rat mTERFm was used, the controls contained the same amount of a non-programmed reticulocyte lysate.

In the case of purified human mTERF, transcription termination and S1 protection analysis were performed as described previously (10,15). The transcription reactions were carried out at 30°C for 30 min in a 25 µl volume using EcoRI- and HindIII-digested pTER plasmid (8) at 20 µg/ml, 10% (v/v) h-mtS100 lysate and 10 ng purified natural human mTERF in transcription buffer. S1 protection assays were performed using, as specific probe, unlabeled RNA synthesized utilizing BamHI-linearized BSAND plasmid and T3 RNA polymerase (9). Termination activity was calculated as the percentage of terminated transcripts relative to total transcripts, after correction for the different content of uridine.

Two-dimensional (2D) gel electrophoresis

Proteins were loaded in an IEF Ready Gel pH 3–10 (Bio-Rad) and subjected to isoelectrofocusing according to the instructions of the manufacturer. After isoelectrofocusing, the lanes were equilibrated for 30 min in 0.123 M Tris–HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS and 7 mM DTT before being applied for electrophoresis by 12% (w/v) SDS–PAGE.

Immunodetection analysis

The anti-phosphoserine (anti-P-S; Zymed), anti-phosphothreonine (anti-P-T; Sigma) and anti-phosphotyrosine (anti-P-Y; UBI) antibodies were used at 1/200, 1/100 and 1/1000 dilutions, respectively, for immunoblot analyses. Blots were developed using alkaline phosphatase-conjugated secondary antibodies (Sigma) at 1/5000 dilution and nitro blue tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (NBT-BCIP) as substrates.

Phosphatase treatments

Human mtS100 lysate (15) and rat [^{35}S]mTERFm were dephosphorylated at 37°C for 20 min with 5 U calf intestinal alkaline phosphatase (CIAP) and then loaded in IEF gel. Purified rat his-mTERF was incubated at a final concentration of 750 nM with agarose (5 µg/µl) or 5 U agarose-conjugated CIAP (Sigma) at 37°C for 20 min. Following incubation, the reaction mixture was centrifuged for 1.5 min at 10 000 *g* and the supernatant used for mitochondrial transcription termination and DNA-binding assays at a final concentration of 120 nM. When purified natural human mTERF was used, 2.5 ng/µl of protein was incubated at 37°C for 20 min with 5 U agarose-conjugated CIAP, centrifuged and then used for transcription termination and S1 protection analysis.

Measurement of equilibrium dissociation constant (K_d)

A series of binding reactions containing a fixed concentration of labeled termination probe was performed at different concentrations of recombinant proteins. The bound and free oligonucleotides were fractionated by mobility shift assay and the amounts were determined by densitometry. The dissociation constant (K_d) corresponding to the dissociation in the equilibrium DNA + protein ↔ DNA–protein was calculated by fitting the values to the following equation: $Y = 1 + [\text{protein}]/K_d$, where Y is the fraction of saturated binding sites ([DNA–protein]/([DNA] + [protein])). The binding isotherms were fitted by computer.

Isoelectric point (pI)

Predicted pIs were obtained using the ProtParam tool provided on the ExPASy Molecular Biology Server (http://www.expasy.org/). The pI values of IEF standards (Bio-Rad) were fitted, using a linear regression algorithm, to the equation $pI = A + B \times R_f$, where R_f is defined as the distance of a band relative to pH 10 ($R_f = 0$) and the position at pH 3 ($R_f = 1$). The pI values of phosphorylated proteins were estimated for the position of the band with respect to the non-phosphorylated one, using the equation $\Delta pI = -4.47 \times \Delta R_f$, derived from the aforementioned linear regression. Finally, the theoretical pI values of proteins with various numbers of phosphates were calculated using the algorithm from Scansite (http://scansite.mit.edu/calc_mw_pi.html).

RESULTS

Structural features of rat mTERF

The ORF of rat mTERF cDNA predicts a 374 amino acid protein with a molecular mass of ~43 kDa and an estimated isoelectric point (pI) value of 9.45. The structure of the rat mTERF sequence conserves the modular organization observed in the human ortholog (Fig. 1): two separated basic regions (B1 and B2), involved in directly binding the protein to its target sequence, and three leucine zipper motifs (Lz1a/b, Lz2 and Lz3), required to bring the two basic domains into close register with the target DNA sequence. It contains a putative mitochondrial targeting sequence of 37 amino acids, with the potential cleavage site placed between P(–1) and F(+1) according to the 'R(–2) rule'. Based on this assignment the mature rat mTERF should be 337 amino acids long and have a calculated molecular mass of ~38.3 kDa and a pI of 9.20. The deduced amino acid sequence of rat mTERF reveals 90, 75 and 72% overall sequence identity and 96, 89 and 88% overall sequence similarity to those of mouse (accession

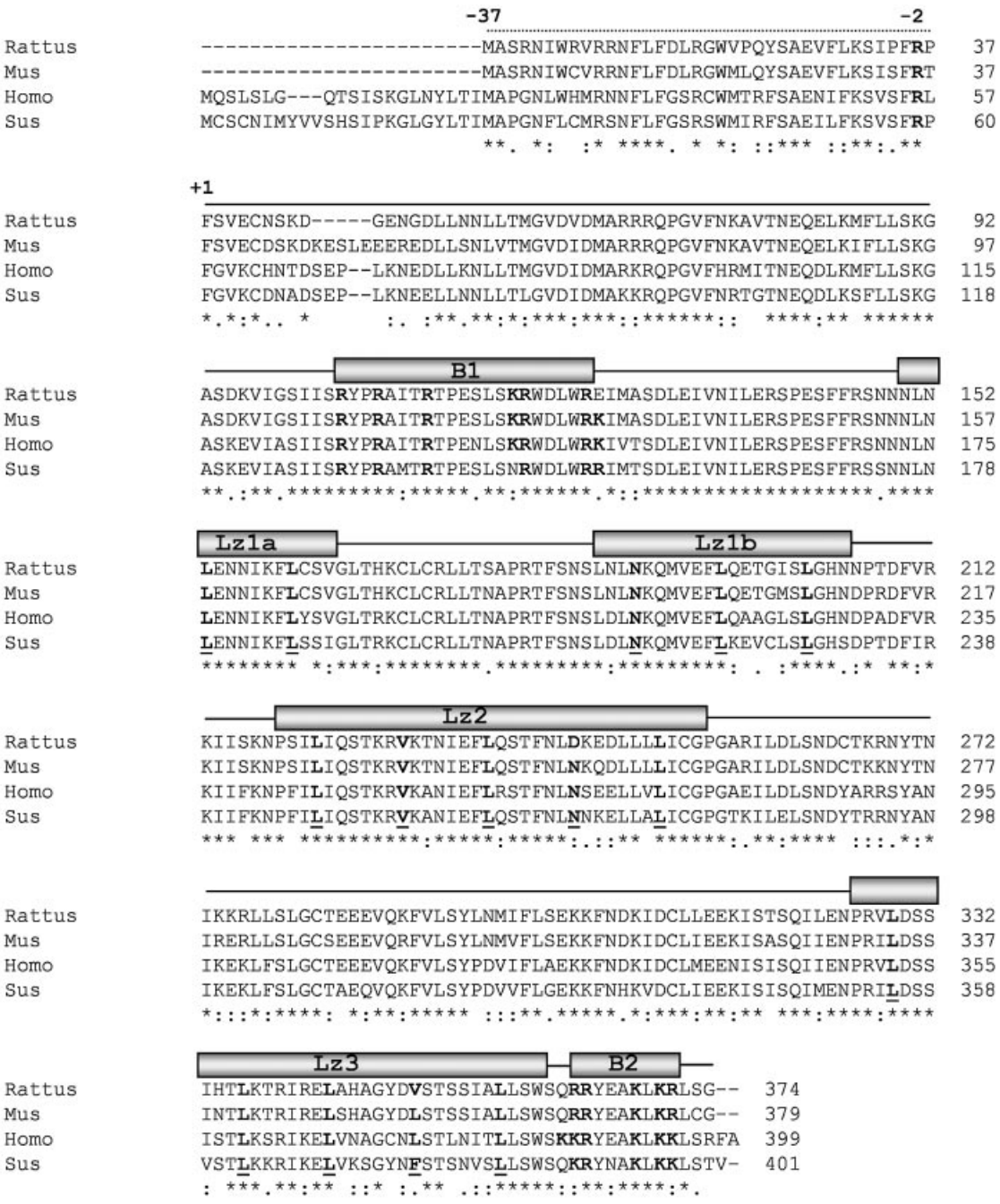

Figure 1. Sequence alignment of mTERFs. The presequence of 37 amino acids is shown by dotted line. The position of leucine zippers (Lz) and the two basic regions (B1 and B2) are shown. Alignment of the amino acid sequence of rat (*Rattus norvegicus*), mouse (*Mus musculus*), human (*Homo sapiens*) and pig (*Sus scrofa*) mTERF is shown. Asterisks indicate identical amino acids; colons and dots indicate very similar residues (31).

no. AC068609), human (accession no. Y09615) and pig (accession no. AC92875) mTERF, respectively. The marked sequence homology among human, mouse, pig and rat cDNA suggests that mTERF is a highly conserved protein in mammals. However, rat mTERF displays only 18 and 15% amino acid identity with *Paracentrotus lividus* mitochondrial transcription termination factor (mtDBP) (17) and the recently cloned *Drosophila melanogaster* mitochondrial transcription termination factor (DmTTF) (18), respectively (data not shown). These data indicate that mitochondrial transcription termination factors are not highly conserved between mammals and non-mammals.

In order to obtain direct evidence of the mitochondrial localization of rat mTERF, *in vitro* import/processing assays were carried out (Fig. 2A). A construct containing the entire ORF and another having a deletion of the fragment that codifies the putative mitochondrial target sequence were expressed *in vitro* in a rabbit reticulocyte lysate to give the precursor rat mTERF and the mature protein rat mTERFm, respectively. The rat mTERF was labeled with

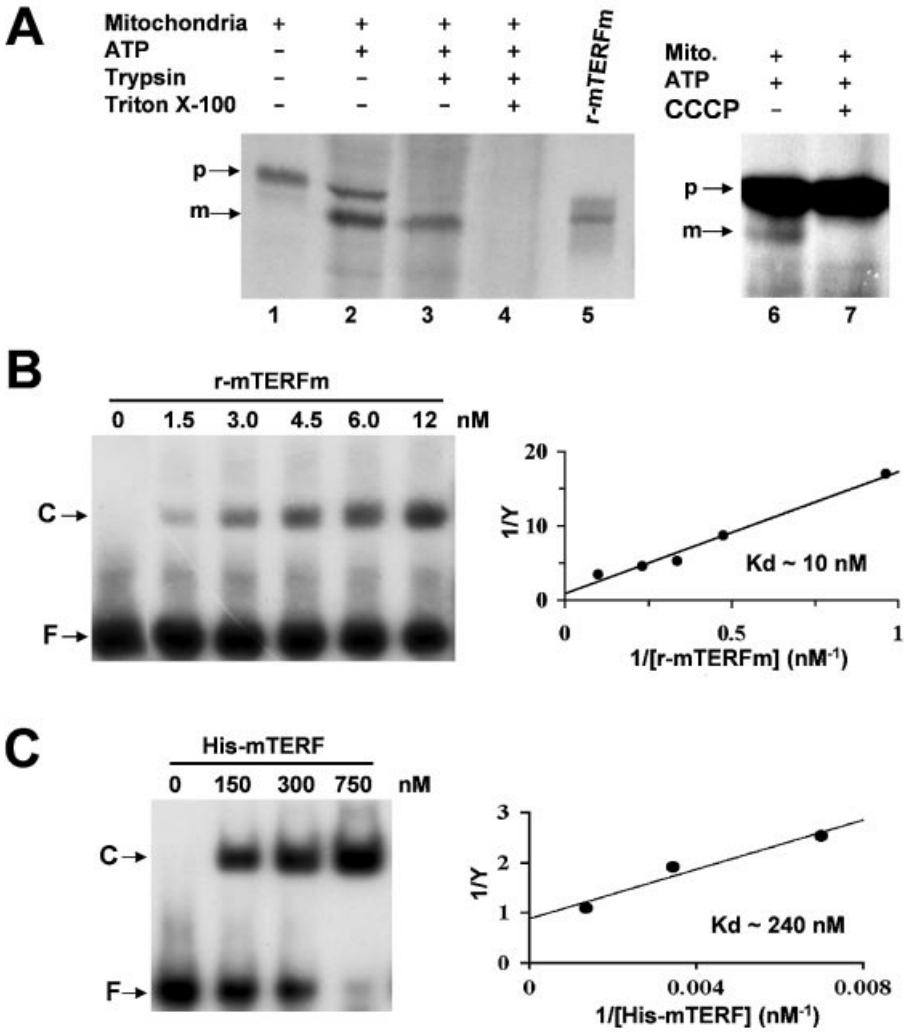

Figure 2. Functional properties of rat mTERF. (**A**) Analysis of rat mTERF after incubation with isolated rat mitochondria for 1 h at 37°C under various conditions (lanes 1–4). ATP (lanes 2–4), trypsin (lanes 3 and 4) and Triton X-100 (lane 4) were used. The *in vitro* synthesized mature protein rat mTERFm is shown in lane 5. Import of rat mTERF after incubation with rat mitochondria for 30 min at 37°C in the presence (lane 7) or absence (lane 6) of the uncoupler CCCP. p, precursor; m, mature. Mobility shift assay using the *in vitro* synthesized rat mTERFm (**B**) or his-mTERF expressed in bacteria (**C**) and ^{32}P-labeled termination probe. Lineweaver–Burk plot analysis was used to measure K_d (right panels). C, protein–DNA complex; F, free probe.

[^{35}S]methionine and then incubated with isolated mitochondria (lane 1). After incubation in the presence of ATP, rat mTERF yielded a second, shorter band where the mitochondrial leader peptide had been cleaved off (lane 2). This band had the same electrophoretic mobility as observed for rat mTERFm (lane 5). Protein internalization into mitochondria was then assayed by exposure to trypsin. The processed product (mature protein), in contrast to the precursor, was resistant to trypsin treatment (lane 3), but became protease sensitive after solubilization of the membranes with Triton X-100 (lane 4). This result demonstrates that the mature protein was internalized within the inner mitochondrial compartment, which is impermeable to trypsin. Furthermore, import was inhibited by the uncoupler CCCP (Fig. 2A, lane 7), as expected from the requirement for a membrane potential for mitochondrial import. All these properties are characteristic of a typical mitochondrial import reaction (19).

Functional analysis of rat mTERF

In order to investigate the functional capacity of the recombinant rat mTERF, its DNA-binding properties and its transcription termination-promoting activity in an *in vitro* system were analyzed. The binding capacity of rat mTERFm was determined by gel shift assays using a ^{32}P-labeled probe that contained the putative rat mTERF binding site (Fig. 2B). The recombinant mature protein was able to bind the probe producing a single retarded band. Increasing the amount of the protein resulted in an increase in the intensity of the retarded band, without the appearance of any more slowly moving secondary band. The amount of rabbit reticulocyte lysate was kept constant even in the absence of rat mTERFm. Finally, the equilibrium constant of binding (K_d) to DNA of recombinant rat mTERFm was estimated to be ~10 nM.

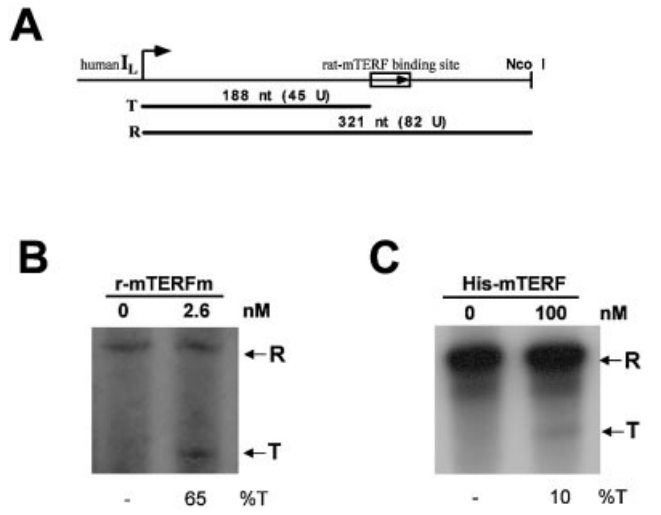

Figure 3. Transcription termination activity of rat mTERF. (**A**) Schematic diagram of pGHLT template used in transcription termination assays is shown. Transcription termination activity of rat mTERFm (**B**) or his-mTERF (**C**) when it was initiated by human mitochondrial RNA polymerase (h-mtS100). R, run-off transcripts; T, terminated transcripts; %T, percentage of termination activity.

A full-length rat mTERF for the mature protein was expressed in *E.coli* as a histidine N-terminal tagged fusion protein and this recombinant his-mTERF protein was purified by Ni^{2+} affinity chromatography (data not shown). In order to analyze the his-mTERF functional capacity, the DNA-binding activity was determined by gel shift assay (Fig. 2C) and the equilibrium binding constant calculated. A value of K_d ~240 nM was obtained.

Termination activity of the recombinant rat mTERFm and his-mTERF proteins was characterized using a human mtS100 lysate as a source of mtRNA polymerase activity and using a chimeric template (pGHLT plasmid) containing the human L-strand promoter (I_L) and the rat mTERF binding site (Fig. 3A). The recombinant proteins were able to support transcription termination activity when added to the system, producing a transcript of ~188 nt which was not present in the absence of the protein. When rat mTERFm was present in the reaction, a high percentage (~65%) of transcripts was stopped at the termination site (Fig. 3B), while only ~10% of termination was produced in the presence of his-mTERF (Fig. 3C). In order to investigate the ability of rat mTERF to interfere with RNA chain elongation catalyzed by bacteriophage RNA polymerases, another template (pGTER plasmid) carrying the rat mTERF binding site in either orientation relative to the bacteriophage promoter (Fig. 4A) was linearized downstream of the selected promoter and then transcribed with the proper bacteriophage RNA polymerase, in the absence and presence of rat mTERF. When the termination activity was tested with SP6 RNA polymerase and pGTER(NcoI) template, two classes of transcripts were generated in the presence of rat mTERFm: a longer run-off transcript and a shorter truncated transcript which corresponds to transcription termination (Fig. 4B). With a protein concentration of 2.6 nM a 50% level of termination was obtained. Transcription with T7 RNA polymerase and pGTER(SalI) template yielded a 40% level of termination (Fig. 4C). Finally, transcription with bacteriophage RNA polymerase generated terminated transcripts in the presence of his-mTERF when the termination site was placed either in the forward orientation with respect to the SP6 promoter or in the reverse orientation with respect to the T7 promoter (Fig. 4D). Thus, the recombinant rat mTERF expressed in *E.coli* is able to bind to the termination site and produce termination of transcription.

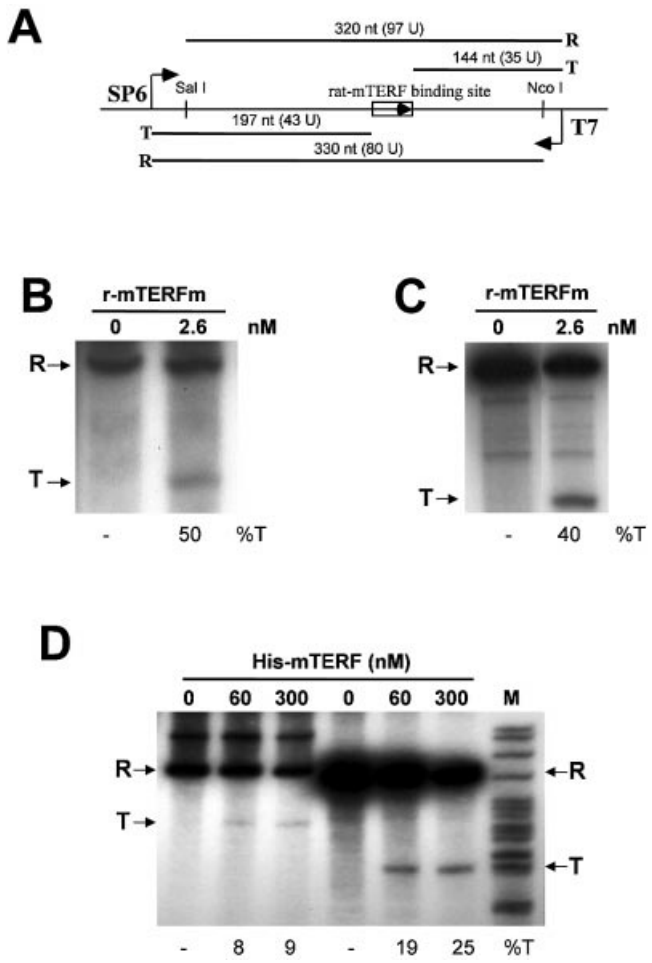

Figure 4. Transcription termination activity of rat mTERF. (**A**) Schematic diagram of pGTER template is shown. Transcription termination activity of rat mTERFm when it was initiated by SP6 RNA polymerase (**B**) or T7 RNA polymerase (**C**). (**D**) Transcription termination activity of rat his-mTERF when the transcription was initiated by SP6 (first three lanes) or T7 RNA polymerase (last three lanes). R, run-off transcripts; T, terminated transcripts; M, markers (MspI-digested plasmid pBR322); %T, percentage of termination activity.

Rat mTERF is phosphorylated at multiple sites

The activity of rat mTERF could be regulated by post-transcriptional modification, and phosphorylation is one of the major mechanisms for regulating protein function in eukaryotic cells. As a first step to address this issue, the migration of [^{35}S]methionine-labeled rat mTERFm was analyzed by 2D gel electrophoresis (Fig. 5A). In the first dimension (isoelectrofocusing), the proteins were separated by charge, whereas in the second dimension (SDS–PAGE) the proteins were separated by size. On 2D gels, two populations were observed

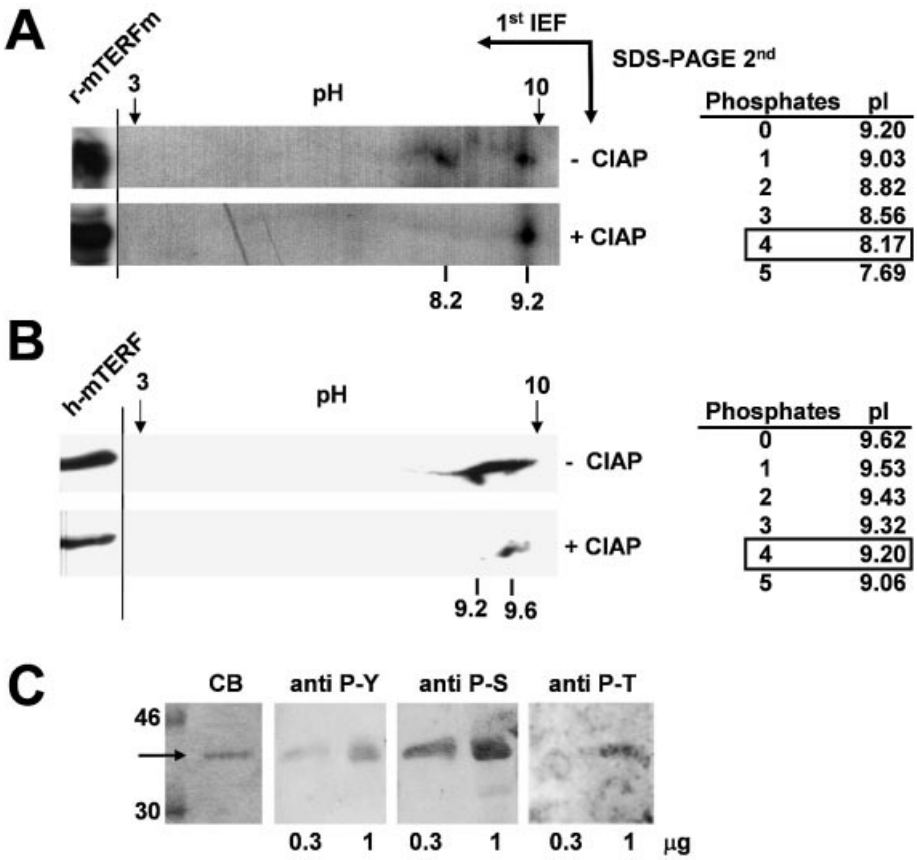

Figure 5. mTERF is phosphorylated at multiple sites. (**A**) Two-dimensional gel electrophoresis (2D) of *in vitro* synthesized and [^{35}S]methionine-labeled rat mTERFm before (–) and after (+) treatment with CIAP. (**B**) Another similar experiment carried out with human mtS100 lysate (human mTERF) and revealed with a polyclonal antibody anti-mTERF. Both methods showed a phosphorylated spot at pI ~8.2 for rat mTERFm and ~9.2 for human mTERF, which correspond to four phosphorylated residues (right panel). The left sides show rat mTERFm and human mTERF when they were running in a well in parallel in SDS–PAGE. (**C**) The bacteria expressed and purified rat his-mTERF was revealed using antibodies against phosphotyrosine (anti P-Y), phosphoserine (anti P-S) and phosphothreonine (anti P-T). CB was stained with Coomassie blue.

with different pI values. After treatment with CIAP, only the population of the unmodified protein with a theoretical pI of 9.2 remained. The other population, with a calculated pI of 8.28, corresponded to the protein with four phosphate groups (Fig. 5A, right panel). Another similar experiment carried out with h-mtS100 lysate and revealed with a polyclonal antibody anti-mTERF indicated that natural human mTERF also has four phosphates (Fig. 5B). Taken together, these results provide convincing evidence that rat mTERF is susceptible to being phosphorylated on four amino acid residues. In order to investigate if rat his-mTERF was also phosphorylated, the protein was expressed in bacteria, purified and resolved by SDS–PAGE and the phosphoamino acid content was determined by immunoblot analysis. For this purpose antibodies specifically detecting phosphotyrosine (anti-P-Y), phosphoserine (anti-P-S) and phosphothreonine (anti-P-T) were used. As shown in Figure 5C, the three antibodies recognize a protein with an apparent molecular mass of 40 kDa that represents the phosphorylated form of rat his-mTERF.

Inhibition of transcription termination activity by dephosphorylation of his-mTERF

As a first step to find out whether phosphorylation may regulate mTERF termination activity, rat his-mTERF expressed in bacteria and then purified was treated with agarose-coupled CIAP and used in transcription termination assays (Fig. 6A). When untreated protein was added to the assay (lane 2), a clear termination band appeared, which was not present in the absence of the protein (lane 1). Most significantly, CIAP treatment produced a total inhibition of his-mTERF transcription termination activity (lane 3) and no termination band was present. This inhibitory effect was produced by dephosphorylation and not by the interference effect of agarose (lane 4). A low abundance RNA species shorter than the run-off transcripts was present regardless of the presence of mTERF and this was probably the result of either degradation of the run-off transcripts or mTERF-independent pausing or termination. The reduced overall transcription observed in lanes 2–4 seems to be due to an inhibition of T7 RNA polymerase by his-mTERF. To test whether the DNA-binding activity of rat his-mTERF is also controlled by phosphorylation, recombinant and CIAP-treated protein was subjected to band shift assays using ^{32}P-labeled termination probe. As shown in Figure 6B, the protein produced a single retarded band (lane2), which remained unaffected after dephosphorylation (lane 4 versus lane 3). The reduction in transcription termination activity (lane 4) and the lower signal of retarded bands in lanes 3 and

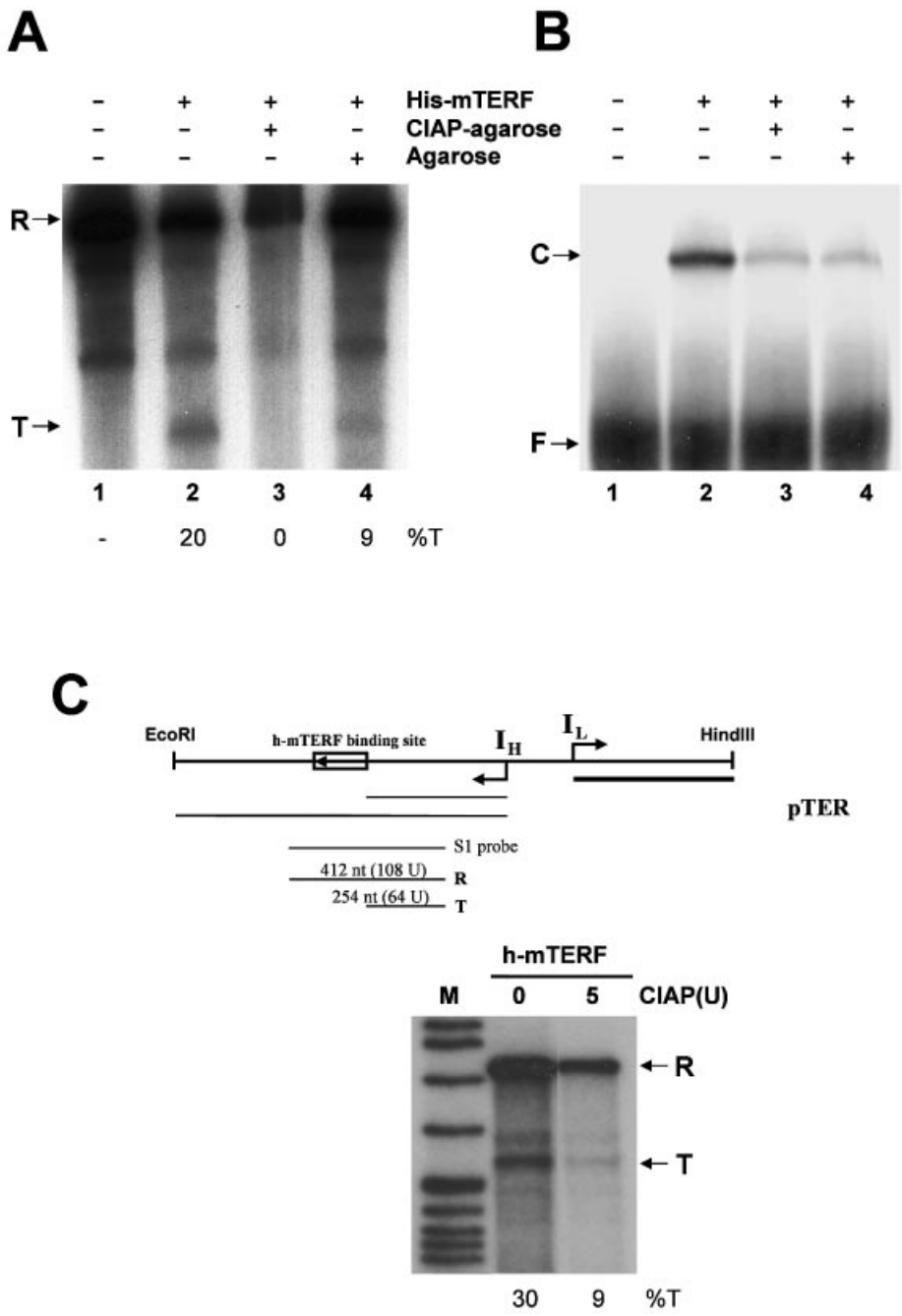

Figure 6. Phosphatase treatment abolishes transcription termination activity. Recombinant his-mTERF protein (120 nM) was treated with agarose-coupled CIAP and then (**A**) used in a transcription termination assay initiated by T7 RNA polymerase or (**B**) DNA binding assay with termination probe. Lane 1, no his-mTERF protein added; lane 2, untreated protein; lane 3, CIAP-treated protein; lane 4, agarose-treated protein. (**C**) Schematic diagram of the template (pTER) and probe used in the transcription termination assays and S1 protection analysis. Only the upstream initiation site for H-strand transcription is shown in the pTER map. The picture shows the S1 protection products from assays of transcription termination activity using CIAP-treated or untreated purified human mTERF. R, run-off transcripts; T, terminated transcripts; %T, percentage of termination activity; C, protein–DNA complex; F, free probe.

4 with respect to the control (lanes 2) were due to the partial loss of protein after agarose treatment. These results provide direct evidence that dephosphorylation inhibits the transcription termination activity of rat mTERF, but that it does not affect its DNA-binding capacity (Fig. 7). More conclusive evidence concerning termination activity regulation by phosphorylation was obtained using purified natural human mTERF (Fig. 6C). Figure 6C shows the results of termination assays carried out with equivalent amounts of natural human mTERF proteins, both CIAP-treated and untreated. It is clear that the CIAP-treated protein almost completely lacks transcription termination-promoting activity, the remaining termination activity being due to endogenous mTERF present in h-mtS100.

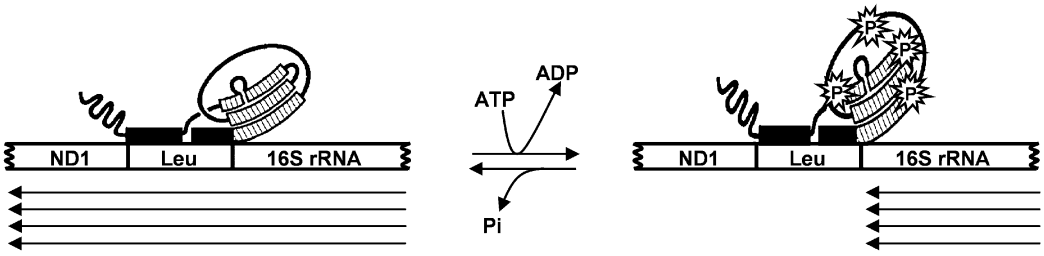

Figure 7. Proposed model of mTERF regulation by phosphorylation. In the dephosphorylated state, the HSP-initiated transcripts proceeded to the full length of the mtDNA. The phosphorylation of mTERF may cause structural remodeling of the DNA–protein complex, causing an increase in termination activity. The circles labeled P represent phosphorylated residues.

DISCUSSION

The amino acid sequence of rat mTERF conserved the modular organization previously observed in human mTERF (10), with two widely separated basic regions and three leucine zipper motifs; the same organization is also conserved in the mouse and pig orthologs. This feature supports the hypothesis that the four proteins share the same 3-dimensional structure. mtDBP from the sea urchin *P.lividus* (17) and *Strongylocentrotus purpuratus* (20) also presents a modular organization with two leucine zipper motifs and two DNA-binding domains.

The recombinant mature protein expressed *in vitro* (rat mTERFm) or expressed in a bacterial system (his-mTERF) conserves the specific DNA-binding capacity to the termination region, with K_d values of 10 and 240 nM, respectively. This difference could be due to the presence of a large percentage of inactive or misfolded molecules of rat his-mTERF from bacterial expression or to possible differences of post-translational modification between the two expression systems. It could also be an effect of the polyhistidine tag on the N-terminus of his-mTERF, because the importance of this region in the binding activity of human mTERF (10) and mtDBP (17) has been demonstrated.

The most important point concerning termination-promoting activity was the capacity of both recombinant mature proteins (rat mTERFm and his-mTERF) to support transcription termination, differently from recombinant human mTERF, which lacks termination activity (10). The lack of termination activity of human mTERF was explained by the absence of a secondary modification of the primary translation product or another additional factor(s) (10). In the case of rat mTERF, the protein expressed in *E.coli* and purified (his-mTERF) was active for mitochondrial transcription termination. This observation rules out the need for an additional factor(s) and points to a secondary modification being required for termination activity.

Furthermore, the recombinant rat mTERF was able to terminate transcription when initiated by human mtRNA polymerase, SP6 or T7 RNA polymerase. This behavior is similar to that shown by human mTERF (21) and mtDBP (22). Therefore, rat mTERF, like human mTERF and mtDBP, is not RNA polymerase-specific.

Both recombinant proteins, rat mTERFm synthesized in rabbit reticulocyte lysate and rat his-mTERF expressed in *E.coli*, are phosphorylated (Fig. 5A and C). The origin of the phosphorylation in these two different systems, one prokaryotic and the other eukaryotic, is presently under investigation in our laboratory.

The most important result presented in this study is the demonstration that the transcription termination activity of rat mTERF is regulated by phosphorylation while the DNA-binding activity of the protein is not. Rat mTERFm resolved into two clear bands in a 2D gel, with the band of lower pI being a phosphorylated form of the protein with four phosphate groups. The data also provide novel evidence that natural human mTERF is mostly phosphorylated at four sites, although the phosphorylation is more heterogeneous. This observation indicates that mTERF is phosphorylated physiologically by mitochondrial kinases. Evidence for mitochondrial protein phosphorylation in various cell types has been previously reported (23,24). Both recombinant rat mTERF and natural human mTERF are active in promoting transcription termination and both are phosphorylated. Furthermore, the non-phosphorylated forms of mTERFs were transcriptionally inactive for termination (Fig. 6A and C). These results demonstrate that the termination activity of mTERF is regulated by phosphorylation and suggest that phosphorylation/dephosphorylation may be an important physiological regulatory mechanism for the activity of this protein. However, whereas transcription termination activity is inhibited by dephosphorylation, the DNA-binding activity of mTERF remains unaffected. Furthermore, phosphatase treatment of human mTERF does not affect the DNA-binding activity of the protein (9). Therefore, both activities are separable and the binding of mTERF to DNA is necessary but not sufficient to terminate transcription, as was previously shown with recombinant human mTERF (10). Phosphorylation is, by any criterion, the most widely used regulatory mechanism for modulating enzyme activity in eukaryotic cells. There are countless examples in which the reversible addition of a phosphate moiety serves as a key switch to activate or inactivate enzyme activity. Thus, phosphorylation of yeast mitochondrial transcription factor A (Sc-mtTFA) (25) and mouse transcription termination factor I (TTF-1) (26) regulates binding of the protein to DNA. Moreover, the protein RNA polymerase I and transcript release factor (PTRF), which interacts with RNA polymerase I, TTF-1 and the 3′-end of pre-rRNA, is phosphorylated at multiple sites and fractionates into transcriptionally active and inactive forms (27). Likewise, the phosphorylated human La antigen (a RNA-binding protein that facilitates transcriptional

termination and reinitiation by RNA polymerase III) binds RNA efficiently but does not support efficient transcription and the phosphoprotein can be reactivated for transcription by dephosphorylation (28).

Therefore, mTERF is the first protein having a role in mammalian mitochondrial gene expression that appears to be controlled by phosphorylation (Fig. 7). Furthermore, mTERF phosphorylation may modulate the mitochondrial synthesis ratio mRNA:rRNA in response to intra-mitochondrial signals (29).

Recently, a new model of mTERF regulation by oligomerization has been proposed (30). In this model, the activity of the protein is modulated by the transition between an active monomer and an inactive trimer, which has no DNA-binding activity.

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