The human mitochondrial transcription termination factor (mTERF) is fully active in vitro in the non-phosphorylated form.

Jordi Asin-Cayuela‡, Thomas Schwend, Géraldine Farge, and Claes M. Gustafsson§

From the Department of Medical Nutrition, Karolinska Institutet, Novum, Huddinge Hospital, S-141 86 Huddinge, Sweden

The human mitochondrial transcription termination factor (mTERF) is a 39-kDa protein that terminates transcription at the 3′-end of the 16 S rRNA gene and thereby controls expression of the ribosomal transcription unit of mitochondrial DNA. The transcription termination activity of human mTERF has been notoriously difficult to study in vitro, and it has been suggested that the activity of the protein is regulated by posttranslational modifications or by protein polymerization. We here characterize the activity of recombinant human mTERF expressed in insect cells. We observed that mTERF efficiently promotes sequence-specific termination in a completely recombinant and highly purified in vitro system for mitochondrial transcription. The termination activity has a distinct polarity, and we observed complete transcription termination when the mTERF-binding site is oriented in a forward position relative the heavy strand promoter but only partial transcription termination when the binding site is in the reverse position. We analyzed the biochemical characteristics of the active mTERF protein and found that it is a stable monomer at physiological salt concentration. Structural analysis, including phosphostaining, two-dimensional electrophoresis, and electrospray mass spectrometry, detected no evidence of phosphorylation. We conclude that the monomeric human mTERF is fully active in its non-phosphorylated form and that the protein does not require additional cellular factors to terminate mitochondrial transcription in vitro.

Transcription of the heavy (H) strand of mitochondrial DNA (mtDNA) involves two overlapping transcription units (1, 2). One unit starts directly upstream of the tRNAPhe gene and spans the tRNAAsp, 12 S rRNA, 16 S rRNA, and tRNAVal genes (initiation site H1). The other transcription unit starts ~100 bp further downstream (initiation site H2) at the boundary between the tRNAAsp and 12 S rRNA genes and produces a single polycistronic RNA that encompasses almost the entire length of the heavy strand. The ribosomal transcription unit is transcribed at a much higher rate compared with the other transcription unit, and control of its expression is exerted at both the initiation and termination levels (3, 4). A central role in the control of termination has been attributed to the mitochondrial transcription termination factor (mTERF), a 39-kDa protein that binds to a 28-base pair region of mtDNA located within the tRNALeu(UUR) gene at a position immediately downstream of the 16 S rRNA gene (5, 6). Previous characterization of recombinant mTERF expressed in bacteria or in coupled in vitro transcription and translation systems produced proteins with sequence-specific DNA binding activity but no transcription termination activity (7). These observations indicated that mTERF requires other cellular factors and/or a posttranslational modification to efficiently terminate transcription. Furthermore, a recent study of recombinant rat mTERF suggested that phosphorylation of four amino acids is required for transcription termination activity (8). Alternatively, mTERF activity may be regulated by its polymerization state, because native human mTERF is present in HeLa mitochondrial lysates in two forms, an active monomer and an inactive polymer (9). Native mTERF has a high tendency to polymerize, thereby explaining a dramatic loss of activity during purification.

We here characterize mTERF-dependent transcription termination in a highly purified and recombinant in vitro system for mitochondrial transcription (10). Previous investigations of mTERF function were performed in partially purified mitochondrial extracts, and the possible contribution of yet to be identified cellular factors was therefore difficult to address. Because bacterial expression in the past had failed to generate an active human protein, we expressed recombinant mTERF in insect cells using the baculovirus system. The advantages of this eukaryotic expression system are the abundant expression of recombinant protein combined with the possibility of cotranslational and posttranslational modifications, including N-glycosylation and phosphorylation (11–14). We found that baculovirus-expressed human mTERF efficiently terminates transcription in vitro and displays distinct strand specificity. The mTERF protein does not require other cellular factors for its termination activity, and a comprehensive structural analysis revealed that mTERF is a stable monomer in solution that actively promotes transcription termination in its non-phosphorylated form.

MATERIALS AND METHODS

Expression and Purification of Recombinant Human mTERF—The gene encoding mTERF (GenBank™ accession number BC000965) was amplified from cDNA by PCR and cloned it into the pBacPAK9 (Clon-§ This work was supported by grants from the Swedish Research Council, the Swedish Cancer Society, the Swedish Foundation for Strategic Research, the Swedish Agency for Innovation Systems (VINNOVA), and the European Union Sixth Framework Program (EU-MI-TOCOMBAT) (to C. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.‡ Recipient of a Marie Curie Intra-European Fellowship from the European Union Sixth Framework Program. § To whom correspondence should be addressed. Tel.: 46-8-5858-3974; Fax: 46-8-779-5383; E-mail: claes.gustafsson@mednut.ki.se.

1 The abbreviations used are: mTERF, mitochondrial transcription factor; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; HSP, heavy strand promoter; IEF, isoelectric focusing; LSP, light strand promoter; MALDI, matrix-assisted laser desorption ionization; Ni2+ -NTA, nickel-nitritolriatic acid; PVD, polyvinylidene fluoride; TOF, time-of-flight.
The recombinant mTERF protein was purified by Ni²⁺-NTA chromatography. The frozen cell pellets were resuspended in 10 ml of lysis buffer (25 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, 0.6 μM leupeptin, and 2 mM benzamidine) and incubated on ice for 20 min. The cells were homogenized with a tight-fitting pestle in a Dounce homogenizer, and the homogenate was brought to 15 ml with lysis buffer. After the addition of 3 ml of 5 mM NaCl, the solution was incubated at 4 °C for 45 min in an orbital shaker. The insoluble material was removed by centrifugation at 100,000 × g for 40 min at 4 °C. The supernatant containing mTERF was passed 10 times through an 18-gauge (1 M NaCl) column of Ni²⁺-NTA Superflow resin (Qiagen) previously equilibrated in buffer A supplemented with 10 mM imidazole (1 ml Ni²⁺-NTA resin per 400 ml of cell culture). The mixture of supernatant and resin was incubated at 4 °C for 1 h in an orbital shaker, and the beads were then collected by centrifugation for 10 min at 1,500 × g. After removal of the supernatant (flow-through), 10 resin volumes of buffer A supplemented with 40 mM imidazole were added to the resin. After 15 min of incubation, the mixture was centrifuged for 10 min at 1,500 × g, after which the supernatant (wash) was removed and the resin transferred into a Poly-Prep® chromatography column (Bio-Rad). The resin was incubated for 15 min with 12 ml of elution buffer (buffer A supplemented with 400 mM imidazole), and the mTERF protein was subsequently eluted in 1-ml fractions that were snap frozen in liquid nitrogen and stored at −80 °C.

**Gel Filtration Chromatography**—The gel filtration chromatography of Ni²⁺-NTA-purified mTERF was performed as described (9) with the following modifications. The column (Superose 12; Amersham Biosciences) was equilibrated in running buffer (25 mM HEPES (pH 7.6), 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, and 0.1% Tween 20, and 500 mM KCl) at a flow rate of 0.3 ml/min. Following the instructions of the column’s manufacturer, a calibration curve was prepared with blue dextran 2000, ferritin (440 kDa), catalase (232 kDa), ovalbumin (47 kDa), and insulin (5.8 kDa), all from Amersham Biosciences (except for insulin from Sigma, catalog number I9278). The protein was dialyzed against the running buffer immediately prior to injection. The injected sample volume was 1 ml, the flow rate was 0.4 ml/min at 4 °C, and 0.2-ml fractions were collected. Fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The mTERF-containing fractions were snap-frozen in liquid nitrogen and stored at −80 °C. The mTERF protein concentration was determined with Bradford reagent (Sigma).

**In vitro Transcription Assay**—The termination activity of mTERF was tested in a fully reconstituted in vitro transcription system described previously (10). Four different DNA templates were used. To construct pHSP1, a DNA fragment corresponding to bp 499–742 of human mtDNA (containing both HSPs) was cloned into pUC18. To construct pHSP1-TERM, a fragment corresponding to bp 3063–3594 of human mtDNA (containing the binding site for mTERF, between nucleotides 3229 and 3268) was cloned immediately downstream of HSP1 fragment in pHSP1. For the generation of pHS1-MRET, the human mtDNA 3063–3595 fragment was cloned into the same position as in pHSP1-TERM, but in the reverse orientation. For pLS-PRET, a LSP-containing fragment spanning bp 477–1 of human mtDNA was cloned into pUC18 followed by the mTERF-binding site fragment of the 477–5373 fragment immediately downstream. After linearization (pHS1-TERM and pHSP1-TERM with BsrI and pLS-PRET with EcoRI), the plasmids were used to measure promoter-specific transcription in a runoff assay as described previously (10). The protein concentrations used in the in vitro transcription experiments were 250 fmol of His-POLRTM, 500 fmol of TFBBM, 2.5 pmol of TFAM and, when indicated, variable amounts of mTERF or an equivalent volume of buffer.

For the pulse-chase experiment, pHSP-TERM was used as template, and the in vitro transcription reaction was scaled up to a final volume of 100 μl. The in vitro transcription reaction contained 0.3 μM mTERF (100 μl), and 5% of the reaction mixture was removed at 32 °C and labeled with [3H]UTP. After completion of transcription, 0.8 μl of phenol, 0.2 ml of chloroform, and 80 μl of 0.3 M sodium acetate (pH 5.2) were added to a final concentration of 2.5 mM. Samples (25 μl) were collected 0, 30, 60, and 90 min after the addition of UTP, and the transcription products were analyzed as described previously (10).

**Phosphoprotein Blot Staining**—The indicated amounts of recombinant mTERF, ovalbumin, and bovine serum albumin were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel, and the proteins were electroblotted to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was subsequently stained with the Pro-Q® Diamond phosphoprotein blot stain kit (Molecular Probes) following the instructions of the manufacturer. The stained bands were revealed by direct UV light transillumination and photographed with a charge-coupled device camera, model ICD-42E (Ikegami Tsushinki Co.).

**Two-dimensional Electrophoresis**—The mTERF protein was separated by isoelectric focusing (IEF) in the first dimension and by SDS-PAGE in the second dimension. An mTERF protein purified by gel filtration over a Superose 12 column and dialyzed against a running buffer (25 mM HEPES (pH 7.6), 5 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 0.1% Tween 20, 1 mM DTT, and 100 μM KCl) was used. IEF was carried out essentially according to the instructions of the manufacturer (Amersham Biosciences). The IEF strips (Immobiline DryStrip pH 7–11 NL, 7 cm) were rehydrated overnight in 200 μl of IPG buffer (8% rehydration buffer and 2% non-rehydration buffer). Forty microliters of 2 μM of the PVDF-bound material was loaded onto the IEF strip anodically in a sample cup. The IEF was performed on an IPGPhor (Amersham Biosciences) IEF unit at 20 °C with a 50-μA current per strip during the following schedule: step 1 (step and hold), 6 days, 0–400 V; step 2 (gradient), 1000 V and 704 V-h; step 3 (gradient), 5000 V and 4500 V-h; and step 4 (step and hold), 4000 V and 3000 V-h.

For the alkaline phosphatase treatment experiments, 20 μl of mTERF protein (1 μmol) were incubated with 2.6 μl of 10× dephosphorylation buffer (Roche Applied Science) and 4 μl of calf intestine alkaline phosphatase (1 unit/ml; Roche Applied Science) at 37 °C for 30 min. As a negative control, the calf intestine alkaline phosphatase was incubated with 4 μl of H₂O. After incubation, the samples were immediately diluted in UTC buffer, DTT, and IPG buffer and loaded onto the IEF strip as described above.

After IEF, the strips were incubated for 15 min at room temperature under constant shaking in 10 ml of equilibration solution (50 mM Tris-HCl (pH 8.5), 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) supplemented with 10 mg/ml DTT, followed by a second equilibration step in the same solution containing 25 μl of 250 mM iodoacetamide. The strip was next placed on top of a 12.5% SDS-polyacrylamide mini-gel that had been covered with a solution of 0.5% agarose and 0.002% bromophenol blue in SDS running buffer. After electrophoresis, the polyacrylamide gels were stained with Coomassie Blue or silver as indicated. Precision Plus protein standard plug (Bio-Rad) was used as a molecular mass marker.

**Protein Analysis with Mass-Spectrometry**—Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) analysis of in-gel digested proteins was carried out with an Ultraflex II TOF/TOF mass spectrometer from Bruker (Billerica, MA). The samples were prepared as described previously (15). Mass spectra were obtained in the positive ion mode at an acceleration voltage of 25 kV and a pulsed ion extraction time of 80 ns. Typically, 300 shots were combined for one spectrum. Data base searches were done with the MS BIOTOOLS software from Ciphergen by using the Mascot search engine (Matrix Science, London, UK).

For electrospray ionization mass spectrometry, 20 μl (1.6 μg) of Ni²⁺-NTA-purified mTERF was loaded on a reversed phase trapping column (Poros R1; Applied Biosystems) equilibrated in 0.1% trifluoroacetic acid. The column was washed for 2 min with 0.1% trifluoroacetic acid at a flow rate of 200 μl/min, and mTERF was subsequently eluted with 10 μl of 0.1% trifluoroacetic acid at a flow rate of 20 μl/min. The protein samples were analyzed with an electrospray ionization-quadrupole TOF mass spectrometer (Applied Biosystems) using the standard conditions for the electrospray interface. Mass calibration was performed with 100 pmol of aprotinin. Deconvolution of the spectra was done with the software supplied with the quadrupole TOF mass spectrometer. The S.D. of the estimated masses were calculated as described.
RESULTS

Recombinant mTERF Efficiently Terminates Transcription in a Fully Reconstituted in Vitro Transcription System—Previous studies have suggested that mTERF activity is regulated by phosphorylation or protein polymerization. We wanted to investigate the requirements for mTERF activity in vitro and therefore generated a recombinant baculovirus encoding the mature form of the human mTERF protein fused in-frame with a carboxyl-terminal His6-tag. Recombinant mTERF was expressed in insect cells and purified by Ni2+/NTA chromatography and gel filtration to near homogeneity (Fig. 1A). The purified protein was used in the experiments illustrated in panels B–E. B, in vitro termination activity of mTERF on pHSP-TERM. Increasing amounts of mTERF were added as indicated. Lane 1, basal transcription reaction; lane 2, mTERF storage buffer. C, in vitro termination activity of mTERF on pHSP-MRET. The protein content in each lane was as in panel B. The transcription reactions in panels B and C were performed in parallel, and band intensities can therefore be compared directly. D, in vitro termination activity of mTERF on pLSP-TERM. The protein content of each lane was as in panel B, E, the mTERF protein, and not POLRMT RNA polymerase pausing, promotes true termination on the pHSP-TERM template. After a 5-min pulse of radiolabeled UTP, a 8000-fold excess of cold UTP was added. At the times indicated, 15-ml aliquots were removed and analyzed as described under "Materials and Methods." MW, 32P-5′-end-labeled DNA ladder (100-bp DNA ladder; New England Biolabs).

Fig. 1. Effects of recombinant human mTERF on mitochondrial transcription in vitro. Transcription reactions were performed as described under "Materials and Methods" and, for clarity, schematic presentations of the DNA templates used are depicted on the right. A, the mTERF protein purified over Ni2+/NTA-agarose by gel filtration was separated by SDS-PAGE (12.5%) and revealed with Coomassie Brilliant Blue staining. The purified protein was used in the experiments illustrated in panels B–E. B, in vitro termination activity of mTERF on pHSP-TERM. Increasing amounts of mTERF were added as indicated. Lane 1, basal transcription reaction; lane 2, mTERF storage buffer. C, in vitro termination activity of mTERF on pHSP-MRET. The protein content in each lane was as in panel B. The transcription reactions in panels B and C were performed in parallel, and band intensities can therefore be compared directly. D, in vitro termination activity of mTERF on pLSP-TERM. The protein content of each lane was as in panel B, E, the mTERF protein, and not POLRMT RNA polymerase pausing, promotes true termination on the pHSP-TERM template. After a 5-min pulse of radiolabeled UTP, a 8000-fold excess of cold UTP was added. At the times indicated, 15-ml aliquots were removed and analyzed as described under "Materials and Methods." MW, 32P-5′-end-labeled DNA ladder (100-bp DNA ladder; New England Biolabs).
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(pHSP-TERM), we failed to observe complete termination even at the highest mTERF concentrations (Fig. 1B). If we instead placed the mTERF-binding site in the reverse orientation (pHSP-MRET), we observed almost complete termination of transcription already at a molar ratio of about 1:1 of mTERF to DNA template (Fig. 1C, lane 6).

The mTERF protein has been shown previously to stimulate HSP transcription (5, 9). The stimulation is only observed when mTERF binds to its cognate binding site and both the promoter and the mTERF-binding site are in the same orientation. In our recombinant in vitro transcription system, we noted that the overall level of transcription was higher on the pHSP-TERM template (Fig. 1D) relative to the pHSP-MRET template. We could not, however, detect specific stimulation of transcription upon the addition of increasing amounts of mTERF to either of these two templates (Fig. 1, B and C).

To further characterize the mTERF protein, we analyzed transcription termination with the LSP followed by the mTERF-binding site in the reverse orientation (that is, as in vivo). We observed effective and complete mTERF-dependent termination but no stimulation of the overall levels of transcription (Fig. 1D). The observed results were essentially identical to what had been observed with the pHSP-MRET template (Fig. 1C).

Finally, we wanted to see if the shorter transcript observed in Fig. 1B was derived from true termination of transcription and not only polymerase pausing. To distinguish between these two possibilities, we performed a transcription reaction with the pHSP-TERM template. After a 5-min pulse of radiolabeled UTP, we added an 8000-fold excess of cold UTP and followed the transcription reaction for 90 min. If mTERF caused RNA polymerase pausing, we expected that the ratio of terminated-to-runoff transcripts would decrease progressively with time due to the resumption of elongation by the stalled enzyme. We found that the shorter transcript persisted for up to 90 min of incubation without any obvious change in the ratio between terminated and runoff transcripts (Fig. 1D). We concluded that the shorter transcripts formed in the presence of mTERF are likely due to true transcription termination and not to POL-RMT RNA polymerase pausing.

Recombinant mTERF Is a Monomer—The mTERF protein purified from mammalian cells has been shown previously to exist in two distinct states, as an active monomer and as an inactive polymer. We determined the multimeric state of our recombinant mTERF with gel filtration chromatography using Superose® 12. The mTERF protein eluted as a single peak with an apparent molecular mass of ~40 kDa, closely corresponding to the theoretical mass of the protein (Fig. 2A). Because the gel filtration analysis had been performed at high salt concentrations (0.5 M KCl) that might disrupt a potential polymer, we pooled peak fractions from the gel filtration chromatography. We subjected the pooled material to gel filtration through Superose® 12 at lower ionic strength (0.15 M KCl). The open arrow over panel A marks the expected elution volume for a mTERF trimer.

We concluded that our recombinant mTERF remains in the active monomeric conformation and does not polymerize to the same extent as demonstrated previously for native mTERF.

Recombinant human mTERF is a stable monomer in solution. A, a peak fraction of mTERF from Ni²⁺-NTA agarose was subjected to gel filtration through Superose® 12 at high ionic strength (0.5 M KCl). Individual fractions were separated with SDS-PAGE and revealed by Coomassie Blue staining. B, peak fractions from gel filtration at high ionic strength (panel A) were subjected to gel filtration through Superose® 12 at lower ionic strength (0.15 M KCl). The open arrow over panel A marks the expected elution volume for a mTERF trimer.

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FIG. 4. Determination of the molecular mass of recombinant mTERF by electrospray ionization-mass spectrometry. A, 40 pmol of Ni$^{2+}$-NTA-purified recombinant human mTERF were desalted by reversed phase chromatography (Poros R1) and analyzed on an electrospray ionization-quadrupole TOF mass spectrometer as described under “Materials and Methods.” B, the molecular mass of human mTERF was calculated by deconvolution. C, magnification of the area of interest depicted in panel B. The mass of individual peaks is indicated.
The Recombinant mTERF Protein Is Not Phosphorylated—

Previous reports of rat mTERF and its regulation by phosphorylation prompted us to investigate the existence of phosphorylated amino residues in our active mTERF. We first used a small molecule organic fluorophore (Pro-Q Diamond dye) that binds directly to the phosphate moiety and has been reported to detect as little as 2 ng of phosphoproteins directly on PVDF membranes (16). We separated our purified mTERF (from fraction 69 in Fig. 2B) on a SDS-polyacrylamide gel alongside equal amounts of ovalbumin (containing two phosphorylated sites and used as a positive control) and bovine serum albumin (used as a negative control). After transfer to a PVDF membrane, we stained the proteins with Pro-Q Diamond dye (Fig. 3A). We observed no staining of our mTERF, even when 3 μg of it was loaded, but obtained a strong signal with 1 μg of ovalbumin.

Phosphoprotein staining is a sensitive technique, and comparisons between autoradiography and Pro-Q Diamond staining on the same 32P-labeled protein samples have revealed very small differences (16). We did, however, want to verify our findings with unrelated techniques and therefore investigated the migration pattern of mTERF during two-dimensional gel electrophoresis. For the first dimension IEF we used DeStreak® Rehydration Solution (Amersham Biosciences) instead of DTT in the running buffer to avoid streaking, a common artifact caused by oxidation of basic proteins. In the second dimension we separated the mTERF protein with SDS-PAGE and stained with Coomassie Blue (Fig. 3A). Under these conditions, we detected a major single spot (spot I) that migrated in excellent agreement with the theoretical isoelectric point (pH 9.26) and molecular mass of mTERF. Two minor spots (spots II and III) could be observed directly above and below the major spot. Another minor spot (spot IV) was also visible. All spots were identified as mTERF by MALDI-TOF analysis. The mTERF protein is known to run as a doublet in SDS-PAGE gels, presumably due to slight conformational changes, which might explain the appearance of extra spots immediately above or below the major spot. We could not detect any spots to the left, the expected location for a phosphorylated form of mTERF. In a second set of experiments, we analyzed mTERF after alkaline phosphatase treatment (Fig. 3B, bottom section). As a control, we exposed the mTERF protein sample to identical conditions but without adding the alkaline phosphatase (Fig. 3B, top section). In these two experiments we observed one major spot at exactly the same position. The presence of mTERF in the spots was confirmed by MALDI-TOF mass fingerprint analysis. Therefore, alkaline phosphatase treatment did not alter the migration pattern of mTERF, arguing against the presence of phosphorylated residues.

In a final effort to identify phosphorylated amino acid residues, we analyzed mTERF by electrospray mass spectrometry, a technique that allows for the determination of the exact molecular mass of a peptide with high accuracy. From the multiple charged protein ions obtained (Fig. 3B), the molecular mass of the protein was determined by deconvolution (Fig. 4, A and C). The determined mass for the dominant species was 40233.2 (S.D. ± 0.6 Da), which coincides precisely with that predicted for the mTERF molecule (40190.5 Da) with an N-terminal acetylation (42.7 Da). Two additional peaks of 40256.1 and 40278.0 Da fit nicely with sodium adducts of the major peak. Our electrospray mass spectrometry analysis also revealed a minor protein peak at 40308.8 (S.D. ± 0.9 Da) with a putative sodium adduct of 40352.4 Da. The difference in mass of 76.6 Da relative to the major mTERF peak at 40233.2 Da is consistent with a disulfide adduct between the protein and β-mercaptoethanol (expected mass increase of 76.0 Da) and efficiently rules out phosphorylation, which should cause an 80-Da increase in mass.

**DISCUSSION**

We used here a highly purified and recombinant transcription system to characterize recombinant human mTERF in vitro. Our defined experimental system minimized the interference of contaminants and led us to conclude that mTERF does not require any additional factors present in mitochondrial extracts to actively promote transcription termination. As observed previously with the native protein, recombinant mTERF terminates transcription bidirectionally of both HSP- and LSP-initiated transcripts (17). The purified mTERF protein is highly active, and we can detect transcription termination already at protein concentrations well below the concentration of the DNA template. We observed near complete transcription termination at about equimolar concentrations of mTERF relative to that of the DNA template. Another relevant conclusion from our study is that non-phosphorylated mTERF is active in transcription termination. We use phosphostaining, two-dimensional electrophoresis, and electrospray mass spectrometry analysis to demonstrate that the active mTERF protein is not phosphorylated. It could, of course, be argued that a small, non-detectable fraction of mTERF is modified and that this fraction is responsible for the termination activity observed in vitro. But because the mTERF protein promotes almost complete transcription termination at a molar ratio slightly above 1:1 of protein to DNA template, this possibility appears extremely unlikely.

Even if human mTERF does not require phosphorylation to terminate transcription in vitro, we do not want to exclude a role for posttranslational modifications in the regulation of mTERF activity in vivo. Specific modifications of mTERF could e.g. promote polymerization and inactivate the protein or influence the DNA binding sequence specificity. Previous studies of the native mTERF protein have shown that the protein does not only terminate transcription at the mTERF-binding site but also stimulates transcription initiation at HSP (5, 9). The stimulation was only observed when mTERF binds to its cognate-binding site and both the promoter and the mTERF-binding site are in the same orientation. We failed to observe mTERF-specific stimulation of transcription in our reconstituted in vitro transcription, which could indicate a difference between the native and recombinant protein in this respect.

Mitochondrial transcription termination factors have been isolated and characterized from many different eukaryotes, including sea urchin (18), Drosophila (19), rat, and human. We, for first time, analyzed here the biochemical characteristics of one such termination factor in a fully defined in vitro transcription system. In future studies we will use our experimental system and combine biochemical experiments with genetics to further elucidate the regulatory aspects of mTERF-dependent transcription termination.

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