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A Monomer-to-Trimer Transition of the Human Mitochondrial Transcription Termination Factor (mTERF) Is Associated with a Loss of *in Vitro* Activity*

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The human mitochondrial transcription termination factor (mTERF) is a nuclear-encoded 39-kDa protein that recognizes a mtDNA segment within the mitochondrial *tRNA$^{Leu(UUR)}$* gene immediately adjacent to and downstream of the *16 S rRNA* gene. Binding of mTERF to this site promotes termination of rDNA transcription. Despite the fact that mTERF binds DNA as a monomer, the presence in its sequence of three leucine-zipper motifs suggested the possibility of mTERF establishing intermolecular interactions with proteins of the same or different type. When a mitochondrial lysate from HeLa cells was submitted to gel filtration chromatography, mTERF was eluted in two peaks, as detected by immunoblotting. The first peak, which varied in proportion between 30 and 50%, appeared at the position expected from the molecular mass of the monomer (41 ± 2 kDa), and the gel filtration fractions that contained it exhibited DNA binding activity. Most interestingly, the material in this peak had a strong stimulating activity on *in vitro* transcription of the mitochondrial rDNA. The second peak eluted at a position corresponding to an estimated molecular mass of 111 ± 5 kDa. No mTERF DNA binding activity could be detected in the corresponding gel filtration fractions. Therefore, we propose that mTERF exists in mitochondria in two forms, an active monomer and an inactive large size complex. The estimated molecular weight of this complex and the fact that purified mTERF can be eluted from a gel filtration column as a complex of the same molecular weight strongly suggest that this inactive complex is a homotrimer of mTERF.

Transcription of the heavy (H)-strand of mitochondrial DNA involves two overlapping transcription units starting at two closely located initiation sites in the D-loop region (1–3). One of these transcription units produces an oligo(dT)-cellulose unbound RNA species (u4a) spanning the whole rDNA region, *i.e.* the genes for the two mitochondrial rRNAs, 12 S and 16 S, the tRNA^{Phe} and the tRNA^{Val}, and ending at the border between the *16 S rRNA* and the *tRNA$^{Leu(UUR)}$* genes (4). The other transcription unit produces a single polycistronic oligo(dT)-cellulose bound RNA molecule (b4), encompassing almost the entire length of the H-strand. The u4a and b4 transcripts are subsequently processed to yield, respectively, the two mature rRNAs, the tRNA^{Phe} and tRNA^{Val}, and the H-strand encoded other tRNAs and mRNAs. The rRNA genes are expressed at a 20–50-fold higher rate relative to the downstream genes (5, 6). This differential expression is regulated not only at the level of transcription initiation, but also at the level of termination at the 3′-end of the rDNA transcription unit. A central role in the attenuation phenomenon taking place at this site is played by the mitochondrial transcription termination factor (mTERF),[1] a 39-kDa DNA-binding protein that binds to a 28-base pair region (nucleotides 3229 to 3256) within the *tRNA$^{Leu(UUR)}$* gene, at a position immediately adjacent to the *16 S rRNA* gene. This protein promotes transcription termination at the 3′-end of the *16 S rRNA* gene, as shown by an *in vitro* transcription assay (7, 8).

The primary structure of mTERF shows some relevant features, namely, three putative leucine-zipper motifs and two basic potential DNA-binding domains. Both types of motifs are essential for binding of mTERF to its DNA target sequence, and, consequently, for promoting transcription termination (9). Despite leucine zippers being typical protein-protein interaction motifs, it has been clearly demonstrated that mTERF binds DNA as a monomer (9). This has led to the suggestion that mTERF acquires DNA binding activity by means of intramolecular leucine-zipper interactions, required to bring the two basic DNA-binding domains together (9).

In the present work, we have explored the possibility that mTERF interacts with other proteins of the same or different type when not bound to DNA, and we have found that mTERF exists in HeLa cell mitochondrial lysates in two forms. One is a monomeric form, exhibiting DNA binding and transcription-termination activities, as expected from previous results (9), whereas the other is a novel high molecular weight complex lacking DNA binding activity. The evidence obtained has strongly suggested that the novel complex form of mTERF is a homotrimer. We, therefore, propose that the activity of mTERF is modulated by the transition between an active monomer and an inactive trimer.

EXPERIMENTAL PROCEDURES

Preparation of Polyclonal Anti-mTERF Antibody—His-tagged mTERF expressed in *Escherichia coli* and purified by nickel column chromatography, followed by SDS-PAGE, and electroelution from the mTERF-containing excised band was used for the immunization of a rabbit as described in Ref. 10.

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[1] The abbreviations used are: mTERF, mitochondrial transcription termination factor; HMW, high molecular weight; LMW, low molecular weight.

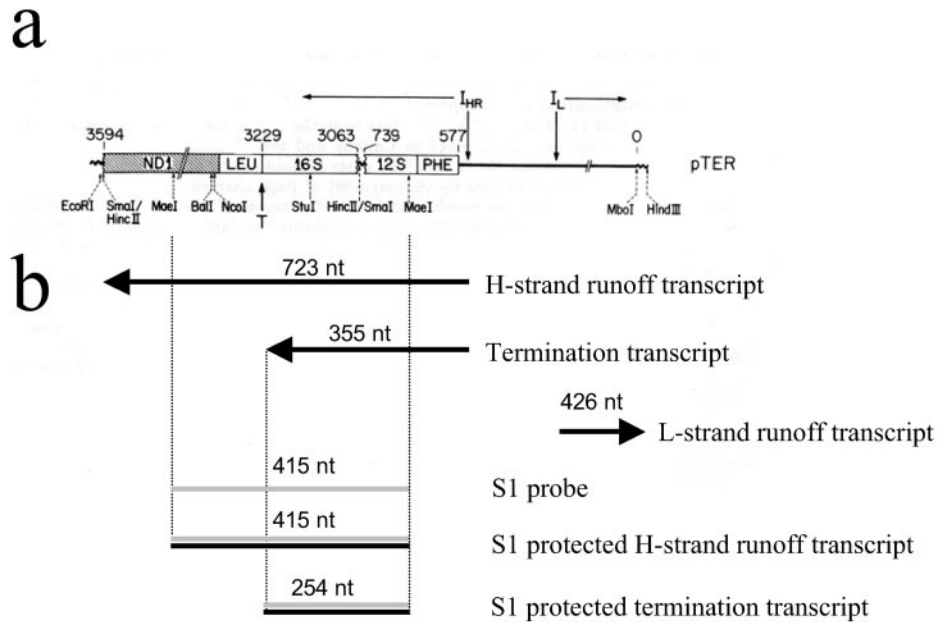

FIG. 1. **Determination of mTERF termination activity.** *a*, map of the clone pTER used as DNA template. Shown are the initiation sites for the H-strand rDNA transcription (I_{HR}) and for L-strand transcription (I_L) and the termination site for rDNA transcription at the 3′ end of the *16 S rRNA* gene (T). *b*, diagram of the *in vitro* transcription assay and the S1 protection assay, showing the map position of the expected transcripts (*thick arrows*), the map position of the MaeI-MaeI RNA probe (S1 probe), as well as the map position of the expected protected products.

The first immunization was carried out by subcutaneous injection of 400 μg of pure His-tagged mTERF dissolved in 0.6 ml of phosphate-buffered saline, 0.1% SDS mixed with 1 volume of Complete Freund's adjuvant (Sigma). Subsequent immunizations were carried out by subcutaneous injection of 200 μg of pure His-tagged mTERF dissolved in 0.6 ml of phosphate-buffered saline, 0.1% SDS mixed with 1 volume of incomplete Freund's adjuvant (Sigma) 3, 7, and 11 weeks after the first immunization. 10–30-ml blood samples were taken prior to each immunization and at week 15, and the animal was exanguinated by cardiac puncture at week 20. The sera obtained were tested for the presence of anti-mTERF antibody by enzyme-linked immunosorbent assay (10). Antibodies were already detectable 3 weeks after the first inoculation and reached a plateau at week 15.

Purification of mTERF from HeLa Cells—mTERF was purified from the S-100 fraction of a HeLa cell mitochondrial Tween 20 lysate (prepared from six 3-liter balloons of HeLa cell suspension cultures grown in modified Eagle's medium supplemented with 5% calf serum to late exponential phase at 37 °C) by heparin chromatography followed by DNA-affinity chromatography (using a DNA affinity resin prepared by ligation of multiple units of a 44-mer double-stranded oligodeoxynucleotide carrying the binding site of mTERF (7) present within the *16 S rRNA/tRNA$^{Leu(UUR)}$* boundary region, followed by coupling to Sepharose) essentially as described previously (11) with two minor modifications: (*a*) the sample was diluted with buffer A without KCl (25 mM HEPES-KOH, pH 7.6, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 μM pepstatin A, 10% glycerol, 0.1% Tween 20) instead of being dialyzed with buffer A containing 100 mM KCl, prior to heparin chromatography, and (*b*) the final elution from the DNA affinity column was carried out with a step gradient of buffer B (25 mM HEPES-KOH, pH 7.8, 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 μM pepstatin A, 20% glycerol, 0.1% Tween 20) containing 0.35, 0.5, 0.8, and 1 M KCl.

Gel Filtration Chromatography of S-100 Fraction from a Mitochondrial Lysate—Gel filtration chromatography of the S-100 fraction from a HeLa cell mitochondrial lysate was carried out on a FPLC system (Pharmacia) in the cold room. The column used was a HiPrep® Sephacryl S-200 (Pharmacia). For each run, the column was equilibrated in running buffer (25 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% Tween 20, 150 or 500 mM KCl) at a flow rate of 1 ml/min. A calibration curve was prepared, following the instructions of the manufacturer of the column, by running blue dextran 2000, cytochrome *c* (12 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), and catalase (232 kDa), all from Amersham Biosciences. The elution of these markers was monitored by UV photometry (280 nm) with the full scale set to 0.05 absorbance units, and the elution volume was measured from the start of the sample application to the apex of the elution peak. The logarithm of molecular weight was plotted against K_{av} that was calculated for each protein as follows: $K_{av} = V_e - V_o/V_t - V_o$, where V_e = elution volume for the protein; V_o = column void volume = elution volume of blue dextran 2000; V_t = total bed volume (120 ml for HiPrep® Sephacryl S-200). Prior to the injection of a sample, a set of three markers (cytochrome *c*, ovalbumin, and catalase) was injected to test the performance of the column. When the K_{av} of the markers was consistent with the calibration curve, 1.5–2 ml of S-100 were loaded into the column, and the eluate was collected in 1-ml fractions, flash frozen in liquid nitrogen, and stored at −80 °C until further analysis.

Analysis of the fractions by SDS-PAGE followed by immunoblotting (12), using anti-mTERF antiserum as primary antibody, goat anti-rabbit IgG (H+L) horseradish peroxidase-linked (Promega) as secondary antibody, and SuperSignal® West Pico (Pierce) as chemiluminescent substrate, allowed the determination of the elution volume of mTERF by autoradiography and quantification of the autoradiogram by densitometry, and the calculation of the K_{av} of the mTERF as described above. Its molecular weight was finally estimated by interpolation of its K_{av} in the calibration curve.

Native Polyacrylamide Gel Electrophoresis—Native PAGE of the eluates from the gel-filtration chromatography was run from anode to cathode under acidic conditions, as described in Ref. 13. The gel consisted of a 3.75% acrylamide, 0.7% acetic acid-KOH stacking gel (pH 6.8) and a 8–15% acrylamide, 2% acetic acid-KOH gradient resolving gel (pH 4.3). The running buffer composition was 0.8% acetic acid, 0.35 M β-alanine (pH 4.5). The samples were mixed with 1 volume of sample buffer (3.4% acetic acid, 0.1 M KOH, pH 4.3, 20% glycerol, and 0.005% methyl green), centrifuged for 1 min at 12,000 × g_{av} and loaded. The run took place at 8 °C at 20 mA until the dye front reached the resolving gel. Then the current was increased to 30 mA, and electrophoresis was continued for 5–10 h.

After the run, the gel was silver stained or transferred to the polyvinylidene difluoride (Bio-Rad) membrane for subsequent immunoblotting. The procedure for immunoblotting of the native gel was essentially identical to that used for SDS-PAGE, except for the fact that the gel was incubated in 0.1% SDS transfer buffer (0.1% SDS, 39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol, pH ~8.3) for 30 min prior to transfer.

In Vitro Transcription and S1 Protection Assays—Transcription-termination experiments were performed in 50× concentrated gel filtration fractions essentially as described previously for S-100 from mitochondrial lysates (8, 11), using 0.5 μg of the transcription-termination clone pTER (Ref. 7 and Fig. 1*a*) as DNA template. S1 protection assays were carried out on the RNA products of the transcription reaction using, as a specific probe, the unlabeled RNA synthesized utilizing BamHI-linearized pBSVM plasmid (containing the MaeI-MaeI fragment of pTER (Fig. 1*a*)) and T3 RNA polymerase, as described (8, 11). A scheme of the assay is shown in Fig. 1*b*.

Band-shift Assays—Mobility shift assays were carried out using as a probe a 5′-end ^{32}P-labeled version of the same double stranded 44-mer oligodeoxynucleotide used in the DNA affinity purification (7). A stand-

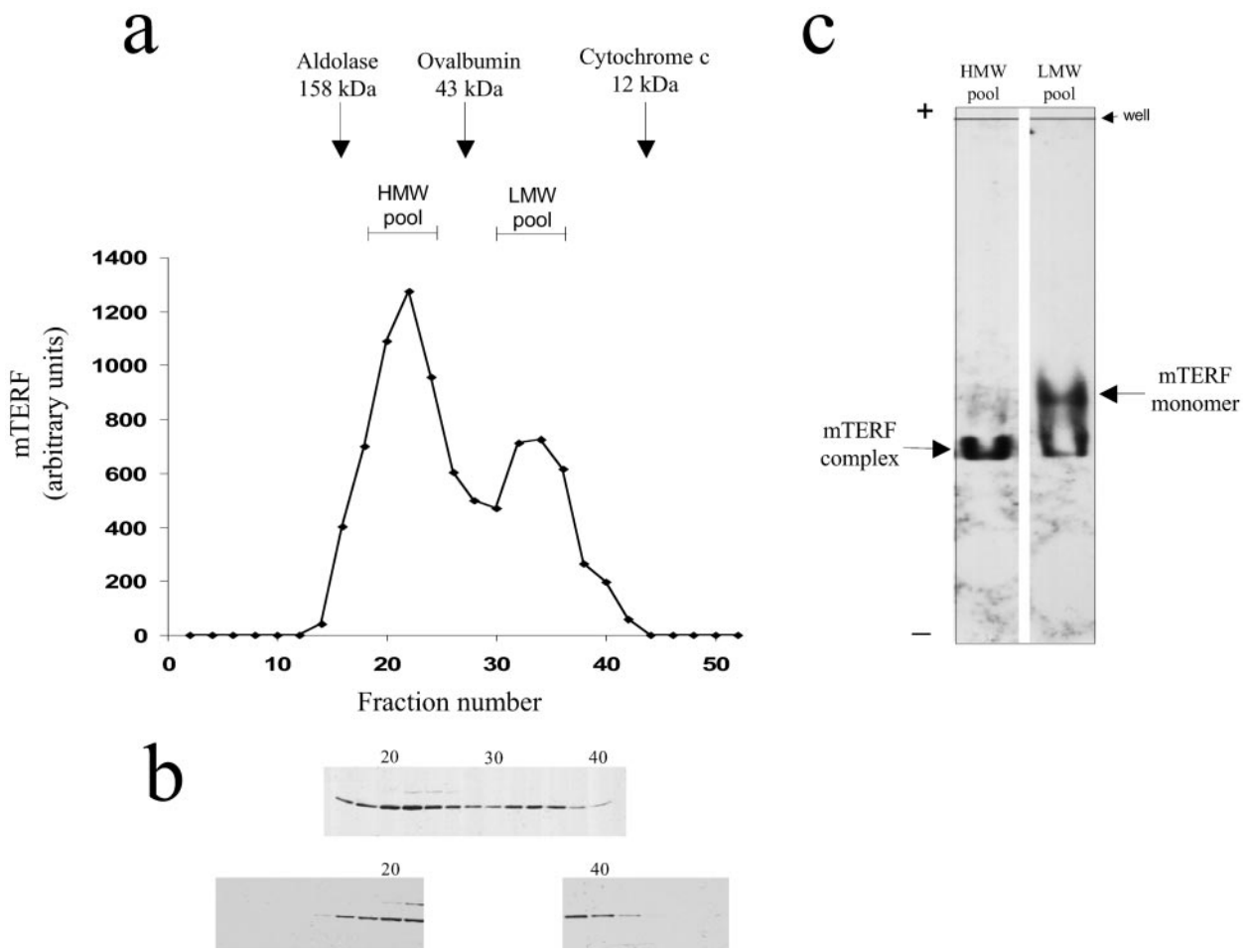

FIG. 2. **Results of gel filtration chromatography of 1.5 ml of S-100 (corresponding to 7.5–10 mg of total protein) using a HiPrep® Sephacryl S-200 column and 0.5 M KCl in the elution buffer.** *a*, elution profile of mTERF determined by immunoblotting of the even fractions. A mixture of aldolase, ovalbumin, and cytochrome *c* was loaded into the column prior to the injection of the mitochondrial S-100. *b*, actual immunoblots used to trace the elution profile shown in *a*. The *upper blot* allowed the demonstration of the existence of the two mTERF peaks, whereas the *lower blots* confirmed the absence of other peaks before or after the ones described. *c*, immunoblotting of a native-PAGE gel. Gel filtration chromatography eluates 18–24 (HMW pool) and 30–36 (LMW pool) were pooled, 50× concentrated, and 25 μl of each pool were mixed with 1 volume of sample buffer (0.1 M KOH, 3.4% acetic acid, 20% glycerol, and 0.005% methyl green, pH 4.3) and loaded. Immunoblotting was carried out as described under "Experimental Procedures."

ard band-shift assay reaction mixture contained 0.5 μg of poly(dI-dC)·(dI-dC), previously heated at 90 °C for 5 min, 20 fmol of 5′-end ^{32}P-labeled probe, 5 μg of bovine serum albumin (New England Biolabs), a variable volume of sample, and buffer C (25 mM HEPES, pH 7.5, 50 mM KCl, 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 20% glycerol, 0.1% Tween 20) to a final volume of 25 μl. The reaction mixture was incubated at room temperature for 20 min, placed on ice, and immediately loaded and run in a native 10% polyacrylamide gel in the cold room as described (11). After the run, the gel was dried and analyzed by autoradiography. In some experiments, especially those involving a subsequent immunoblotting of the shifted bands, the reaction mixture was upscaled to 100 μl.

Supershift experiments were carried out as described above, except that 1 μl of anti-mTERF antiserum was included in the reaction mixture. A control in which 1 μl of preimmune serum was added was run in parallel.

RESULTS

Gel Filtration Chromatography of the Mitochondrial S-100 Reveals Two Forms of mTERF—The S-100 fraction of a mitochondria lysate is known to contain mTERF, and it has been used in the past as the primary source for the purification of mTERF from HeLa cells (7). Preliminary experiments aimed at identifying high molecular weight (HMW) forms of mTERF involved a sedimentation of the mitochondrial S-100 from HeLa cells through a 15–35% linear glycerol gradient (14), using bovine liver catalase as a sedimentation marker and SDS-PAGE followed by Western blots with anti-mTERF antiserum to detect the presence of mTERF. These experiments indicated the presence of a form of mTERF having a higher sedimentation constant than that of the monomeric form. For further analysis of this potential HMW form of mTERF, the S-100 fraction from HeLa cells was fractionated by gel filtration chromatography using a HiPrep® Sephacryl S-200 column. The presence of mTERF in the chromatography fractions was revealed by SDS-PAGE, followed by Western blotting using anti-mTERF antiserum. Because mTERF is known to be stable and dissociated from DNA at high salt concentration, gel filtration chromatography was first carried out with a running buffer containing 0.5 M KCl. As shown in Fig. 2, *a* and *b*, mTERF was eluted in two peaks, the first encompassing ~⅔ and the second ~⅓ of the antibody-reactive material. Gel filtration chromatography of the same mitochondrial S-100 preparation carried out in 0.15 M KCl yielded identical results (data not shown). In contrast, in chromatographic runs of different mitochondrial S-100 preparations a certain variability in the proportion of the two peaks was observed (see below). To estimate the molecular weight of each form, their K_{av} values were calculated and interpolated on a calibration curve previously performed on the same column, as described under "Experimental Procedures." The molecular mass values obtained for

the mTERF present in the two peaks were 41 ± 2 and 111 ± 5 kDa (expressed as mean ± S.D. of three independent experiments). The estimated molecular weight of the lighter form (LMW) closely matched that of mature mTERF (39,000), but the other peak at 111,000 indicated that mTERF coexists in the mitochondrial lysate as part of a HMW complex. When pooled gel filtration fractions of each mTERF form were submitted to native-PAGE electrophoresis from anode to cathode under acidic conditions, as specified under "Experimental Procedures," and subsequent immunoblotting with anti-mTERF antibody, a clear difference in migration was observed between the two forms (Fig. 2*c*). The HMW pool showed a single band, whereas the LMW pool showed two bands, a less intense one migrating exactly as the HMW form, and a second one migrating clearly behind it. The presence of the faster migrating band in the LMW pool is most probably because of a contamination with the HMW form, whereas the slower migrating band is the actual LMW form. The unexpected relative migration of the two mTERF forms by native PAGE electrophoresis is commented upon under "Discussion." The relative intensity of the HMW and LMW bands is consistent with the relative amount of both forms observed by gel filtration chromatography (compare Fig. 2, *c* with *a*).

Transcription-termination Activity Assay of Gel Filtration Fractions—The transcription-termination activity of the two forms of mTERF was next determined. For this purpose, the gel filtration fractions of the two mTERF-containing eluate peaks, that in this experiment were of approximately equal size, were divided into 9 pools, concentrated 50-fold, and tested in an *in vitro* transcription system, followed by an S1 protection assay. As seen in Fig. 3, the monomeric form of mTERF exhibited transcription-termination activity, as expected (9). By contrast, the transcription-termination assay did not provide any information about the activity of the HMW form, because *in vitro* transcription was almost completely inhibited in the pooled fractions containing this form.

Analysis of the run-off transcripts from fractions containing the monomeric form showed that the rate of L-strand transcription remained at the same level as that shown in the control sample (in which no gel filtration eluate was added to the *in vitro* transcription mixture), whereas the rate of H-strand transcription dramatically increased (Fig. 3). These results suggested that the monomeric form of mTERF may enhance H-strand transcription from the I_{HR} initiation site, in agreement with previous observations (7).

Only the Monomeric Form of mTERF Has DNA Binding Activity—Because the termination activity assays of the HMW form of mTERF revealed complete absence of transcription in the presence of this form, the DNA binding activity of both mTERF forms was investigated. A band-shift assay using a double-stranded 44-mer oligodeoxynucleotide probe containing the mTERF DNA-binding site (7), carried out on fractions from another gel filtration chromatography experiment (which again yielded two peaks of mTERF-containing eluate of approximately equal size), resulted in a series of shifted bands (Fig. 4*a*). Only two of these (indicated by *arrows*) were present in fractions that contained mTERF. Fractions containing the HMW form exhibited almost exclusively the upper one of the two bands, whereas fractions containing the monomeric form exhibited both bands. As this observation suggested the possibility that both forms of mTERF had specific DNA binding activity, the analysis was refined by a supershift assay carried out on a fraction (number 27) containing both bands (Fig. 4*b*). The lower one of the two bands almost disappeared when the anti-mTERF antiserum was present in the reaction mixture, being replaced by a slower moving band, whereas the upper

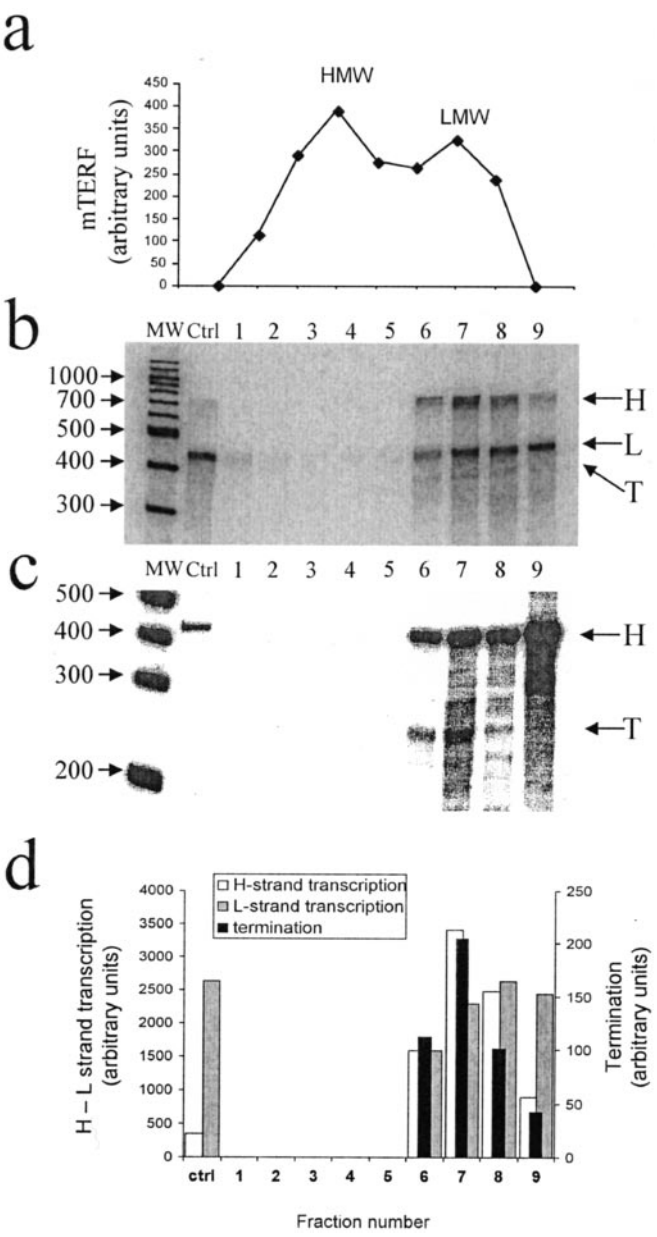

FIG. 3. **Transcription-termination activity assays.** *a*, elution profile of mTERF from the gel filtration of the mitochondrial S-100. Nine 5-ml fractions were collected, 50× concentrated, and analyzed by SDS-PAGE and immunoblotting using anti-mTERF antiserum. *b*, ^{32}P-labeled products from an *in vitro* transcription assay of each fraction were run on a 5% acrylamide, 7 M urea gel. *H*, H-strand runoff transcript; *L*, L-strand runoff transcript; *T*, H-strand terminated transcript. *c*, S1 protection products of the same fractions, run on a 5% acrylamide, 7 M urea gel. *H*, H-strand runoff transcript; *T*, terminated transcript. *d*, quantification by densitometry of the autoradiogram obtained from the products of *in vitro* transcription assays shown in *b*. *Ctrl*, control sample, in which the *in vitro* transcription reaction was run in the absence of any added gel filtration fraction. *MW*, molecular weight markers: 100-bp DNA ladder, from New England Biolabs.

band was unaffected. In agreement with this finding, excision of the two shifted bands from another gel fraction (number 26) and analysis by Western blotting of the eluted proteins showed that mTERF was indeed in the lower band, but not in the upper one (Fig. 4*c*).

To confirm the previous observation, after a gel filtration chromatography, pooled fractions containing either the monomeric or the HMW form of mTERF were submitted to heparin chromatography, followed by Western blotting analysis of the flow-through and eluted fractions (Fig. 4*d*). Heparin binds

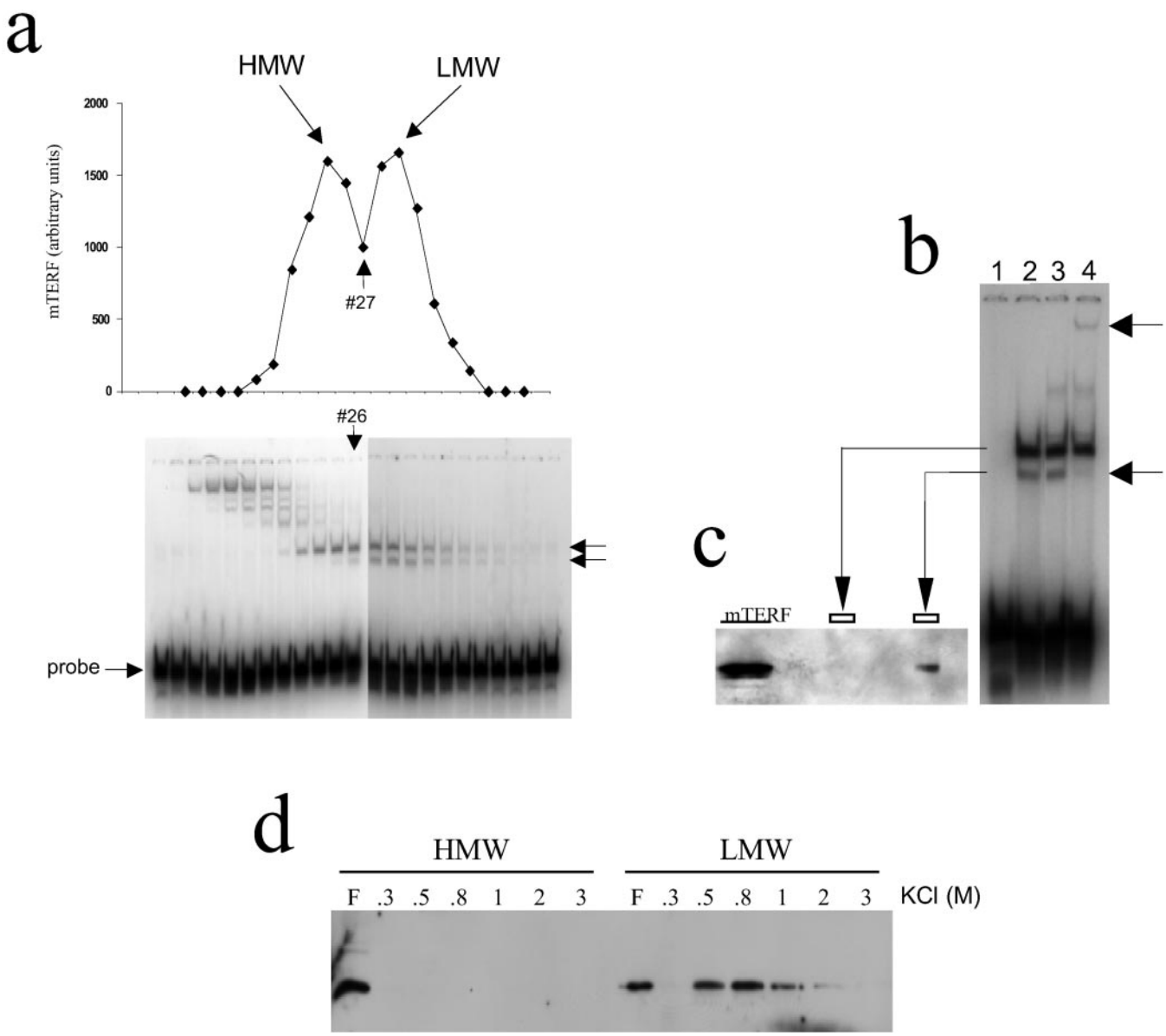

FIG. 4. **DNA binding assays.** *a*, band-shift assay of fractions from a gel filtration chromatography. *Upper half*, elution profile of mTERF, as assessed by SDS-PAGE of the odd gel filtration fractions, followed by immunoblotting using anti-mTERF antiserum. *Lower half*, the even fractions from the same experiment were submitted to band-shift assay as described under "Experimental Procedures." The *arrows* on the *right* of the autoradiography mark the two shifted bands of interest. *b*, supershift assay. *1*, probe only. *2*, fraction 27 from the gel filtration experiment shown in *a*. *3*, fraction 27 in the presence of preimmune serum. *4*, fraction 27 in the presence of anti-mTERF antiserum. The *thick black arrows* on the *right* of the autoradiogram indicate the disappearance of the lower shifted band and the appearance of a new, supershifted band when the anti-mTERF antibody is present in the band-shift assay. *c*, immunoblotting, using anti-mTERF antiserum, of the proteins eluted from the excised bands shown in *b* and run on an SDS-PAGE in parallel with pure mTERF. Only the lower shifted band shows the presence of mTERF. *d*, SDS-PAGE followed by immunoblotting of the flow-through (*F*) and eluates (0.3 M through 3.0 M KCl) from heparin chromatography of the HMW and LMW pools, showing that only the LMW form of mTERF binds to heparin.

DNA-binding proteins non-specifically, and is a commonly used first step in the purification of DNA-binding proteins (15). The monomeric form of mTERF showed the typical behavior of a DNA-binding protein. Although a small proportion was present in the flow-through (most probably because of saturation of the resin, or to the presence of a small amount of HMW-mTERF in the LMW pool), most of the mTERF started to be eluted from the column at 0.5 M KCl. In contrast, when the HMW mTERF-containing pooled fractions were loaded onto the heparin column, all the mTERF was present in the flow-through, and none was detected in the eluates. From the band-shift and supershift assays and from the Western blotting and heparin chromatography experiments, we conclude that only the monomeric form of mTERF has DNA binding activity, and that the protein(s) responsible for the upper one of the doublet of shifted bands in Fig. 4*a* is unrelated to mTERF.

Pure mTERF Is Eluted as a HMW Form from the Gel Filtration Column—mTERF from HeLa cells can be purified to a high degree by heparin chromatography followed by DNA-affinity chromatography (7). As shown in Fig. 5*a*, only a pair of low molecular mass contaminants co-purify with the two "34-kDa" mTERF bands (8, 9) under these conditions. When the 0.8 M KCl eluate from the DNA affinity column was submitted to gel filtration chromatography, mTERF was eluted completely as the previously identified HMW form, whereas the contaminating bands were eluted in the first fractions, totally separated from mTERF. The observation that pure mTERF was eluted completely from a gel filtration column as the HMW form

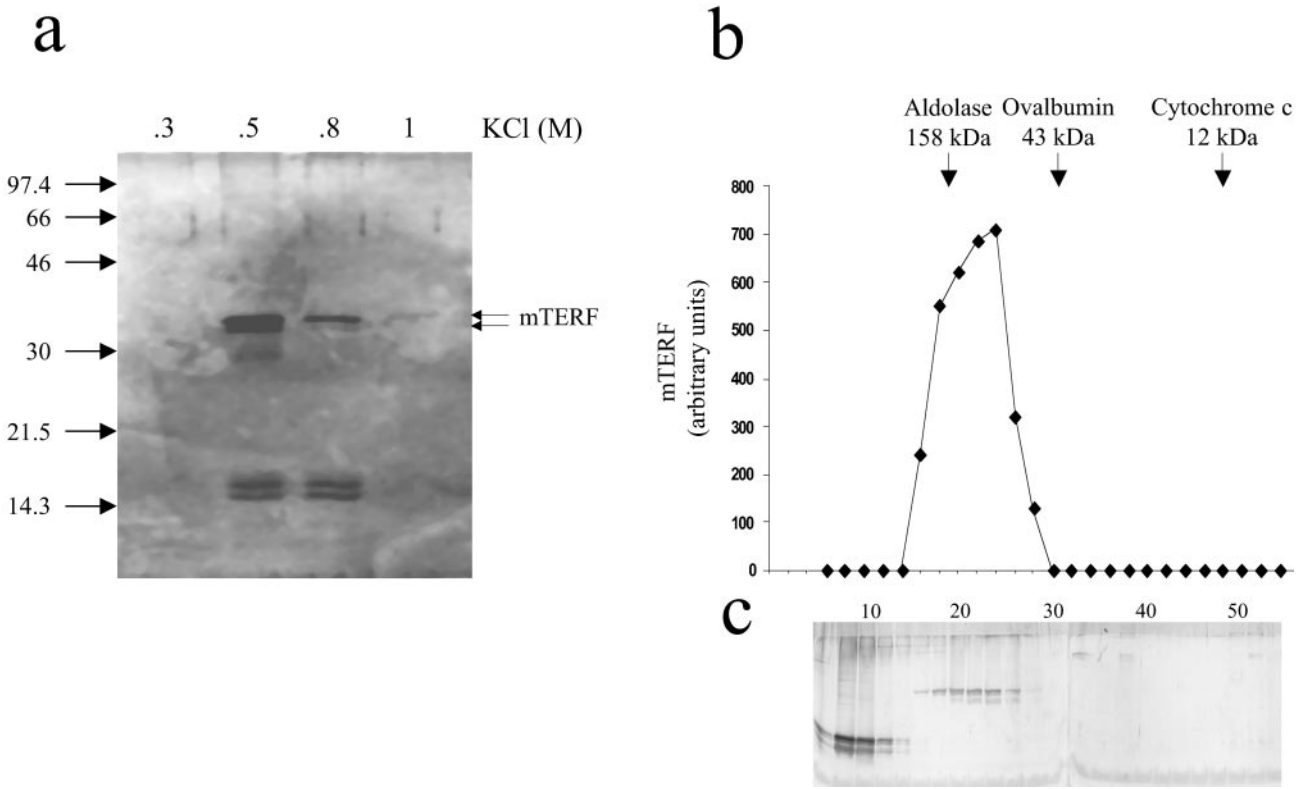

FIG. 5. **DNA affinity purified mTERF from HeLa cells is eluted from a gel filtration chromatography column essentially as the HMW form.** *a*, silver-stained SDS-PAGE gel showing the 0.5 and 0.8 M KCl eluate from a DNA affinity purification (molecular weight marker: High-Range Rainbow™, from Amersham Biosciences). *b*, elution profile of mTERF from a gel filtration chromatography column of 0.5 ml of the 0.8 M KCl DNA affinity chromatography eluate (shown in *a*). *c*, actual silver-stained SDS-PAGE gel of the fractions from the gel filtration chromatography used to plot the elution profile of mTERF shown in *b*.

without trace of the monomer form was unexpected, because the DNA affinity chromatography following heparin chromatography was the previously used approach to purify active mTERF. This question is discussed below.

DISCUSSION

mTERF is known to bind to DNA as a monomer (9). This led to the proposal that intramolecular interactions between the leucine-zipper motifs were required to bring the two basic domains in close register with the mTERF target DNA sequence. This is in contrast with most leucine-zipper proteins, which use their leucine-zipper domains to interact with other proteins. Many of them, like C/EBP, GCN4, c-Fos, and c-Jun are transcription factors like mTERF, and the formation of homo- or heteropolymers regulates their DNA binding activity (16, 17). It was in this context that we decided to explore whether mTERF can form homopolymeric or heteropolymeric complexes with possible regulatory roles.

In the present work, no difference was observed in the separation by gel filtration chromatography of the monomeric form of mTERF and of a complex roughly three times bigger than the monomer, or in their proportion, when high (0.5 M) or low (0.15 M) concentrations of KCl were used during the chromatography. The stability of the HMW form of mTERF at high ionic strength may be indicative of the fact that electrostatic interactions are not essential for the formation of such a structure. In fact, the interaction between leucine-zipper domains is basically maintained by the interaction of the hydrophobic residues at positions **a** and **d** of the heptad repeats (the residues of the heptad being designated as **a**–**g** (17, 18)), and the stabilizing role of the electrostatic interactions between residues in positions **e** and **g** described in some cases is far from being general (19, 20). This interpretation is in accordance with the paucity of charged residues in the leucine-zipper domains of mTERF (6 polar residues of 15 at position **e**, and only 2 of 15 at position **g**) (9).

In vitro transcription experiments clearly showed the expected transcription-termination activity associated with the monomeric form. Surprisingly, however, transcription of both strands was inhibited in the fractions containing the HMW form. Further work is needed to identify the factor(s) causing this effect. It cannot be ruled out that mTERF itself, in its trimeric form, is responsible for the inhibition of transcription initiation. However, transcription experiments carried out with the DNA affinity purified mTERF (which behaved like a trimer in gel filtration chromatography) showed no effect on transcription initiation. Other proteins present in the gel filtration fractions might be responsible for the effect on initiation of transcription. Another surprising result of the *in vitro* transcription experiments was that the termination activity observed in the fractions containing the monomer was accompanied by a strikingly increased rate of H-strand transcription from the rDNA initiation site (I_{HR}). If it is confirmed that the monomeric form of mTERF is responsible for both the termination activity and the stimulation of transcription initiation, the ideas about the mechanism whereby mTERF exerts control of rDNA transcription will have to be revised, and a broader role for mTERF will have to be considered. This role is reminiscent of that described for TTF-I, a protein that promotes transcription termination of the nuclear rRNA genes in mammals (21, 22). TTF-I-mediated termination is accompanied by an increase in initiation because of facilitation of reinitiation after RNA polymerase release from the termination site (23). This mechanism may also apply to mTERF. However, the possibility that the DNA carrying the rDNA transcription unit forms a loop that brings

promoter and termination elements in close contact, so that the RNA polymerase is "handed over" from the termination to the initiation sites, as initially proposed for TTF-I (24), cannot be ruled out. Furthermore, it is also possible that mTERF interacts directly with the H-strand promoter, activating it.

From the *in vitro* transcription experiments it was not possible to obtain any information about the transcription-termination activity of the HMW form. However, the band-shift experiments, the Western blot analysis of the excised shifted bands, and the heparin chromatography assays showed clearly that the monomeric form, but not the HMW form of mTERF, had DNA binding activity. Therefore, it can be concluded that only the monomer has transcription-termination activity, and that the formation of the HMW form blocks DNA binding. In the present work, binding of the HMW form to other mtDNA sequences, besides the segment containing the only known binding site of human mTERF within the $tRNA^{Leu(UUR)}$ gene, was not tested. However, the capacity of the HMW or LMW forms to bind to other mtDNA sites is definitely an important question for future investigations. This is especially true in view of the evidence presented here that suggests a possible role of mTERF in the control of the initiation of rDNA transcription.

Two pieces of evidence strongly suggest that the polymer is a homotrimer. First, the estimated molecular weight of the HMW form (111,000) corresponds closely to three times the molecular weight of mTERF ($3 \times 39{,}000 = 117{,}000$). Second, mTERF highly purified from HeLa cells by heparin chromatography followed by DNA affinity chromatography could be eluted from the gel filtration chromatography column at the same position as the HMW mTERF form (Fig. 5). There are two well documented examples of leucine-zipper proteins whose activity is controlled by homotrimerization. These are the heat shock transcription factor (25) and the influenza virus hemagglutinin (26, 27). In both cases, the interaction between the three polypeptides, which is required for activity, is established through their leucine zippers, which form a triple coiled-coil structure. mTERF, however, would be unique, in the sense that its activity is associated with the monomeric form. In this context, it may seem paradoxical that the HMW form migrated faster than the LMW form in a native PAGE run, because, in principle, the mass/charge ratio of the two forms should be the same if the HMW form is a homotrimer. Indeed, as discussed above, no difference was observed in the ratio of the polymeric to the monomeric form in gel filtration chromatography runs made at 0.5 and 0.15 M KCl. However, the change in secondary and tertiary structure associated with the transition between the two forms might well be accompanied by a differential exposure of other charged residues (not involved in the interaction between the leucine zippers) on the surface of the protein. Hypothetically, changes in shape might also contribute to the paradoxical phenomenon observed. An answer to this issue will require an analysis of a high resolution structure of the two mTERF forms.

In the present work, no evidence was obtained for the existence of a heteropolymeric form of mTERF in the S-100 fraction of a mitochondrial lysate from HeLa cells. However, this negative result does not rule out the possibility of mTERF establishing transient interactions with other proteins. This is a very important point that will require further investigation.

The results of the gel filtration of DNA affinity purified mTERF (Fig. 5) were in a certain sense unexpected, and gave an idea of the complexity of this protein. In fact, the observation that virtually all mTERF migrated as an inactive polymer (transcription-termination experiments carried out on these fractions showed no activity; data not shown) contrasted with previously published experiments in which substantial transcription-termination activity was detected in purified fractions obtained under the same conditions (7, 8). Heat shock transcription factor 1, a leucine-zipper protein with high structural similarities with mTERF, is known to show a high tendency to trimerize spontaneously during purification (28), in a concentration-dependent manner (29). It is therefore not surprising that mTERF also shows a high spontaneous tendency to polymerize. The very elaborate protocol used for purification by DNA affinity chromatography of mTERF indeed offers ample opportunities for its polymerization. This phenomenon was presumably not identified before because of the impossibility of detecting inactive mTERF, because of the lack of anti-mTERF antibody. Moreover, because the HMW form is unable to bind to either heparin or the 44-mer double-stranded oligodeoxynucleotide used in the DNA affinity chromatography, the polymers detected in the last step of the purification must have been formed after the DNA affinity chromatography. Accordingly, the following sequence of events can be envisaged. Heparin chromatography eliminates all the HMW form originally present in the S-100, while concentrating the monomer. This concentration would enhance the polymerization of a fraction of the monomeric form, which would be lost during the DNA affinity step. The remaining pure monomeric form would have a very strong tendency to polymerize, because of high concentration and high purity. The substantial unexplained differences in the mTERF termination activity observed in previous studies (7, 8) suggest that this phenomenon was indeed also happening in the earlier work. On the other hand, also in the present work a certain variability was observed from experiment to experiment in the proportion of mTERF in the S-100 that was in the monomeric form, this proportion varied between ~30 and ~50%. Differences in the initial concentration of cells, length of culture, cell homogenization conditions, to mention just a few of the possible variables, might expose mTERF to different levels of proteases, or other unidentified factors that influence the stability of either form, causing the variability observed. Another possible cause of this variability is the presence of mtDNA contaminating the S-100, which may favor the presence of the monomeric form. It is obvious that further work is necessary to understand the variables controlling the proportions of the monomeric or HMW forms of mTERF in different experiments. However, it seems clear that pure mTERF can be eluted from the gel filtration column at the position of the HMW form, and this observation provides a very strong indication that the HMW form is indeed a homopolymer of mTERF.

Fernandez-Silva and colleagues (9) proposed that the leucine zippers of mTERF form a triple coiled-coil structure that brings the two basic domains in close register with its target DNA sequence. Some examples of leucine zippers establishing intramolecular interactions had previously been described, like spectrin (30) and seryl-tRNA synthetase (31). In view of the results obtained in the present work, we now extend this hypothesis, proposing that a rearrangement in the interaction between leucine zippers is responsible for the control of the activity of mTERF. The tertiary structure of the active monomer would be maintained by intramolecular interactions, whereas that of the inactive HMW form, in the form of a homotrimer, would depend on intermolecular interactions.

Although the work described in this paper has extended our knowledge on the role of mTERF in the regulation of transcription of the mitochondrial rDNA, it is clear that numerous critical questions have been raised by the results obtained. Does the mTERF trimer have a function, or is it a means for controlling the amount of active mTERF monomer in the mi-

tochondrial matrix? Is there a physiological mechanism controlling the formation of the trimer and its conversion back to monomer? How much trimer is normally occurring *in vivo*? Future work will be aimed at assessing the *in vivo* relevance of our findings, at studying the molecular mechanism responsible for the transition between the active mTERF monomer and the inactive trimer, as well as at dissecting the putative role of mTERF in the control of transcription initiation.

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