MATERIAL AND METHODS

1) Preparation of anti-mTERF antiserum

The anti-mTERF antiserum used for immunoblotting was obtained by injection of purified His-tagged mTERF to a rabbit, and the efficiency of the immunization assessed by Enzyme-Linked-Immunosorbent-Assay (ELISA).

1.1) Purification of His-Tagged mTERF

1.1.1) Bacterial culture

A glycerol stock of GI724 bacterial strain transformed with a pLEX vector containing the cDNA of His-tagged mTERF was used as the primary source of this protein. Bacteria were streaked out on a RMG-Ampicillin plate (1x M9 salts*, 2% Bacto® Casamino acids, from DIFCO Laboratories, 0.5% glucose, 1 mM $MgCl_2$, 100 μg/ml ampicillin, 1.5% agar) after scraping the surface of the frozen glycerol stock with a loop and then were incubated at 30°C overnight. A single colony was picked up from the plate and inoculated in 50 ml RM medium (1x M9 salts, 2% Bacto® Casamino acids, 1% glycerol, 1 mM $MgCl_2$, 100 μg/ml ampicillin). This culture was incubated at 30°C overnight in a shaking incubator at 200-225 rpm. The next day, OD_{550} of the bacterial culture was checked, and a volume of it was transferred to a 2 liter Erlenmeyer flask containing 500 ml induction medium (1x M9 salts, 0.2% Bacto® Casamino acids, 0.5% glucose, 1 mM $MgCl_2$, 100μg/ml ampicillin) to bring OD_{550} to 0.1. This culture was incubated at 30°C. When OD_{550} reached 0.5, 1 ml culture was taken (t=0) and L-Triptophan was added to the remaining to a final concentration of 100 μg/ml to induce the expression of His-tagged mTERF. At this point, the culture was transferred to a 37°C rotary shaker and incubated for 5 h. 1 ml samples were taken at times 2.5 h and 5 h. (t=2.5 and t=5, respectively). After reading OD_{550}, the three samples were centrifuged for 5 min. at 12,000xg, the supernatants removed and the pellets kept at –20°C until further analysis.

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* 10x M9 salts composition: 113.2 g $Na_2HPO_4 \cdot 7H_2O$, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl in 1 liter of H_2O.

In parallel, the 500 ml culture was divided into two 250 ml bottles, centrifuged for 10 min. at 5,100xg, the supernatants discarded and the pellets stored at –20°C .

In order to test the efficiency of expression of His-tagged mTERF, the three pellets from the 1 ml samples taken from the culture at times 0, 2.5 and 5 hours were resuspended in 500 µl TE buffer and bacteria were lysed by three sessions of sonication (three pulses of 10 sec., 21% amplitude, using a Branson Sonifier, model 250), freezing and thawing. The lysates were centrifuged for 10 min. at 12,000xg at 4°C and supernatants (soluble fractions, SF) were separated from the pellets (insoluble fractions, IF). IF were dissolved in a volume of 1xSDS sample buffer (see Material and Methods-6 for composition) according to this formula:

$$Vol_{(t=x)} = OD_{550}(t=x) \times 50/OD_{550}(t=0) \quad \text{(in µl)}$$

A volume of SF, calculated with this formula:

$$Vol_{(t=x)} = OD_{550}(t=0) \times 50 / OD_{550}(t=x) \quad \text{(in µl)},$$

was mixed with three volumes of aceton, incubated for 30 min at –20°C, centrifuged 30’ at 12,000xg and the pellets resuspended in 20 µl of 1xSDS sample buffer. 20 µl of each sample were boiled for 4’ and run on a 12.5% PAGE gel (see Material and Methods-6). After the run, the proteins were visualized with Coomassie Blue staining (see Material and Methods-6). His-tagged mTERF always appeared in the insoluble fraction, probably due to the formation of inclusion bodies during its expression *in bacteria*. Figure 12 shows a typical example of expression of His-tagged mTERF.

Once the efficiency of expression was assessed, each pellet from the 500 ml culture was resuspended in 12.5 ml 20 mM phosphate, 500 mM NaCl and 10 mM imidazole, pH 7.4, sonicated as described above and centrifuged for 10 min. at 12,000xg at 4°C. The supernatants were removed, a 20 µl aliquot was kept apart and the rest was frozen at –20°C. The pellets were thoroughly resuspended in 5 ml/g wet weight of lysis buffer (20 mM phosphate, 500 mM NaCl, 10 mM imidazole and 8M urea, pH 7.4), using a Pasteur pipette and then centrifuged at 16,000xg, for 10 min. at 4°C. A 20 µl aliquot of the supernatant was taken and the rest was frozen at –20°C.

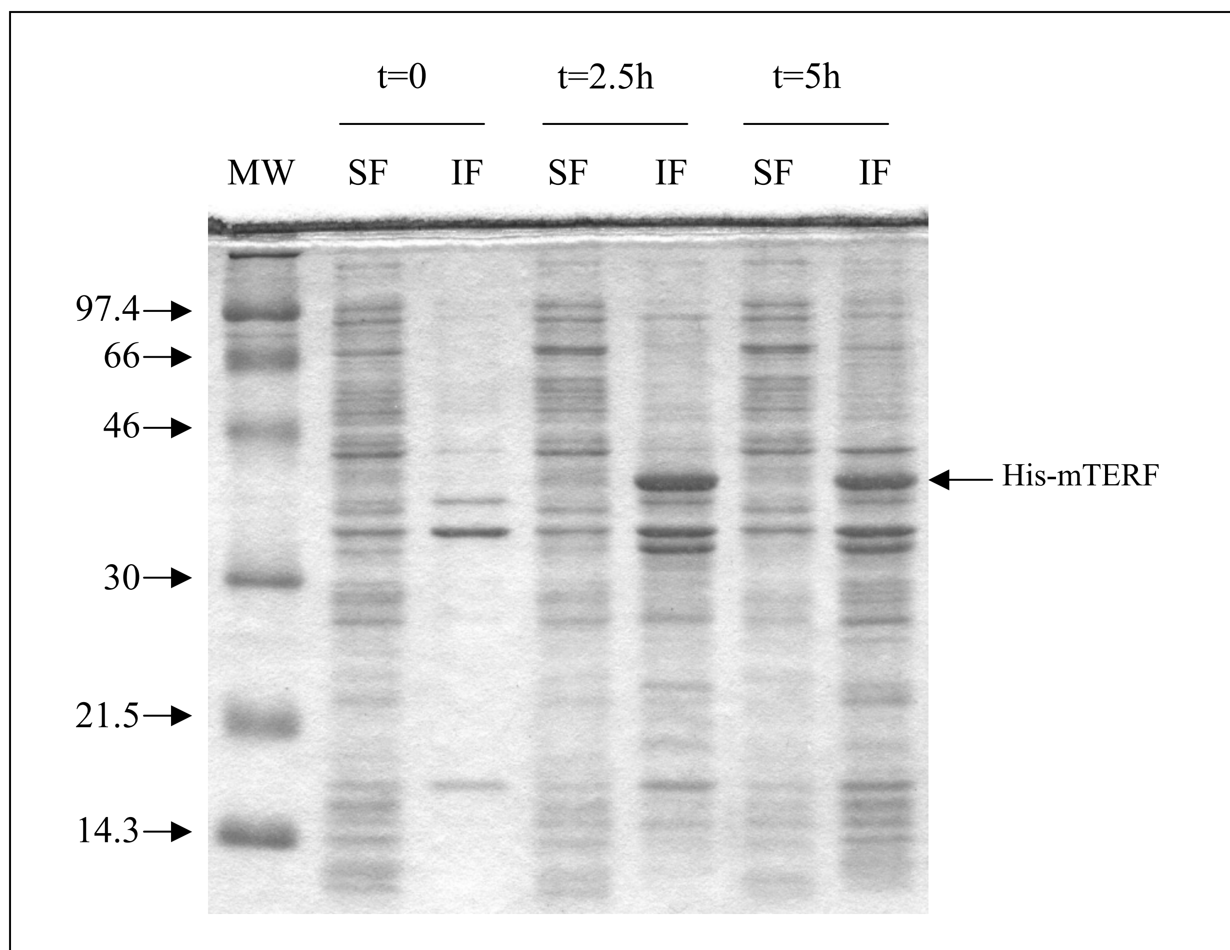

Fig.12. SDS-PAGE – silver staining analysis of samples obtained after the induction of the expression of his-mTERF *in bacteria*. MW: molecular weight marker. SF: soluble fraction. IF: insoluble fraction.

Then the 20 µl aliquots were run on a 12.5% SDS-PAGE gel and stained with Coomassie Blue to confirm that His-tagged mTERF was present in the insoluble fraction, as expected from the efficiency test.

1.1.2) Nickel column chromatography

For the purification of His-tagged mTERF, a nickel column was used (HIS-TRAP, Amersham), following the protocol provided by the manufacturer:

1.1.2.1) Column preparation

- Fill a 5 ml syringe with distilled water.

- Remove upper stopper from the column and connect the column to the syringe with a “drop to drop” adaptor to avoid introducing air in the column.

- Remove twist-off bottom end from the column and wash it with 5 ml distilled water.

- Disconnect syringe, fill it with 5 ml 0.1 M $NiSO_4$ and load it into the column.

- Wash the column with 5 ml distilled water.

1.1.2.2) Purification

- Prepare 24 ml Binding Buffer, pH 7.4:

- 3ml 8x phosphate buffer (provided with the column)
- 0.12 ml 2M imidazole
- 11.5 g urea
- distilled water to 24 ml

- Prepare 8 ml Elution Buffer, pH 7.4:

- 1 ml 8x phosphate buffer
- 2 ml 2M imidazole
- 3.84 g urea
- distilled water to 8 ml

- Using syringe, equilibrate column with 10 ml Binding Buffer.

- Apply the sample, using a peristaltic pump. Flow rate: 1-3 ml/min. Collect Flowthrough.

- Wash with 10 ml Binding Buffer. Collect Wash.

- Elute with 5 ml elution buffer. Collect in 1ml fractions.

- In order to test the efficiency of the purification, take 50 µl of flowthrough, wash and 5 eluates, and precipitate with 3 volumes of aceton incubating 30 min. at –20°C. Centrifuge for 15 min. at 12,000xg, remove supernatant and resuspend the pellets in 1xSDS sample buffer. Boil for 4 min., load on a 12.5% SDS-PAGE gel and stain it with Coomassie Blue (see figure 13 for a typical example).

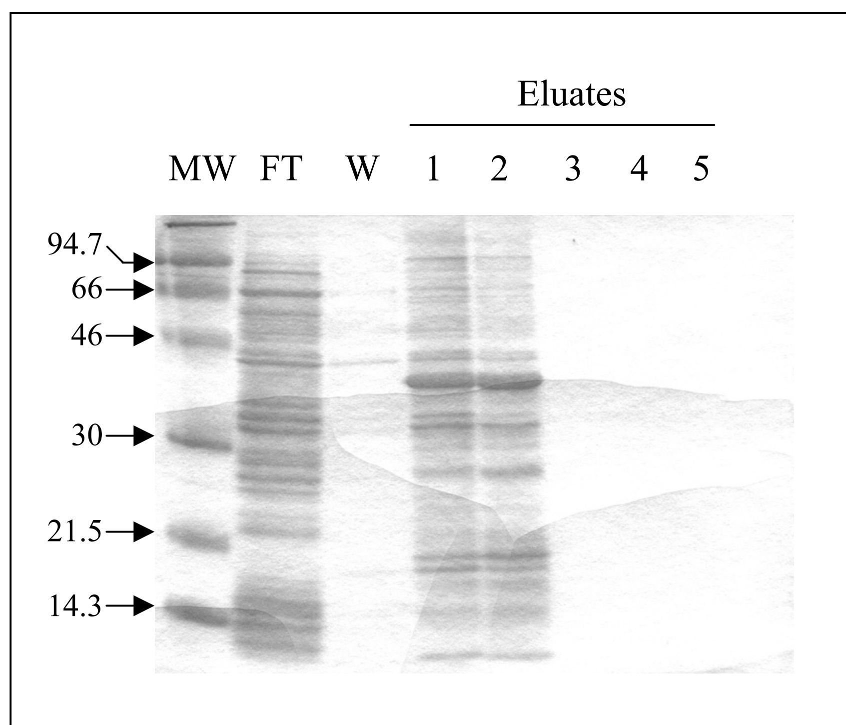

Fig. 13. SDS-PAGE – silver staining analysis of different fractions from a nickel column purification of His-mTERF. MW: molecular weight marker. FT: flowthrough. W: wash.

1.1.3) Electroelution of the protein from the gel slice

As a second step in the purification of His-tagged mTERF, the band corresponding to this protein was cut out from a Coomassie blue stained SDS-PAGE gel, and the protein was extracted from it by electroelution using a Elutrap® chamber (Schleicher & Schuell). This device consists of two chambers (A and B, B being much smaller) separated by a membrane permeable to proteins (BT2). Both chambers are closed on the outer edges by membranes impermeable to proteins (BT1). Therefore, when an electric current is allowed to flow through the Elutrap^{R}, the proteins contained in one chamber will pass to the other chamber and will be retained in it. Elutrap^{R} was placed inside a horizontal electrophoresis cuvette, and both filled with elution buffer (15 mM NH_4HCO_3, 0.1% SDS, pH 8.2). The gel slice was pre-equilibrated in elution buffer for 2-5 min. and then placed in chamber A of the Elutrap® close to BT2 membrane. Electoelution was allowed to proceed at 200 V for 16 hours at room temperature. Before collecting the sample from chamber B, polarity was inverted for 30 sec. to detach any proteins from the BT1 membrane. After

electroelution, the sample was precipitated with 3 volumes of aceton, and then resuspended in PBS/0.1% SDS. The protein was now ready for injection to the rabbit.

1.2) Immunizations and bleedings of the rabbit

The immunization of the rabbit was achieved following the schedule below:

Week 0: 10 ml of blood were extracted from the rabbit prior to the immunization. After formation of the clot, the blood sample was kept at 4°C overnight, in order to allow the clot to shrink. The next day, the serum was removed and centrifuged at 10,000xg for 10 min. at 4°C, aliquoted and stored at –40°C. This was thereafter referred to as pre-immune serum (also A3-1)

While the blood sample was being processed, 400 µg of pure His-tagged mTERF (see Material and Methods-2) dissolved in 0.6 ml PBS/0.1% SDS were mixed with one volume of Complete Freund's adjuvant (Sigma) and injected subcutaneously to the rabbit in ten different sites.

Week 3: 10 ml of blood were extracted from the rabbit, and serum from it was obtained and processed in the same way as described in week 0. This serum was labeled A3-2.

A first boost of 200 µg pure His-tagged mTERF dissolved in PBS/0.1% was mixed with one volume Incomplete Freund's Adjuvant (Sigma) and injected in the same way as described in week 0.

Week 6: 30 ml blood were obtained and processed as described (A3-3).

Week 7: 10 ml bleeding (serum labeled as A3-4) and a second boost of 200 µg of pure protein was injected as described in week 3.

Week 11: 30 ml bleeding (serum labeled as A3-5). Third boost performed in the same way as week 7.

Week 15: same as week 11 (serum labeled A3-6).

Week 20: terminal bleed by cardiac puncture was performed with the rabbit under general anesthesia. 150 ml blood were collected, and the serum obtained from it was labeled as A3-7).

1.3) Testing the efficiency of immunization by Enzyme-Linked-Immunosorbent-Assay (ELISA).

The ELISA test was performed in 96-well Falcon plates, according to the following schedule:

1) Coat the wells needed with 200 µl of His-tagged mTERF (final concentration: 0.001 mg/L) diluted in PBS, pH 6.0 (8g NaCl, 0.2g KCl, 1.44g Na_2PO_4, 0.24g KH_2PO_4. Adjust to pH 6.0 with HCl and bring the volume to 1 L with water). Allow coating to proceed for 2 hours at room temperature or overnight at 4^oC. Plates can be stored at 4^oC up to one week.

2) Wash the wells twice with PBS, pH 6.0.

3) Block extra binding sites with 0.1% ovalbumin, 0.1% Tween-20 in PBS, pH 7.5 for 2 h. at room temperature.

4) Wash six times with 0.1% Tween-20 in PBS, pH 7.5

5) Add 50 µl of serum diluted in PBS/0.1% Tween-20, pH7.5. The following dilutions are analyzed for each serum tested: 1/10, 1/100, 1/1,000, 1/5,000, 1/10,000, 1/50,000, 1/100,000, 1/500,000 and 1/1,000,000. All serums are tested in duplicate. Incubate for 2 h. at room temperature.

6) Wash four times with PBS/0.1% Tween-20, pH 7.5.

7) Add 50 µl diluted peroxidase conjugated anti-rabbit IgG (H+L), affinity purified from goat (Boehringer Mannheim). Dilution: 1:3,000. Incubate for 1-2 h.

8) Wash six times with PBS/0.1% Tween-20, pH 7.5.

9) Add 150 µl substrate solution (0.04% OPD, 25 ml, 0.015% H_2O_2, 0.1 M Na_2HPO_4, 35 mM citric acid. Prepare fresh daily). Incubate in the dark for 30 min.

10) Stop the reaction with 50 µl 3M H_2SO_4 solution.

11) Read OD_{490} immediately using a microplate reader (Bio-Rad, model 450).

2- Purification of mTERF from HeLa cells

2.1) Preparation of S-100 from HeLa cell mitochondria (from Kruse *et al*, 1995)

S-100 is the primary source of mTERF from HeLa cells, and to obtain it requires the lysis of the cells, purification of mitochondrial fraction, lysis of mitochondria and high speed centrifugation of the mitochondrial lysate.

For each preparation, 3 liters of HeLa suspension cultures of the S3 clonal strain were grown in modified Eagle's medium* supplemented with 5% calf serum to late exponential phase at 37°C. All steps from the collection of the cells onwards were carried out on ice or at 4°C, unless otherwise specified. All solutions were sterilized and precooled at 4°C.

Suspension cultures were divided in 1 L bottles and centrifuged at 280xg for 9 min. After disposing of the supernatants, cells in each bottle were resuspended in 30 ml 1xNKM buffer (130 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4), transferred to a 250 ml centrifuge bottle and centrifuged at 250xg for 9 min. The pellet was resuspended in 30 ml 1xNKM buffer, transferred to two 50 ml polycarbonate graduated tubes and centrifuged at 370xg for 7min. After the spin, the volume of packed cells was determined, and an extra wash with 1xNKM was carried out in the same tubes. At this point, cells were divided into a number of polycarbonate graduated tubes containing 4-6 ml packed cells each. The lysis of the cells was performed in one tube at a time as follows: each cell pellet was quickly resuspended in 6 volumes of Special 1xRSB buffer (10 mM KCl, 0.15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4), transferred to a Thomas homogenizer (size C) and incubated for 2 min. on ice. Cells were then broken with 6 strong strokes with a motor-driven pestle set to

* Composition of modified Eagle's medium (for 20 L): 168.2 g deficient DME-High phosphate, from Irvine Scientifics, 90 g glucose, 28 g $NaH_2PO_4 \cdot H_2O$, 74 g $NaHCO_3$, 2.1 g L-isoleucine, 0.6 g L-methionine, 6.4 g penicillin, 20 g streptomycin, 1.4 g kanamycin, 20 ml antimycotic solution (0.2 g n-butyl-p-hydroxybenzoate to 1 liter of water. Autoclave).

rotate at 1600 rpm. Breakage was monitored under a phase-contrast microscope and considered optimal when 60-70% cells were broken. Then the homogenate was immediately transferred to a 100 ml Erlenmeyer flask containing 1 volume of 2M sucrose, vigorously mixed and poured into a 50 ml centrifuge tube. After spinning at 1,160xg for 3 min. to sediment nuclei, unbroken cells and large cytoplasmic debris, the supernatants were collected and poured into another 50 ml polycarbonate tube, leaving a 2-3 mm layer above the pellet to prevent contamination. This operation was repeated a second time, after which the supernatants were transferred to 50 ml round-bottom Nalgene polyethylene centrifuge tubes and centrifuged at 6500xg for 10 min. The pellets were then thoroughly resuspended in 10 ml 1x suspension buffer (0.25 M sucrose, 10 mM KCl, 0.15 mM $MgCl_2$, 10 mM Tris-HCl, pH 6.7) using a Pasteur pipette and each transferred to a 50 ml polycarbonate tube and spun at 1,000xg for 3 min. The supernatants were each poured into a 50 ml round-bottom Nalgene polyethylene tube and centrifuged at 5000xg for 10 min. The pellet now contains the bulk of mitochondria, as well as elements of the rough endoplasmic reticulum and smooth membrane components. Lysis of mitochondria started by resuspending the mitochondrial pellet in ¼ volume of total packed cells of lysis buffer (10% glycerol, 25 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiotreitol, 1mM phenylmethylsulfonyl fluoride) and homogenizing with 5 light strokes in a Thomas homogenizer (size B, rotation speed of pestle: 1,600 rpm). Immediately, Tween-20 was added to a final concentration of 0.5% and 5 more light strokes were carried out. Then KCl was added to a final concentration of 0.5 M, followed by 10 light strokes and a 10 min. incubation on ice, after which the sample was transferred to a ultracentrifuge tube and centrifuged at 100,000xg for 1 hour. After ultracentrifugation, the supernatant (S-100) was collected after discarding the lipid layer on top, aliquoted, flash-freezed in liquid nitrogen and stored at $-80^{o}C$. Protein concentration was measured following the protocol described in Material and Methods-11.

2.2) Preparation of DNA affinity column (from Kadonaga and Tjian, 1986)

DNA affinity column preparation requires the purification, annealing, 5'-end phosphorylation and ligation of two partially complemetary oligonucleotides, followed by the coupling of the ligation product to Sepharose. Two 44-bases long oligonucleotides corresponding to complementary sequences of the H and L strand of human mtDNA in the 16S rRNA/tRNA$^{Leu(UUR)}$ boundary region were used for this purpose:

5'-AGAACAGGGTTTGTTAAGATGGCAGAGCCCGGTAATCGCATAAA-3' L-strand

3'-GTATTTTCTTGTCCCAAACAATTCTACCGTCTCGGGCCATTAGC–5' H-strand

These oligonucleotides contain the binding site of mTERF to mtDNA, and when annealed, present complementary 3'-end protruding hexanucleotide stretches, thus allowing the ligation of multiple units.

Optimum ligation required the purification of the oligonucleotides by running 150 µg of each on a 2mm thick, 30 cm long, 10% acrylamide/7M urea gel, visualizing the DNA on the gel over a Eastman TLC plate 13254 with short UV light, cutting the bands from the gel and eluting them as described in Material and Methods-5.

After purification, 220 µg of each oligonucleotide were mixed and precipitated in the presence of 0.2 M NaCl and 2.5 volumes of ethanol at –20°C for 1 h. After centrifugation at 12,000xg for 30 min, the pellets were resuspended in 78 µl TE (10 mM Tris, 1 mM EDTA), pH 7.6, added to 12 µl 10x PNK buffer containing 100 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 10mM DTT, 2 mM spermidine and 2 mM EDTA, and allowed to anneal by incubating at 88°C for 2 min, 65°C for 10 min, 37°C for 10 min and room temperature for 5 min.

After annealing, the 5' ends of each strand were phosphorylated by adding 3.6 µl 100 mM ATP, pH 6.8, 3.6 µl 100 mM DTT, 2.5 µl γ-^{32}P-ATP (2 mCi/ml), 12 µl T4 polynucleotide kinase (NEB, 10U/microliter) and 8.3 µl of water, and the mixture was incubated at 37°C for 2 h. The reaction was stopped by adding 60 µl 10M NH_4Oac, pH 5.5, and 120 µl of water, and heating at 65°C for 15 min. After taking an aliquot to test ^{32}P incorporation (see Material and Methods-10), the 5'-end phosphorylated,

double stranded DNA was precipitated by adding 2.5 volumes of ethanol and incubating for 1h at –20°C. After 30 min centrifugation at 12,000xg, the pellets were resuspended in 225 µl TE, pH 7.6, and DNA was extracted by adding 250 µl phenol/chloroform/isoamylalcohol (25:24:1), vortexing for 1 min and spinning at 12,000xg for 5 min, followed by a second extraction of the upper layer with Chloroform/isoamylalcohol (24/1) to remove traces of phenol. At this point, DNA was ethanol-precipitated by adding 25 µl 3M NaOAc pH 5.5 and 3 volumes ethanol, and incubating at –20°C for 1 h. After spinning at 12,000xg for 30 min., the pellets were washed with 800 µl 75% ethanol, spun briefly and allowed to dry.

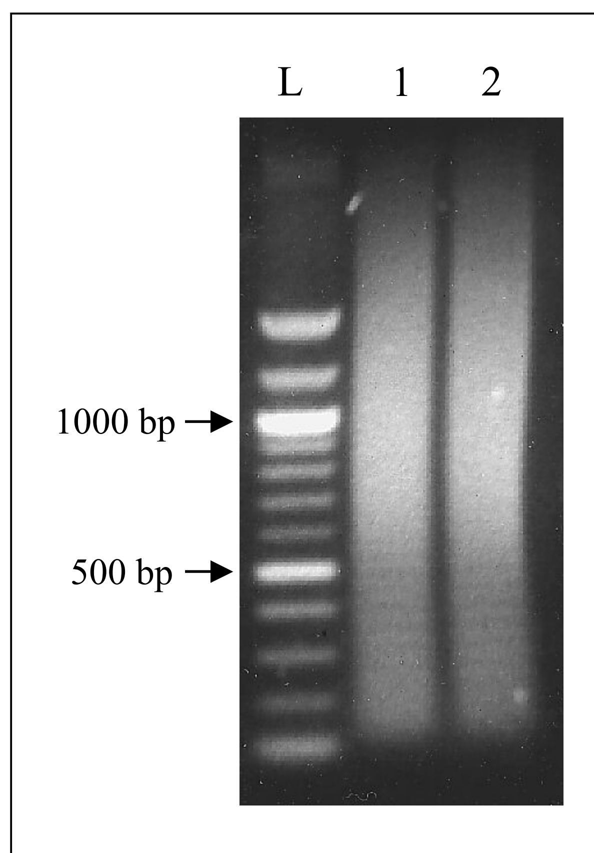

Fig. 14. Example of the expected products of a ligation reaction run on a 0.8% agarose gel. L: DNA ladder. 1 and 2 are samples of two ligation reaction mixtures.

The ligation was performed after resuspending the previous pellet in 65 µl of water and adding 10 µl 10x linker-kinase buffer (660 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 10 mM spermidine), 8 µl 100 mM ATP, pH 6.8, 2 µl 200 mM DTT and 15 µl T4 DNA ligase (NEB, 90 Weiss units), and subsequent incubation at 16°C overnight. The ligated product was extracted with one volume of phenol-chloroform-

isoamylalcohol (25:24:1) and one volume of chloroform-isoamylalcohol (24:1) as described above. The DNA was precipitated with 33 µl 10 M ammonium acetate and 133 µl 2-propanol and incubated at –20°C for 1 h. After 30 min. at 12,000xg, the pellet was dissolved in 225 µl of TE, pH 7.6, and the DNA was reprecipitated with 25 µl sodium acetate and 750 µl ethanol. After 1 hour at –20°C and 30 min. at 12,000xg, the final pellet was washed with 75% ethanol, allowed to dry, dissolved in 50 µl of water and stored at –20°C. In order to assess the efficiency of ligation, an aliquot was run in a 0.8% agarose gel in parallel with size markers (see Material and Methods-7). The ligation products should cover a size range up to more than 2,000 bp, with the majority being between 400 and 1,000 bp in size (see figure 14).

Coupling of the ligated product to Sepharose required the following steps:

a) activation of Sepharose:

-Wash 15 ml Sepharose CL-2B (Pharmacia) in 500 ml water for 15 min. under constant stirring. Allow to sediment.

-Suspend in water to give a 20 ml slurry. Pour into a 50 ml polypropylene tube (Corning) and equilibrate to 15°C in a water bath.

-Dissolve 1.1 g CnBr in 2 ml N,N-dimethylformamide and add dropwise over 1 min to the Sepharose under constant stirring. CnBr is extremely toxic and should be handled in a fume hood.

-Add 1.8 ml 5m NaOH dropwise over 10 min. pH should not exceed 10. Stop by adding 100 ml ice-cold water, followed by gentle suction filtration (a coarse-sintered glass funnel was used for this purpose, and for all subsequent washes). Special care should be taken not to overdry the resin.

-Wash extensively in 300 ml ice-cold water (20 min, under magnetic stirring).

-Wash in 100 ml 10 mM potassium phosphate, pH 8.0.

b) Coupling

-Transfer 5 ml of resin to a 15 ml polypropylene screw-cap tube.

-Add 2 ml 10 mM potassium phosphate, pH 8.0 to give a thick slurry.

-Add the ligated DNA and incubate for 16 h at room temperature in a rotary shaker.

-Transfer the resin to a filter unit and wash twice with 100 ml water and once with 100 ml 1M ethanolamine-HCl, pH 8.0.

-Transfer the resin to a 15 ml polypropylene screw cap tube and add 1 M ethanolamine-HCl, pH 8.0 until the mixture is a smooth slurry. Then incubate on a rotating wheel at room temperature for 4-6 h.

-Wash the resin with:

-100 ml 10 mM potassium phosphate, pH 8.0

-100 ml 1M potassium phosphate, pH 8.0

-100 ml 1M KCl

-100 ml water

-100 ml column storage buffer (10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 10 mM EDTA, 0.02%.

-Store the resin at 4°C in one volume of storage buffer with 0.02% NaN_3.

The efficiency of coupling was determined by measuring the incorporation of ^{32}P into the resin, following the protocol described in Material and Methods-10 and comparing the signal with that measured in the ligated DNA prior to coupling. Average efficiencies were around 50%.

2.3) Heparin chromatography

A typical heparin chromatography for the purification of mTERF was carried out utilizing the S-100 fraction of the mitochondrial lysate prepared from 6 3-liter balloons, containing in total 200 mg protein, approximately. All steps were carried out in the cold room. The lysate was diluted with buffer A (25 mM HEPES-KOH, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 0.1 μM pepstatin A, 10% glycerol, 0.1% Tween 20) minus KCl, to bring the KCl concentration of the S-100 to 150 mM KCl. KCl concentration was determined with a Marksson conductimeter. The diluted S-100 was then applied onto a heparin-agarose (Sigma) column, previously equilibrated with buffer A, at a 0.5 ml/min flow rate, adjusting the ratio protein to resin to around 40 mg/ml bed volume. The column was then washed with 3 column volumes of buffer A, and bound components were sequentially eluted with a 3 column volumes step gradient of buffer A containing 0.3,

0.5 and 0.8 M KCl. 3.5 ml fractions were collected, and KCl concentration of each fraction determined by conductimetry. Those fractions with KCl concentration between 450 and 650 mM were pooled, adjusted to 20% glycerol, frozen under liquid nitrogen and stored at –80°C. Protein concentration of the pooled fractions was determined using the Bradford method (see Material and Methods-11).

2.4) DNA affinity chromatography

All the procedure was carried on in the cold room. The pool of fractions between 450 and 650 mM KCl obtained from the heparin chromatography were diluted with buffer B (25 mM HEPES-KOH, pH 7.8, 12.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 0.1 µM pepstatin A, 20% glycerol, 0.1% Tween 20) until conductivity reached a value corresponding to 175 mM KCl. After addition of poly(dI-dC)·(dI-dC)* (previously heated at 90°C for 5 min and allowed to cool at room temperature), to 8 µg/mg protein, the solution was incubated for 20 min. on ice and then applied, at a flow rate of 0.25 ml min, onto two columns containing in total a volume of DNA affinity resin such that the ratio mg protein/ml resin was kept between 40-50. The resin had been previously equilibrated with buffer B containing 150 mM KCl. The column was then washed with 10-15 column volumes of buffer B containing 150 mM KCl and bound proteins were eluted with 3 column volumes buffer B containing 1 M KCl by mixing the resin with the buffer and incubating for 10 min. This elution step was repeated two more times, and all the eluates were pooled and diluted with buffer B to a KCl concentration of 220 mM. After addition of pre-heated poly(dI-dC)·(dI-dC) to 2 µg/mg protein, the sample was incubated for 15 min. on ice and reapplied onto one of the previously used columns, preequilibrated with buffer B containing 200 mM KCl. The column was then washed with 10-15 volumes of the same buffer and bound proteins were eluted with 1 column volume of buffer B containing 0.35, 0.5, 0.8 and 1 M KCl. Each step of the gradient involved mixing and 15 min. incubation.

Fractions were flash-frozen in liquid nitrogen and stored at –80°C. 20 µl fractions were kept apart in order to test the efficiency of purification by SDS-PAGE

* From Amersham Pharmacia Biotech.

(see Material and Methods-6). A typical example of mTERF purification is shown in figure 15.

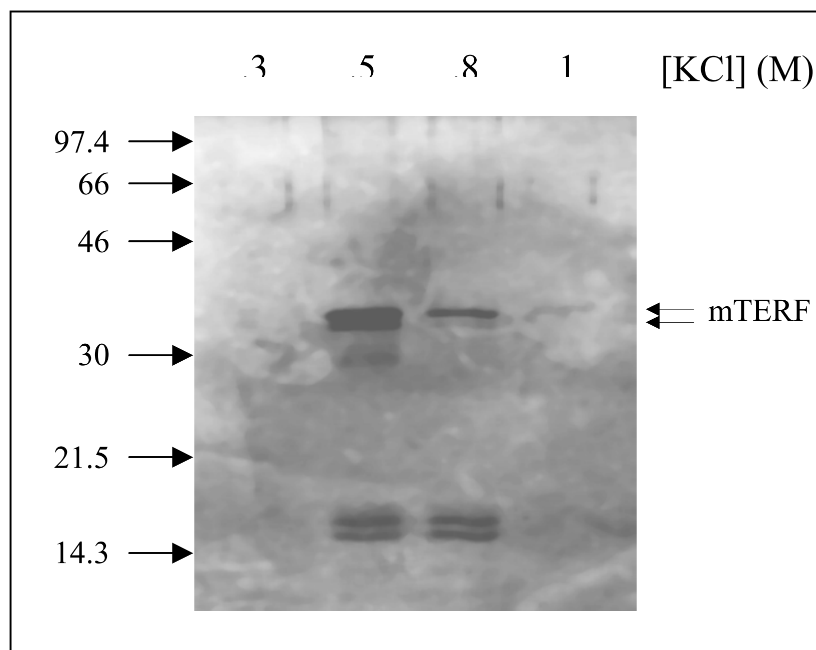

Fig. 15. SDS-PAGE – silver staining analysis of eluates obtained from a DNA-affinity purification of mTERF from HeLa cells.

3) Gel filtration chromatography

Gel filtration chromatography of S-100 from mitochondrial lysate was carried out on a FPLC system (Pharmacia) in the cold room. The column used was a HiPrep^R 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 DTT, 10% glycerol, 0.1% Tween 20, with a KCl concentration that varied for each experiment from 500 mM to 150 mM) at a flow rate of 1 ml/min. A calibration curve was prepared by running, in two different runs, Blue Dextran 2000, cytochrome C (12Kda), ovalbumin (43 Kda), bovine serum albumin (67 Kda), aldolase (158 Kda) and catalase (232 Kda), all from Amersham Pharmacia. The elution of these markers was monitored by UV photometry (280 nm) with an AUFS (absorbance units full scale) of 0.05, 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} (see figure 16). K_{av} for each protein was calculated as follows:

$$K_{av} = Ve - Vo / Vt - Vo$$

where Ve = elution volume for the protein; Vo = column void volume = elution volume of Blue Dextran 2000; Vt = total bed volume (in the case of HiPrep^R Sephacryl S-200 is 120 ml). 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 S-100 were loaded into the column and the eluate collected in 1 ml fractions, flash freezed in liquid nitrogen and stored at -80^oC until further analysis.

Analysis of the fractions by immunoblotting using anti-mTERF antiserum allowed to determine the elution volume of mTERF, and its Kav was calculated as described above. Estimation of its molecular weight was achieved by interpolating its K_{av} in the calibration curve.

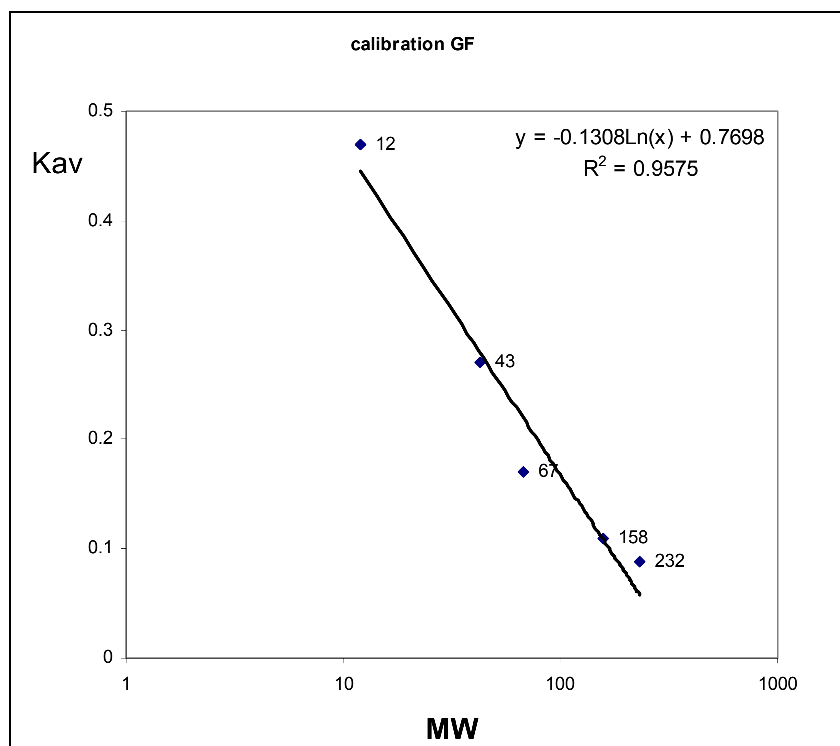

Fig 16. Calibration curve for HiPrep^R Sephacryl S-200 column (Pharmacia). The stardards used were: cytochrome C (12Kda), ovalbumin (43 Kda), bovine serum albumin (67 Kda), aldolase (158 Kda) and catalase (232 Kda)

In order to confirm the results obtained with HiPrep[R] Sephacryl S-200 column, and to improve the precision at high molecular weight level, S-100 were also fractionated in a Superdex 200 HR 10/30 column (Amersham Pharmacia). The same running buffer was used in this case, but the flow was adjusted to 0.5 ml/min, and the sample volume reduced to 0.5 ml.

4- Study of DNA binding activity: Band-Shift analysis

Band-shift experiments allow the detection of proteins in a sample with binding activity against a fragment of DNA used as a probe. After incubation of the sample with the ^{32}P-labelled probe, the mixture is run on a non-denaturing polyacrylamide gel and analyzed by autoradiography. Any DNA-binding activity will generate a shifted band, when compared with the free probe, due to the combined weights of the probe and the protein(s) bound to it.

4.1) 5'-end labelling of the probe

The probe used for band-shift assay was the same double stranded DNA used for mTERF affinity chromatography purification prior to the ligation step (see Material and Methods-2). Each strand is 44 bases long, and when annealed they produce hexameric overhangs. This DNA contains the binding site of mTERF at the 16S rRNA /tRNA$^{Leu(UUR)}$ boundary region.

The labeling reaction, consisting of 4 pmole oligo, 2.5 µl 10x kinase buffer (New England Biolabs), 1.5 µl T4 polynucleotide kinase (New England Biolabs, 10u/ul), 5 µl γ^{32}P-ATP (10mCi/ml, 3000 Ci/mmol) for a total volume of 25 µl, was incubated at 37°C for 15 min., heated at 65°C for 5 min. and precipitated by adding 2.5 µl 3M NaOAc, pH 5.5, 2 volumes of ethanol and 10 µg glycogen. After 2 hours at –20°C, the sample was centrifuged at 12,000xg for 30 min. and the pellets resuspended in 100 µl TE, pH 7.6. A second precipitation was carried out, and the probe was left precipitating at –20°C until the day of analysis. After 30 min. centrifugation at 12,000xg, the pellets were resuspended in 100 µl T0.1E, pH 7.6 (probe is now diluted to 4 pmole/100 µl). The working solution was a 10x dilution of the previous in buffer C (see below for composition).

4.2) Band-shift assay

A standard band-shift assay reaction mixture contained 0.5 µg Poly (dI-dC)·(dI-dC), previously heated at 90°C for 5 min, 5 µl of probe working solution, 0.5 µl bovine serum albumin (New England Biolabs, 100x), a variable volume of sample, and buffer C (25 mM HEPES, pH 7.5, 50 mM KCl, 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.1% Tween 20) to a final volume of 25 µl. The reaction was incubated at room temperature for 20 min., placed on ice and immediately loaded onto a 10% polyacrylamide midi-gel (running buffer, 5x: 0.25 M TrisBase, 1.9 M glycine, 10 mM EDTA, pH 8.5. Gel composition: 9.8% acrylamide, 0.25% bisacrylamide, 5% glycerol, 10 ml 5x running buffer and water to 50 ml. Polymerization was induced by the addition of 250 µl 10% ammonium persulfate and 80 µl TEMED and carried out overnight in the cold room). The gel was pre-run for more than an hour at 200 V in the cold room, and the actual run was carried out at 300 V, also in the cold room. Bromophenol blue (BPB) solution was loaded in one well to follow the run, and when BPB reached ½ of the length of the gel, electrophoresis was stopped, the gel dried and analyzed by autoradiography.

For some experiments, especially those involving a subsequent immunoblotting of the shifted bands, the reaction volume was upscaled to 100 µl.

4.3) Super-shift assay using anti-mTERF antiserum

As an approach to identify which shifted band(s) contained mTERF, band-shift experiments like the one described above were carried out, except for the fact that 1 µl anti-mTERF antiserum was included in the reaction mixture. A control adding 1 µl pre-immune serum was run in parallel. Those bands containing mTERF, already migrating slower than the free probe, were expected to shift upwards, when run in a 10% polyacrylamide gel, due to the added weight of the antibody, or to disappear, due to the inhibition of DNA-binding activity of mTERF caused by the interaction with the antibody.

5) Transcription termination activity test (from Micol *et al*, 1996)

mTERF transcription termination activity was determined using an *in vitro* transcription system, with S-100 of a mitochondrial lysate as a source for the transcription machinery, and pTER, a DNA construct containing the promoter regions for both mtDNA strands, as well as the mTERF binding site (see fig. 17a), as a template. Addition of ^{32}P-labelled UTP to the *in vitro* transcription reaction allowed us to identify the RNA species newly synthesized, after running the transcription products on a denaturing gel followed by autoradiography.

5.1) Preparation of DNA template (pTER)

A glycerol stock of TOP10F' bacterial strain transformed with a pTER-inserted pCRII plasmid was used as a starting material for the obtention of pTER.

Bacteria were streaked out on a LB-Ampicillin plate* after scraping the surface of the frozen glycerol stock with a loop and incubated overnight at 37°C. The next day, a single colony was picked up, inoculated into 4 ml LB-ampicillin medium* and incubated for 8 hours at 37°C with vigorous shaking. 0.2 ml of this culture were added to 100 ml LB-ampicillin medium and incubated overnight at 37°C with vigorous shaking. Bacteria were harvested by centrifugation at 6,000xg for 15 min. at 4°C, and the bacterial pellet was thoroughly resuspended in 10 ml buffer P1 (from Quiagen Maxi-Prep Plasmid Purification Kit, like all buffers used hereafter for the purification of pTER). This buffer contains Rnase A. Then 10 ml buffer P2 (bacteria lysis buffer) were added and mixed by inverting 4-6 times, and the mix was incubated at room temperature for 5 min. After the lysis of bacteria, 10 ml chilled buffer P3 were added to the lysate, mixed immediately but gently by inverting 4-6 times and the mix was incubated on ice for 20 min. This step promoted the precipitation of genomic DNA, proteins and cell debris. These components were pelleted by centrifuging at 20,000xg for 30 min. at 4°C in a polypropylene tube. The supernatant, containing plasmid DNA, was then carefully removed, re-centrifuged at 20,000xg for 15 min. at

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* Composition of LB medium: 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl in 1 liter of water, pH 7.0. Sterilize by autoclaving for 20 min. LB plates where prepared by adding 15 g/liter bacto-agar to LB medium just before autoclaving. Ampicillin was added after autoclaving, letting the solutions cool down to 50°C, to a final concentration of 50 µg/ml.

4°C and applied to a Qiagen-tip 500 column pre-equilibrated with 10 ml buffer QBT, allowing the sample to flow by gravity. The resin was then washed twice with 30 ml buffer QC (gravity flow) and the DNA was then eluted with 15 ml buffer QF. The eluate was collected in a 30 ml Corex® glass tube, and the DNA was precipitated by adding 0.7 volumes room-temperature isopropanol, mixing and centrifuging immediately at 15,000xg for 30 min. at 4°C. The supernatant was then carefully decanted, and the pellet was washed with 5 ml room temperature 70% ethanol and centrifuged at 15,000xg for 10 min. After decanting the supernatant, the pellets, containing the purified plasmid, were air-dried for 5-10 min. and redissolved in 250 µl TE, pH 8.5. The concentration of DNA was determined by measuring OD_{260}, assuming that 1 unit OD_{260} corresponds to 50 µg/ml plasmid (according to Sambrook *et al*, 1989). DNA purity was estimated by determining the ratio OD_{260}/OD_{280}. A ratio of 1.8 or higher was considered optimum.

The release of pTER from the purified vector required the sequential digestion of the plasmid with HindIII and EcoRI (both from New England Biolabs). Buffer 2 from NEB was used for HindIII digestion, and EcoRI buffer (NEB) for EcoRI digestion. Reaction volumes varied from experiment to experiment, but DNA concentration was always kept at 0.2 µg/µl, and the amount of enzyme was adjusted to the amount of DNA and the incubation time. Both digestions were carried out in a 37°C water bath. After the reaction with HindIII, NaCl to 0.2 M and 3 volumes of ethanol were added, and the DNA was allowed to precipitate at –20°C for 1 hour. After 30 min. centrifugation at 12,000xg, the pellets were resuspended in TE, pH 8.5 and an aliquot was run on a 0.8% agarose mini-gel (see Material and Methods-7) to test the efficiency of the digestion. If optimum, the sample was digested with EcoRI as described, and a second test performed in the same conditions, this time to test the efficiency of EcoRI. The sample was then reprecipitated as described above, resuspended in TE, pH 8.5, DNA concentration determined spectrophotometrically as described, aliquoted and stored at –20°C.

In order to purify the insert from the plasmid, several aliquots containing 50 µg DNA in total were thawed and run on a 0.8 % agarose mini-gel without EtBr, dividing the sample into 4 wells. After the run, the gel was submerged in a solution of 0.02% methylene blue for 15 min. under constant agitation, and destained in water for 1 hour, under constant agitation and with several water changes. Methylene blue staining allowed to visualize pTER and distinguish it from the plasmid DNA, the

latter one migrating clearly above pTER. The fragment of agarose containing pTER was excised from the gel and extracted using QIAEX II, a kit from Qiagen. Briefly, the gel slice was weighted, and 3 volumes of buffer QX1 and 30 µl of QIAEX II resin added (if the amount of DNA was higher than 10 µg, 30 extra µl were added for each additional 10 µg of DNA). The mixture was incubated at 50°C for 10 min. to solubilize the agarose and to bind the DNA to the resin. Vortexing every 2 min was required to keep the QIAEX II resin in suspension. Samples were then centrifuged for 30 sec. at 12,000xg, supernatants carefully removed and the pellets were washed with 500 µl buffer QX1, followed by two washes with 500 µl buffer PE. The pellet was air-dried for 30 min, and the elution of DNA was carried out by vortexing the pellet in 20 µl of 10 mM Tris-HCl, pH 8.5, centrifuging for 30 sec. at 12,000xg and recovering the supernatant. A second elution in the same conditions might be added to increase DNA recovery. DNA concentration was determined by spectrophotometry as described above, and samples were used as they were or, if too diluted, ethanol precipitated and resuspended in a suitable volume of TE, pH 8.5.

5.2) *In vitro* transcription assay

All solutions were prepared with DEPC-treated water and sterilized by autoclaving or by filtration through a 20 µm filter when autoclaving was not possible. DEPC-treated water was obtained by adding 0.01% DEPC (diethyl pyrocarbonate, Sigma) to 1 liter of water, stirring for 1 hour and autoclaving for 20 min.

A scheme of this technique is depicted in figure 17a.

The standard transcription assay consisted of 10 µl 5x transcription buffer (50 mM Tris-HCl, pH 8.1, 50 mM $MgCl_2$, 5 mM EDTA, 50% glycerol), 5 µl 10 mM DTT, 1 µl bovine serum albumin (5 mg/ml), 2.5 µl 10 mM ATP, 5 µl 1 mM CTP, 5 µl 1 mM GTP, 5 µl 0.1 mM UTP, 1-2 µl α-^{32}P-UTP (400 Ci/mmol, 10 mCi/ml, Amersham Life Science), 0.5 µg of template DNA (pTER), 5 µl S-100 from mitochondrial lysate, 40 units RNasin (Promega) and DEPC-treated water to a final volume of 50 µl. The reaction mix was incubated at 30°C for 30 min, and the reaction was stopped by the addition of one volume of transcription stop buffer (10 mM Tris-HCl, pH 8.1, 0.15 M NaCl, 20 mM EDTA, 0.5% SDS) plus 5 µg of yeast tRNA. RNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated by adding 3 volumes of ethanol and 2.5 µl 4M NaCl and keeping at –

20^oC for 1 hour. After 30 min centrifugation at 12,000xg, the pellets were dissolved in 100 µl ice-cold DEPC-treated water and RNA was reprecipitated by adding 5 µl 4M NaCl and 300 µl ethanol and keeping the mixture at -20^oC for 1 hour. After centrifuging at 12,000xg for 30 min, the pellets were dissolved in 90 µl DEPC-treated water and 10 µl of those were mixed with one volume of urea dye (7M urea, 0.01% bromophenol blue, 0.01 xylene cyanol in TBE; see below for TBE composition), heated for 10 min. at 80^oC and loaded into a 20 cm long, 1 mm thick 5% polyacrylamide/7M urea gel. To prepare this gel, 21 g urea were dissolved in 6.25 ml 40% acrylamide/bisacrylamyde solution (19:1), 5 ml 10x TBE (0.89 M Tris, 0.89 M boric acid, 0.25 M EDTA, pH 8.3) and water to a final volume of 50 ml. When the urea was dissolved, the gel was filtered through a Whatman #1 filter to remove solid particles, 100 µl TEMED and 250 µl 10% ammonium persulphate were added, poured and allowed to polymerize at room temperature for at least three hours. After polymerization, the gel was pre-run for 1 hour at 20 v/cm. Running buffer was 1x TBE . The samples were loaded next to a 5'-end ^{32}P-labelled DNA molecular weight marker (pBR322/MspI, from New England Biolabs) and run at 20 v/cm until the dye had entered the gel. Then the voltage was raised to 37.5 v/cm. After the run, the gel was rinsed in water for 15-20 min, dried on a gel drier under vacuum at 80^oC for one hour and analyzed by autoradiography.

5.3) S1 protection assay

Although the *in vitro* transcription assay described above is by itself a good method to study transcription initiation from both promoters as well as mTERF-mediated transcription termination, this approach was usually followed by an S1 protection assay. The latter not only helps confirm the results obtained with the transcription assay, but also allows the identification of the transcripts. It consists in incubating the ^{32}P-labeled transcripts generated in the *in vitro* transcription assay with an RNA probe that will hybridize with both the H-strand runoff and the termination transcripts. Subsequent digestion of all single stranded RNA with S1 nuclease will leave only two species of double stranded RNA of different size, one for each transcript (see fig. 17b).

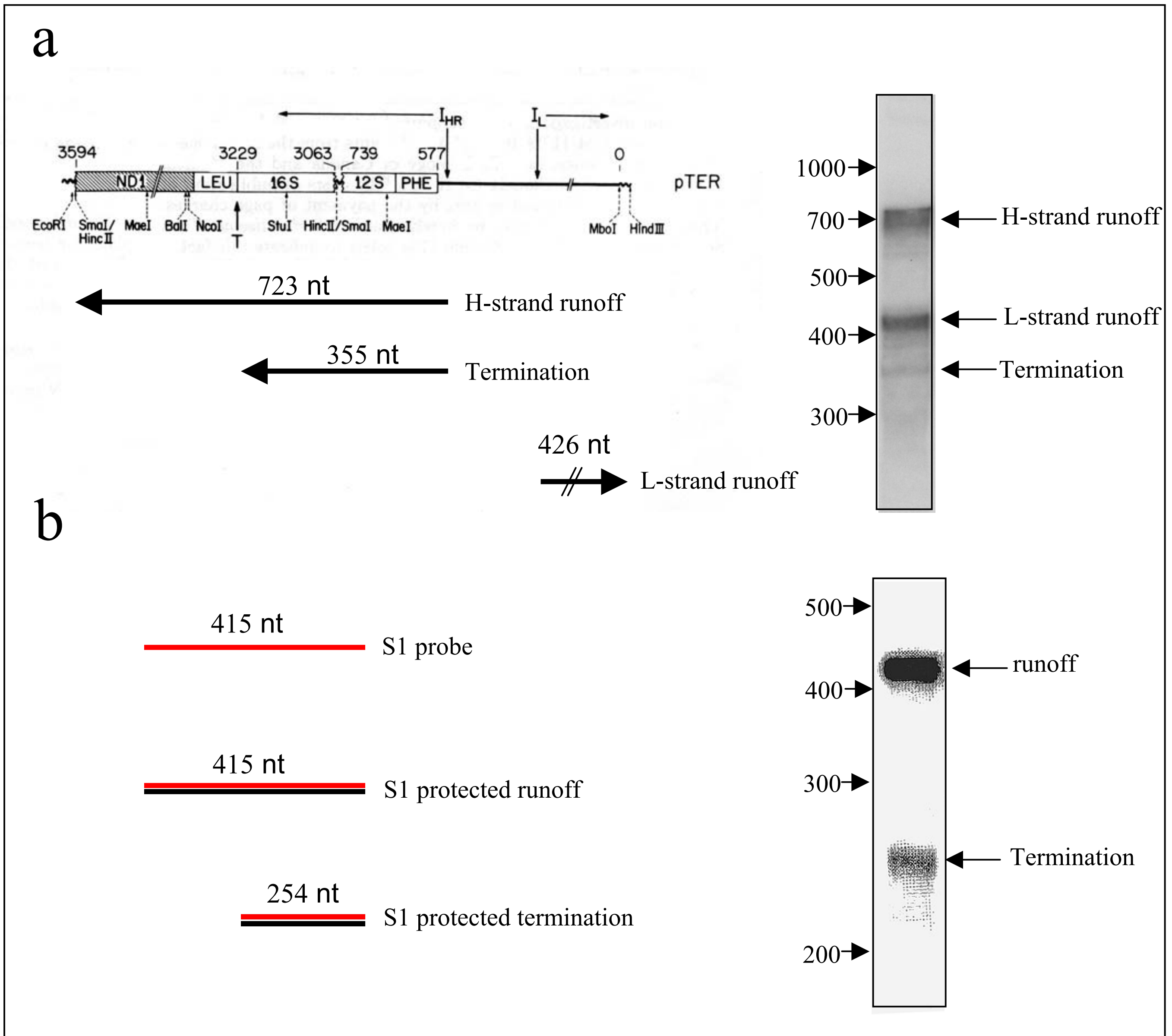


Fig. 17. Determination of mTERF termination activity. (a) scheme of the *in vitro* transcription assay. The figure shows the map of the clone pTER used as DNA template and the map positions of the *in vitro* transcripts. (b) scheme of the S1 protection assay, showing the map position of the MaeI-MaeI probe used in this assay, as well as the map position of the protected products. The right part of both panels show examples of autoradiographies practiced on 5% acrylamide/7M urea gels with products of in vitro transcription (a) and S1 protection (b) reactions.

5.3.1) Preparation of S1 RNA probe

The plasmid pBSVM, containing the MaeI-MaeI fragment of pTER (see fig. 17b) filled in and cloned into pBS KS(+) vector (Promega) and hosted in JM 109 Amp^R cells was amplified and purified in the same way as pTER (see Material and

Methods-5.1). After linearization with BamHI*, 1.5 µg plasmid were added to a reaction mixture containing 10 µl of 5x T3 transcription buffer (200 mM Tris-HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 2.5 mM each of ATP, CTP, GTP and UTP, 8 mM DTT, 40 units RNasin (Promega) and 30 units T3 RNA polymerase (Promega), with a final volume of 50 µl. The mixture was incubated at 37°C for 90 min and then 20 units RNase-free DNase I (Boehringer Mannheim) were added and the mixture was incubated for an additional 15 min at 37°C to destroy the template DNA. After phenol extraction, the synthesized RNA was precipitated at -20 ^{o}C overnight after adding sodium acetate to a final concentration of 0.3 M and 2 volumes ethanol and then recovered by centrifugation at 13,000xg for 30 min and resuspended in 20 µl DEPC-H_2O. The RNA probe concentration was determined by running an aliquot on a 10% acrylamide/7M urea gel and comparing the intensity of the band under UV light to that of molecular weight standards of known concentration (100bp, New England Biolabs).

5.3.2) S1 protection assay

The remaining 80 µl from the in vitro transcription reaction were mixed with 20 µl 5x DNase I buffer (100 mM Tris-HCl, pH 7.6, 50 mM $CaCl_2$, 50 mM $MgCl_2$) and 10 units RNase-free DNase I and incubated at room temperature for 20 min. After phenol extraction and ethanol precipitation in the presence of 0.2 M NaCl, the labeled transcripts were resuspended in 25 µl DEPC-treated water and mixed with 0.4 µg S1 RNA probe and precipitated again in the same conditions. The new pellet was resuspended carefully in 20 µl S1 hybridization buffer (80% deionized formamide, 40 mM PIPES-HCl, pH 6.4, 380 mM NaCl, 0.5 mM EDTA) by pipetting up and down repeatedly. After heating for 10 min at 80 ^{o}C to denature the sample, hybridization was performed at 50 ^{o}C for 8 hours. After hybridization, 200 µl S1 digestion buffer (40 mM sodium acetate, 3 mM $ZnCl_2$, 250 mM NaCl, pH adjusted to 4.6 with HCl) containing 20 µg/ml denatured salmon sperm DNA (Sigma) and 400 units S1

* BamHI digestion was carried out following the same strategy as Eco RI and Hind III digestions described in Material and Methods-5.1, using BamHI buffer from New England Biolabs. Special care was needed for the phenol extraction that followed the digestion, since the presence of traces of phenol inhibited the T3 RNA polymerase. For this reason, a first extraction with 1 volume phenol/chloroform/isoamylalcohol was followed by a second extraction in 1 volume chloroform/isoamylalcohol.

nuclease (Boehringer Mannheim) were added to the sample, and the mixture was incubated at 41 ^{o}C for 30 min. The reaction was stopped by adding 55 µl S1 stop buffer (4M ammonium acetate, 20 mM EDTA, 200 µg/ml yeast tRNA) and the S1-resistant products were precipitated by adding 2 volumes of ethanol. After overnight incubation at -20 ^{o}C, the samples were precipitated for 30 min at 13,000xg and the pellets resuspended in 15 µl DEPC-treated water, mixed with one volume of urea-dye (see Material and Methods-5.2 for composition), heated at 70°C for 5 min, then kept on ice for 5 min and immediately loaded on a 5% acrylamide/7M urea gel (see Material and Methods-5.2 for composition) and run for 2-3 hours at 400 V. After electrophoresis, the gel was washed twice in distilled water for 10 min, vacuum dried for 1 h at 80 ^{o}C and exposed for autoradiography (see fig. 17b for a scheme of the procedure).

6) SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE, from Garfin, 1990)

6.1) General procedure

SDS-PAGE was routinely used throughout this project to separate and analyze mixtures of proteins contained in a sample. Two sizes of gels were used: mini-gels, 8.5 cm long, 1 mm thick, and midi-gels, 20 cm long, 1 mm thick. SDS-PAGE gels consist on two gels, a stacking gel at the top, with a low concentration of acrylamide, and a resolving gel, with higher acrylamide concentration, at the bottom. Proteins run fast in the stacking gel, and separation does not occur. As a consequence, proteins tend to concentrate at the interphase of the two gels, while real separation starts at the top of the resolving gel. This approach makes all proteins in the sample start their separating run from the same point, thus improving the resolution. These are the compositions of stacking and resolving gels:

Resolving gel:

- 12.5% acrylamide (ratio acrylamide:bisacrylamide: 32:1)
- 0.375 M Tris-HCl, pH 6.8
- 0.1 % SDS
- adjust with water to 6 ml (mini-gel) or 30 ml (midi-gel)

Stacking gel:

- 5% acrylamide (ratio acrylamide:bisacrylamide: 32:1)
- 0.125 M Tris-HCl, pH 8.8
- 0.1% SDS
- adjust with water to 2 ml (mini-gel) or 10 ml (midi-gel)

Resolving gel was poured first, after adding 30 µl 10% ammonium persulfate (APS) and 5 µl TEMED (mini-gel) or 150 µl APS and 25 µl TEMED (midi-gel). A layer of water was carefully laid on top of the gel until polymerization was complete. Then the water layer was removed, the stacking gel poured, after adding 20 µl APS plus 5 µl TEMED (mini-gel) or 100 µl APS plus 25 µl TEMED (midi-gel) and the comb positioned on top of the stacking gel. Polymerization was allowed to proceed for about an hour.

The composition of the running buffer was: 25 mM Tris Base, 192 mM glycine and 0.1% SDS. Before loading, the samples were mixed with SDS-loading buffer (5x: 0.125 M Tris-HCl, pH 8.8, 5% SDS, 10 mM EDTA, 42.5% glycerol, 0.05% bromophenol blue) and boiled for four minutes. Rainbow^{TM} coloured protein molecular weight markers (Amersham Life Science) were run in each gel.

After the run, proteins could be visualized by Coomasie blue staining of by silver staining.

6.2) Coomasie blue Staining

Staining was carried out by incubating the gel with gentle shaking for 30 min. at room temperature in a solution containing 0.1 % Coomasie Blue R-250, 40% methanol and 10% acetic acid. Gels were then destained for 1-3 hours in 40% methanol/10% cetic acid. Alternatively, SimplyBlue^{TM} SafeStain, from Invitrogen, was used. In this case, the gel was rinsed 3 times for 5 min. with water, immersed in SimplyBlue^{TM} SafeStain solution for 1 hour at room temperature with gentle shaking and destained in water for 1 to 3 hours.

6.3) Silver staining

The gel was fixed for 30 min. in 50% methanol, 10% acetic acid and 2.5% glycerol (it could be left overnight). After washing twice for 5 min. in 10% methanol, the gel was incubated for 5 min. in Farmer's reducing agent. This solution was prepared right before use by mixing its two components, solution A (5.0 g $K_3Fe(CN)_6$ in 250 ml water) and solution B (8.0 g $Na_2S_2O_3$ in 250 ml water). The gel was then washed exhaustively in water until the yellow background disappeared, and incubated in 0.1% $AgNO_3$ for 45 min. in the dark. After a brief wash in water, the gel was developed in 3% $NaCO_3$/0.02% formaldehyde with gentle shaking. When the intensity of the staining was judged sufficient, developing was stopped by immersing the gel in 1% acetic acid and equilibrated in 3% glycerol/10% methanol/5% acetic acid.

After staining, the gels were dried for 1 hour at 80°C on a Slab Gel Dryer model GD2000 (Hoefer Scientific Instruments) and/or scanned on a UMAX Astra 2200 scanner.

7) Electrophoresis on agarose for DNA analysis (Sambrook *et al*, 1989)

Many procedures throughout this project required separation of DNA on an agarose gel, for assessment of purity, efficiency of restriction enzyme digestion, estimation of molecular weight, or even as a purification step. For a standard

horizontal agarose mini-gel, 0.4 g agarose (Sigma) were mixed with 50 ml 1xTBE buffer (see Material and Methods-5 for composition) and the mix was heated on a microwave oven with occasional stirring until the agarose was completely dissolved. After letting the gel cool down for a few minutes, EtBr was added to a final concentration of 0.01% and the gel poured onto a Bio-Rad mini-sub DNA cell with the comb already in place, where it was allowed to solidify for at least 2 hours. To prevent inhalation of EtBr vapour, the gel casting was carried out in a fume hood. Occasionally, in those circumstances in which DNA damage by EtBr was to be avoided, this compound was omitted from the gel mix. After mixing with Ficoll dye (10x: 0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll [type 400] in water), the samples were loaded in the wells without further manipulation. 1xTBE was utilized as running buffer, and a typical run was carried out at 50 mA for 1-2 hours on a Bio-Rad electrophoresis apparatus. One or more lanes were reserved for molecular weight markers. The ones routinely used were 100 bp and 1 kb ladders, from New England Biolabs.

When EtBr was used, DNA could be directly visualized under UV light. When it was omitted, methylene blue staining (see Material and Methods-5) or direct visualization with short UV light reflected on a TLC plate (see Material and Methods-2) could be used as alternative methods.

8) Immunoblotting (adapted from Sambrook *et al*, 1989)

Following SDS-PAGE (see Material and Methods-6), the gel was released from the plates and the resolving gel was separated from the stacking gel and washed in water for 10 min. with gentle shaking, while the stacking gel was discarded. At the same time, a piece of Immun-Blot© PVDF membrane (Bio-Rad) of the same size as the resolving gel was immersed in 100% methanol for a few seconds and then equilibrated in transfer buffer (39 mM glycine, 48 mM TrisBase, 0.037% SDS, 20% methanol, pH ~8.3) for 5 min. Once the membrane was equilibrated, four pieces of Whatman #1 paper of the same size as the resolving gel were cut and pre-wetted in transfer buffer. The transfer sandwich was set by superimposing (from cathode to anode) two Whatman papers-resolving gel-membrane-two Whatman papers. The

sandwich was placed between two sponges and clamped inside the transfer cassette. The cassette was then submerged into the transfer cuvette filled with transfer buffer and transfer was allowed to proceed overnight at 30 V. The cassette, sponges and cuvette were parts from the Trans-Blot kit from Bio-Rad.

After the transfer step, the membrane was washed briefly in PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1 liter of water), and immediately incubated in blocking solution (5% non-fat dried milk, 0.02% NaN_3, 0.02% Tween-20 in PBS) for 2 hours at room temperature with gentle shaking (at this point, the gel was usually stained with Coomasie blue to test the transfer efficiency). The blocking solution was then discarded and replaced by new blocking solution containing the primary antibody (anti-mTERF antiserum obtained as described in Material and Methods-1) at a 1:1,000 dilution. Incubation with the primary antibody was carried out in the cold room for 4 hours with gentle agitation. The membrane was then washed five times, 10 min. each time, in PBS and once in Washing Solution (150 mM NaCl, 50 mM Tris-HCl, pH 7.5), all at room temperature, followed by incubation in secondary antibody solution (5% non-fat dried milk, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing Anti-Rabbit IgG (H+L) HRP conjugate (Promega) at a 1:20,000 dilution for 1 hour at room temperature, with gentle shaking. After incubation with the secondary antibody, the membrane was washed five times, 10 min. each time, in Washing Solution, and then the membrane was placed on a glass plate and SuperSignal®West Pico Substrate working solution (Pierce) was poured over the membrane and kept for 5 min. (SuperSignal®West Pico Substrate working solution is prepared by mixing equal volumes of Luminol/Enhancer solution and Stable Peroxide Solution. Both solutions are provided by Pierce. The working solution is stable for 24 h at room temperature). After removing the excess of substrate solution, the membrane was placed in a plastic membrane protector, placed against a film and exposed. Kodak-ECL film was used for this purpose. Exposure times ranged from a few seconds to a few minutes.

9) 2D electrophoresis

2D electrophoresis was used as a method to study the interaction of mTERF with other proteins, giving us not only the possibility to visualize them, but also

providing us with a first step for its purification and further identification. The first dimension is run in native conditions, so that protein complexes run as such, and the second dimension is run in denaturing conditions, thus allowing the separation of different components of a protein complex.

9.1) First dimension: Native PAGE (from "Gel Electrophoresis of proteins". Ed: Hames & Rickwood, 1981)

Considering the fact that we aim to identify mTERF and mTERF-binding proteins, and that the isoelectric point (IP) of mTERF is around 9, we decided to work in acidic conditions (in this way we make sure the protein is strongly positively charged) and to run the gel from anode to cathode, unlike SDS-PAGE, in which SDS confers negative charge to all proteins regardless of its IP.

Native PAGE consisted of two parts, a 3.75% acrylamide stacking gel and a 8% - 15% acrylamide gradient resolving gel. Resolving gel was cast first, in the cold room, using a model SG50 gradient maker from Hoefer Scientific Instruments. After the gel was poured, a layer of water was carefully laid on top, and polymerization was allowed to proceed at room temperature for 2-4 hours. Then the layer of water was removed, and the stacking gel was cast on top of the resolving gel, placing a comb on top and allowing to polymerize for an hour. This is the composition of resolving and stacking gels:

	Stacking	Resolving (8%)	Resolving (15%)
40% acrylamide	0.94 ml	3 ml	4.7 ml
Stacking buffer	2.5 ml	-	-
Resolving buffer	-	1.9 ml	1.6 ml
10% APS	250 µl	127 µl	100 µl
TEMED	15 µl	76 µl	60 µl
Final volume	10 ml	15.3 ml	12.7 ml

- Stacking buffer: acetic acid,-KOH (pH 6.8). 48 ml 1M KOH and 2.9 ml glacial acetic acid were mixed and volume brought to 100 ml with water.

- Resolving buffer: acetic acid-KOH (pH 4.3). 48 ml 1M KOH and 17.2 ml glacial acetic acid were mixed and volume brought to 100 ml with water.

Running buffer used was acetic acid-β-alanine, pH 4.5 (31.2 g β-alanine and 8 ml glacial acetic acid were dissolved in and made to 1 liter with water). 40% acrylamide solution used for the stacking and resolving gels was purchased from ICN Biomedical Inc (Liqui-Gel® 37.5:1)

The samples were mixed with one volume of 2x sample buffer (1/5 dilution of resolving gel buffer, 20% glycerol and 0.005% methyl green), centrifuged for 1 min. at 12,000xg and loaded. Occasionally, one well was reserved to run a High Molecular Weight Calibration Kit for native electrophoresis, from Amersham Pharmacia, containing thyroglobulin, ferritin, catalase, lactate dehydrogenase and albumin. The run took place in the cold room at 20 mA until the front reached the resolving gel. Then the amperage was increased to 30 mA, and the electrophoresis was allowed to proceed for times that ranged from 5 to 10 hours.

After the run, the gel could be stained with silver staining (see Material and Methods-6), transferred to a PVDF membrane for subsequent immunoblotting (see Material and Methods-8), or processed for SDS-PAGE (second dimension).

The procedure for immunoblotting of the native gel is essentially identical to the one described for SDS-PAGE (See Material and Methods-6), except for the fact that the gel was incubated in 0.1% SDS transfer buffer for 30 min. prior to tranfer.

When visualization of the native molecular weight markers was desired, the membrane was stained with Ponceau S (Sigma) right after transfer by incubating it with Ponceau S working solution (1/10 dilution of Ponceau S concentrate in water) for 10 minutes and rinsing briefly in 5% acetic acid. This staining method is reversible and perfectly compatible with immunoblotting.

9.2) Second dimension: SDS-PAGE.

After the first dimension, the lane to be analyzed was excised from the gel and incubated in 1% SDS/0.1% mercaptoethanol for 2 hours with gentle shaking (exceptionally, this incubation was carried out overnight, with identical results) and then for 1 hour in 1% SDS only, to remove traces of mercaptoethanol that would inhibit acrylamide polymerization. The lane was placed between the two glass plates

where the SDS-PAGE gel was to be cast, and the resolving gel was poured and allowed to polymerize as described in Material and Methods-6. Water was poured on top of the resolving gel, embedding the lane completely, until complete polymerization of the resolving gel was achieved. Then the water was removed and the stacking gel poured carefully, with special attention not to leave any bubbles under the lane. A one-well comb was placed beside the lane to run a molecular weight marker (Rainbow^{TM} coloured molecular weight markers, from Amersham). This gel was run in identical conditions as described in Material and Methods-6, except for the fact that 400 µl of 1x SDS-sample buffer were loaded on top of the lane to visualize the run, and further help in the denaturation of the proteins present in the lane.

After the run, the gel could be stained with silver staining (see Material and Methods-6) or transferred to a PVDF membrane for subsequent immunoblotting (see Material and Methods-8).

10) Measurememt of ^{32}P incorporation to DNA

In order to determine the percentage of ^{32}P incorporated in a DNA-labeling reaction, 1 µl reaction mixture was mixed with 109 µl 500 µg/ml salmon sperm DNA (sigma) in a 10 ml borosilicate tube, and 10 µl of this mix were spotted on a 25 mm diameter GFC filter (Whatman) and allowed to dry (filter A). 1 ml 1M HCl/0.1 M Na pyrophosphate solution was added to the remaining 100 µl and incubated for 10 min. on ice. The mix was then filtered through a GFC filter (filter B) under vacuum, and the filter was then washed once with 30 ml 1M HCl/0.1 M Na pyrophosphate solution and once with 30 ml ethanol and allowed to dry.

Both filters plus a third one used as a blank were introduced each into a scintillation vial, 5 ml scintillation fluid (High Flash Point Cocktail Safety Solve, from Research Products International Corporation) added to each vial and cpm (counts per minute) determined for each sample using a Model LS 5000CE Beckman coulter. The reading from the blank was substracted from the other two samples. Incorporation was calculated according to this equation:

$$\text{Incorporation (\%)} = cpm_B \times 10 / cpm_A$$

11- Determination of protein concentration

All protein determinations were carried out using Bio-Rad Protein Assay. This is based on the method of Bradford (Bradford, 1976), and involves the addition of an acidic dye to a protein solution and subsequent measurement at 595 nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration.

Dye reagent working solution was prepared right before use by diluting 1 part Dye Reagent Concentrate with 4 parts distilled water. The working solution was filtered through Whatman #1 filter to remove particulates. A standard curve with 0, 10, 20, 40, 60 and 80 µg bovine γ-globulin/100 µl was prepared, while the samples were brought to 100 µl with water. 100 µl of each standard and sample solution were pipetted into a 10 ml borosilicate tube, and 5 ml dye reagent working solution were then added to each tube and vortexed. After 5 min. incubation at room temperature, absorbance at 595 nm was determined for each standard and sample, and the protein concentration in each sample calculated by interpolating on the calibration curve. Incubation with the reagent should not exceed one hour.

12- Concentration of samples

Sample concentration was carried out with Ultrafree® centrifugal filter devices, from Millipore. No pre-rinsing or treatment of any sort was required prior to concentration. For small volumes, Ultrafree®-0.5, with a capacity for 500 µl was used. For bigger volumes, Ultrafree®-4 was chosen. This model has a capacity for 4 ml. Both had nominal molecular weight limits of 5 kDa. Concentration was performed by centrifugation at 4°C. The sample was pipetted into the filter device taking extra care not to touch the membrane. The filter was then placed into the centrifuge rotor. Ultrafree®-0.5 was centrifuged at 10,000xg, and Ultrafree®-4 at 3,500xg. The concentrate could be recovered from the concentrate pocket once the desired concentration was achieved. To maximize recovery, the concentrate was resuspended several times with a pipette. The sample was now ready for further analysis.

RESULTS

1) Preparation of polyclonal anti-mTERF antibody.

The methodological approach designed to address the main objective of this project, namely to study possible interactions of mTERF with other proteins, required the preparation of an antibody against mTERF. For that purpose, His-tagged mTERF expressed *in bacteria* was purified by a multi-step protocol that consisted on nickel column chromatography, SDS-PAGE, and electroelution from the mTERF-containing excised band. In order to induce the production of anti-mTERF antibody, purified His-mTERF was inoculated to a rabbit at weeks 0, 3, 7, 11 and 15, and blood samples were taken at weeks 0, 3, 6, 7, 11, 15 and 20. The serums obtained from these blood samples were designated A3-1 through A3-7, A3-1 being the pre-inmune serum. In order to determine the levels of anti-mTERF antibody, the serums were tested by ELISA. As shown in figure 18, antibodies were already detectable 3 weeks after the first inoculation (A3-2), and reached a plateau at week 15 (A3-6).

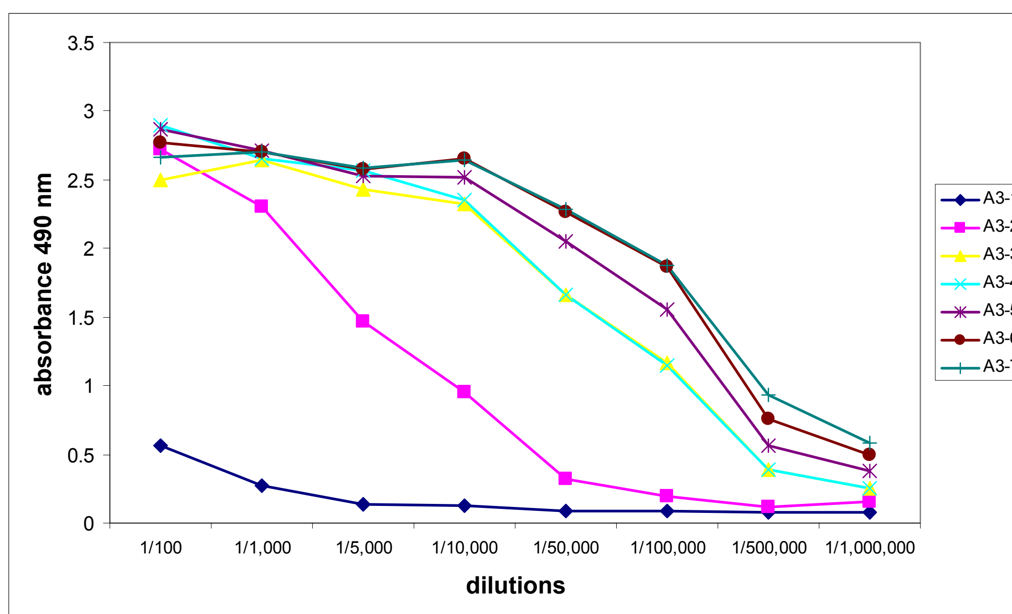


Fig. 18. Results of the ELISA tests performed on the rabbit serums extracted during the immunization procedure.

2) Gel filtration chromatography of S-100 from HeLa mitochondrial lysate.

As a first approach to study the possibility that mTERF is part of a protein complex, the S-100 fraction of a mitochondrial lysate from HeLa cells was analyzed by gel filtration chromatography using a HiPrep^R Sephacryl S-200 column from Pharmacia. S-100 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 (Kruse *et al,* 1989). The presence of mTERF in the chromatography fractions was revealed by SDS-PAGE followed by Western blotting using an anti-mTERF antiserum. Since mTERF is known to be stable at high salt concentrations and essentially detached from DNA, gel filtration chromatography was first carried out with a running buffer containing 0.5 M KCl. The elution profile of mTERF in this condition is shown in figure 19a, and it is clear that mTERF elutes in two peaks. To estimate the molecular weight of each form, their K_{av}s were calculated and interpolated on a calibration curve previously performed on the same column, as explained in Material and Methods. The values obtained for those two peaks from three independent chromatographies were 41 +/- 1.73 Kda. and 111.3 +/- 4.62 kDa
High ionic strenght is known, in some cases, to disrupt the interaction between leuzine zipper domains (Pernelle *et al*, 1993). Therefore, there was a possibility that the previous experiment failed to detect other mTERF-containing protein complexes. To eliminate this possibility, gel filtration chromatography was repeated using 0.15 M KCl in the running buffer (fig. 20). At low ionic strength, the profile was comparable to that obtained at 0.5 M KCl. Therefore, from this point onwards, all the gel filtration chromatography experiments were performed at 0.5 M KCl. The estimated molecular weight of the low molecular weight form (LMW) matches very closely that of mature mTERF, known to be 39 kDa., but the existence of a second peak at around 111 kDa. indicated that mTERF is also part of a larger structure (to which we will hereafter refer to as high molecular weight form, or HMW), and that both forms coexist in the S-100.

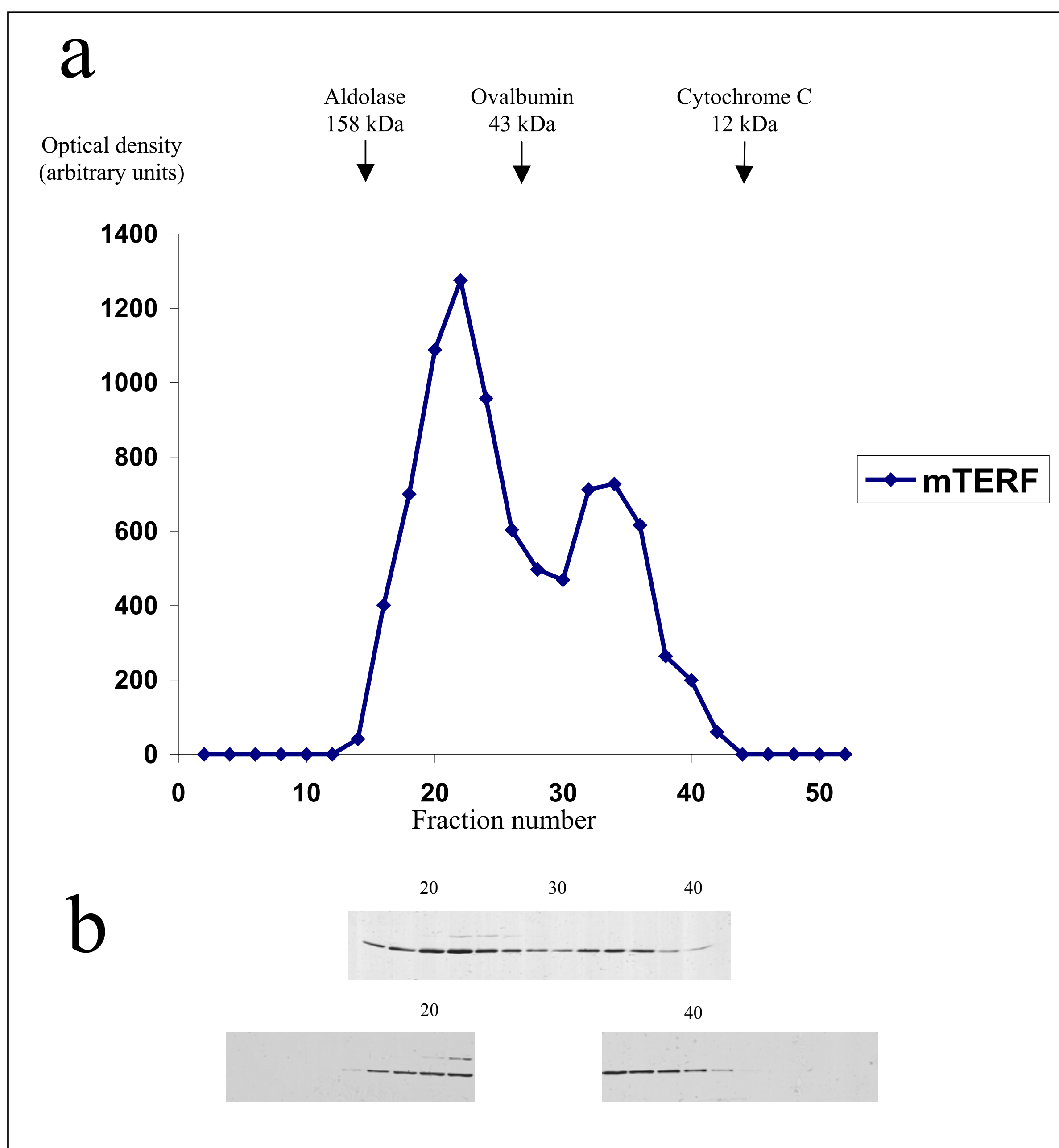


Fig. 19. Results of gel filtration chromatography of 1.5 ml S-100 using a HiPrep^{R} Sephacryl S-200 column and 0.5 M KCl in the elution buffer. (a) elution profile of mTERF determined by Western blot of the even fractions. A mixture of aldolase, ovalbumin and cytochrome c was loaded into the column prior to the injection of the S-100. (b) Actual Western blots used to trace the elution profile depicted in (a). The upper blot allowed the demonstration of the existence of the two mTERF peaks, while the lower blots confirmed the absence of other peaks below or above the ones described.

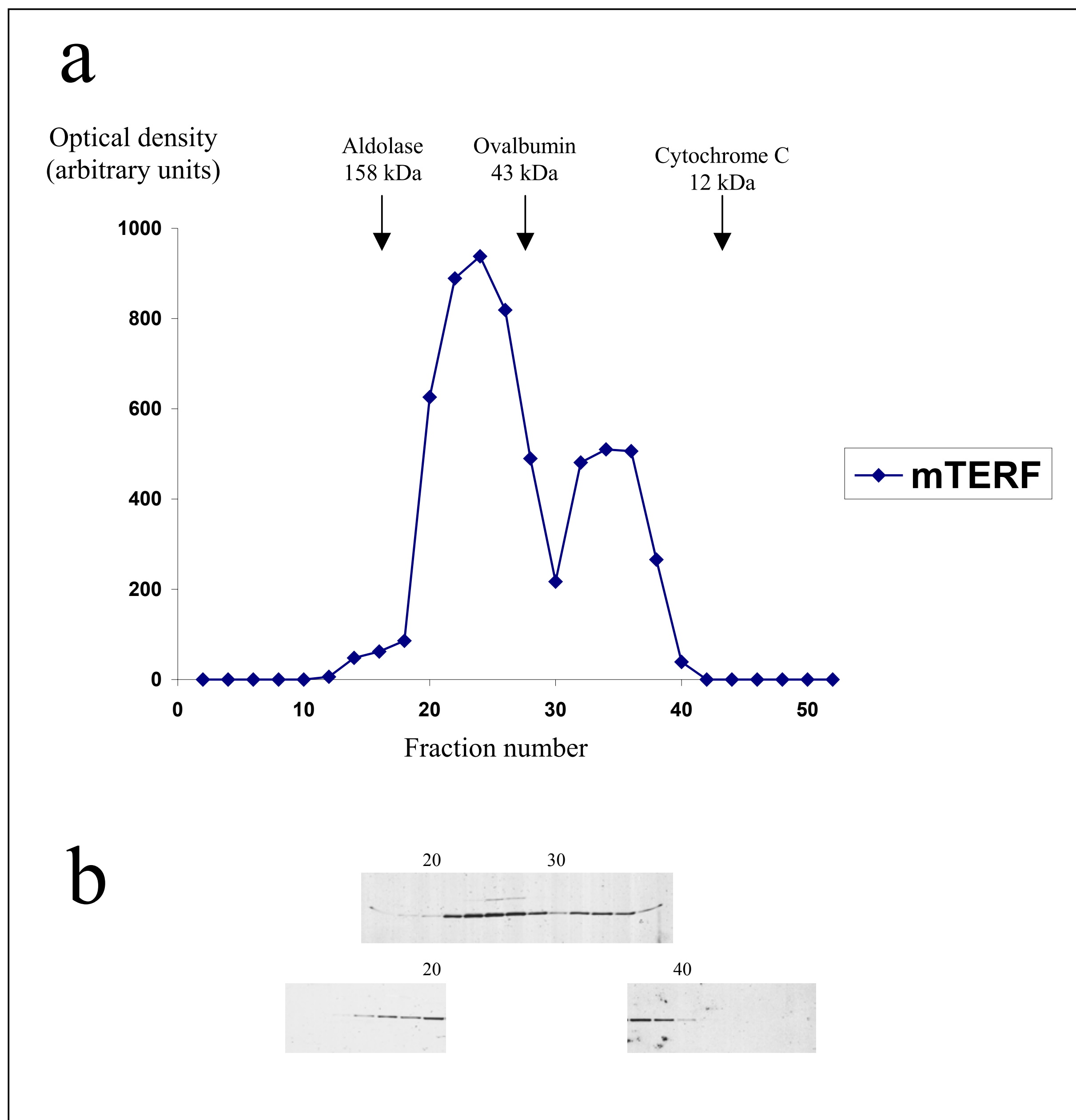


Fig. 20. Results of gel filtration chromatography of 1.5 ml S-100 using a HiPrep^{R} Sephacryl S-200 column and 0.15 M KCl in the elution buffer. The change in ionic strength did not alter the pattern observed at 0.5 M KCl. (a) elution profile of mTERF determined by Western blot of the even fractions. A mixture of aldolase, ovalbumin and cytochrome c was loaded into the column prior to the injection of the S-100. (b) Actual Western blots used to trace the elution profile depicted in (a).

3) Native PAGE of HMW and LMW pools

The migration of both forms of mTERF on a native PAGE was analyzed with two objectives in mind. On the one hand, a differential migration would reinforce the results obtained with the gel filtration chromatography. On the other hand, successful separation of both forms on a gel would be a very useful tool for future experiments, aimed at the characterization of both forms, as well as for the study of the mechanism responsible for the transition between forms. In native gels, proteins migrate according to their charge/mass ratio. Since mTERF has a net positive charge at neutral pH, the native PAGE had to be run from cathode to anode, unlike most electrophoretic procedures. Furthermore, in order to improve the solubility of mTERF, native PAGE was carried out at acidic pH. Under these conditions, concentrated pools of gel filtration chromatography fractions corresponding to HMW and LMW forms were run on native PAGE gels and analyzed by Western blotting with an anti-mTERF antiserum. As seen in figure 21, HMW pool gave a clear single band, while LMW pool gave two bands, a weak band migrating at the same position as the one found in the HMW pool, and a strong band migrating distinctly above it. From these results it was concluded that the lower band, since it's the only one showing up in the HMW pool, must correspond to the HMW form, while the upper band corresponds to the LMW form. The presence of a small fraction of HMW form in the LMW pool might be due to overlapping of both forms in some of the fractions contributing to the LMW pool, or to a partial transformation of LMW form to HMW at some point after gel filtration.

4) Transcription termination activity assay of GF fractions

Once it was confirmed that mTERF is found in S-100 fractions as part of a protein complex, as well as an apparent free monomeric form, the next step in our research was to determine whether these two forms showed transcription-termination activity. For this purpose, fractions from a gel filtration chromatography were divided in 9 pools, 50x concentrated, and samples from each concentrated pool tested in an *in vitro* transcription

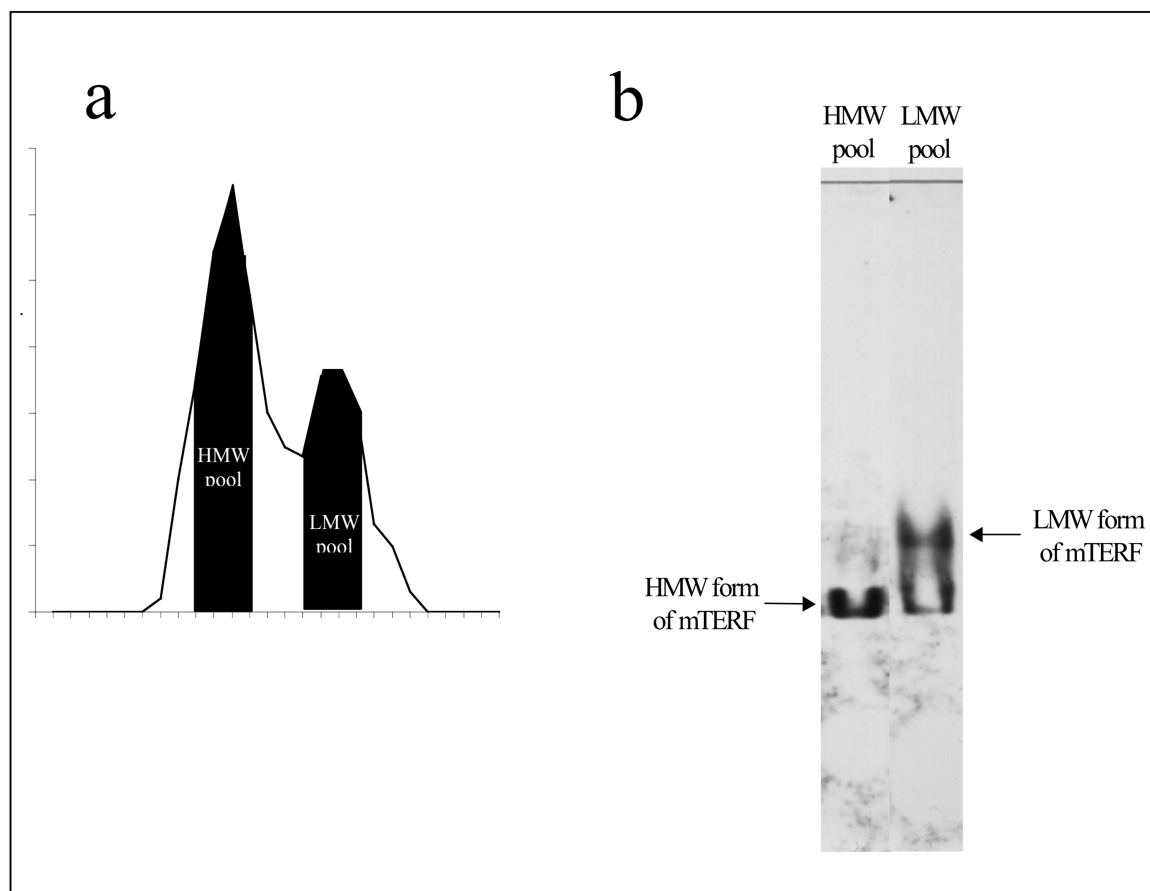


Fig. 21. Separation of both forms of mTERF by native-PAGE. (a) elution profile of mTERF from the gel filtration column. The black areas represent the two pools of fractions analyzed by native-PAGE. (b) Western blot of a native-PAGE using anti-mTERF antibody.

system as described in Material and Methods. As seen in figure 22, the LMW form presented transcription-termination activity. This observation reinforced the hypothesis that this form indeed corresponds to the monomeric form of mTERF, since it was found that mTERF binds DNA, and thus exerting termination activity, as a monomer (Fernandez-Silva *et al*, 1987). However, this approach did not render any information about the HMW form. As shown in fig. 22, *in vitro* transcription was almost completely inhibited in those pools containing this form, probably due to the presence of inhibiting factor(s).

Another interesting observation from these experiments came from the analysis of the run-off transcripts from both strands. While in those reactions containing the LMW

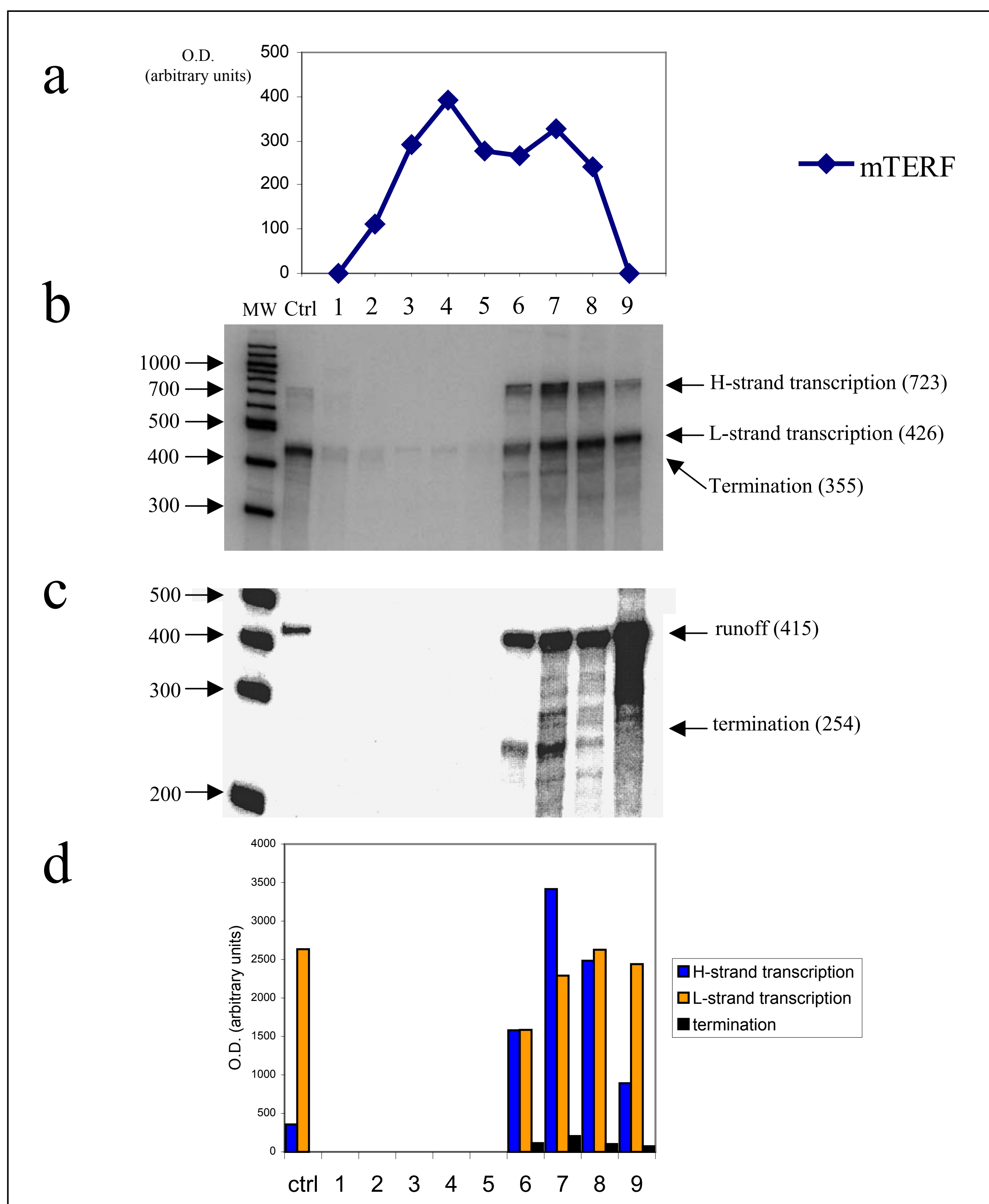


Fig. 22. Transcription termination activity assays. (a) elution profile of mTERF from the gel filtration of S-100. Nine 5-ml fractions were collected, concentrated and analyzed by SDS-PAGE and Western blot using anti-mTERF antiserum. (b*) In vitro* transcription assay. (c) S1 protection assay. (d) Quantification of the results obtained in the *in vitro* transcription assay.

form pools L-strand transcription remains at the same level as the control, H-strand transcription increases, following the same profile as the transcription-termination band and the level of mTERF, as determined by Western blotting (see fig. 22). These results suggest that mTERF is exerting some positive control at the level of initiation of H-strand transcription, and are in agreement with previous observations, in which termination activity of purified mTERF from HeLa cells was commonly accompanied with an increase in H-strand transcription initiation (Kruse *et al*, 1989).

5) DNA-binding activity assays

Since the termination activity assays failed to give any information about the HMW form, we decided to investigate the DNA-binding activity of both mTERF forms. A band-shift assay using a DNA probe containing the mTERF DNA-binding site on fractions from a gel filtration chromatography, resulted in a series of shifted bands (fig. 23). Of these, only two are present in fractions that contain mTERF. The upper band seemed to appear concomitantly with the HMW form of mTERF, whereas the lower one appeared in those fractions corresponding to the LMW form, suggesting, in principle, that both forms presented DNA-binding activity. The question was further approached by carrying out a super-shift assay on a fraction containing both bands. Results showed that the lower band was sensitive to the presence of anti-mTERF antiserum during the band-shift assay. As shown in figure 24a, this band almost disappeared when anti-mTERF antiserum was present, and a new slower-moving band appeared instead. The upper band, though, remained unaltered. A more direct approach was carried out by excising the two shifted bands and running a second dimension on a SDS-PAGE gel, followed by Western blot analysis. This analysis unequivocally revealed that mTERF was present in the lower band, but failed to detect it in the upper one (fig. 24b).

In order to confirm the previous observation, pools containing either form of mTERF were submitted to heparin chromatography, followed by SDS-PAGE and Western blotting analysis of the flowthrough and eluted fractions (fig. 25b). Heparin is a highly positively charged molecule, and binds non-specifically to DNA-binding proteins,

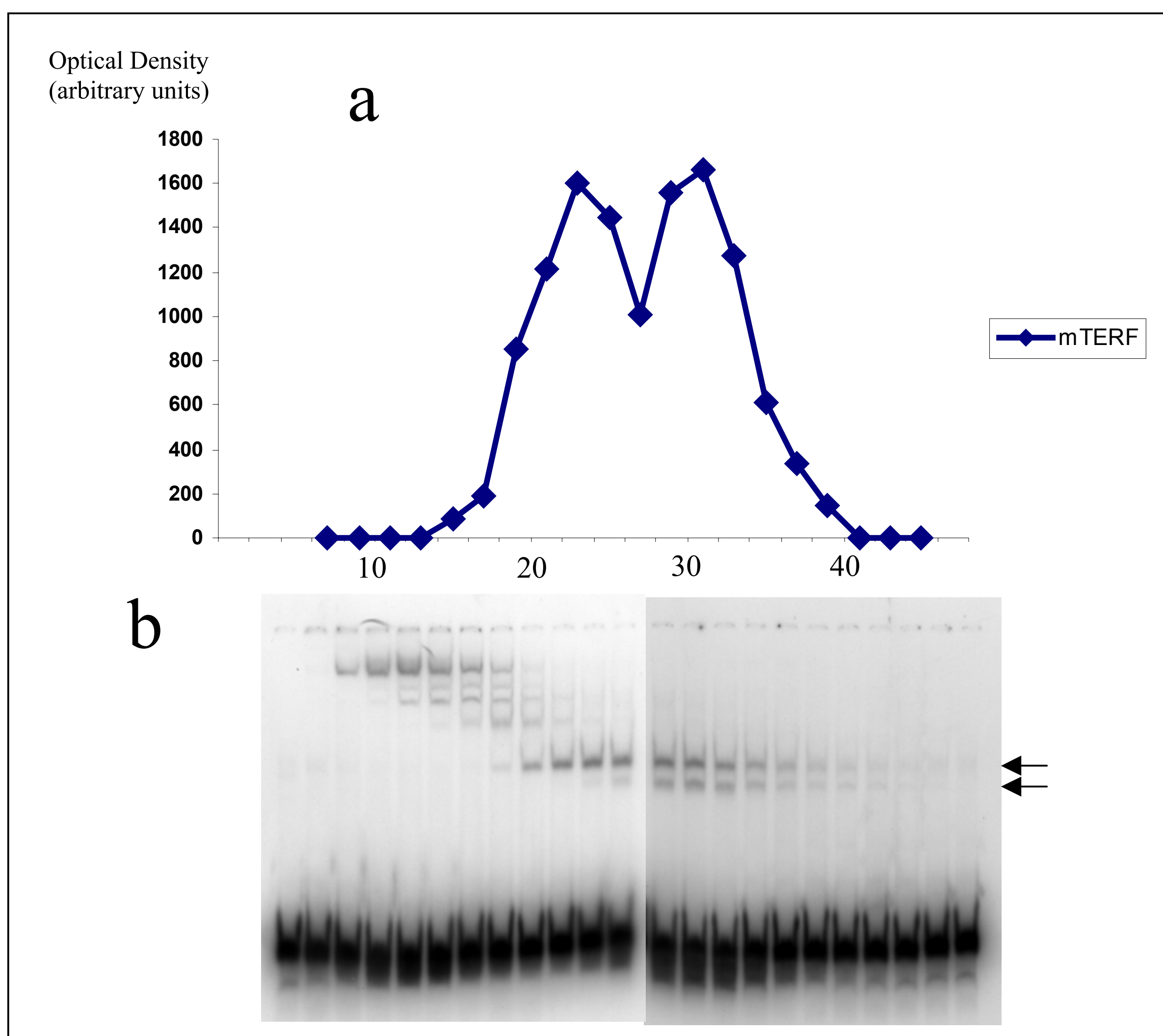


Fig. 23. Band-shift assay. (a) The fractions from a gel filtration chromatography were analyzed by SDS-PAGE followed by Western blotting using anti-mTERF antiserum. (b) the same fractions were submitted to band-shift assay as described in the text. Two shifted bands of interest (black arrows) were identified.

to the point of being commonly used as a first step in the purification of DNA-binding proteins by DNA-affinity chromatography (see Kadonaga and Tjian, 1986). LMW-mTERF showed the typical behaviour of a DNA-binding protein. Although a small proportion was present in the flowthrough (most likely due to saturation of the resin, or to the presence of a small amount of HMW-mTERF in this pool), most of the mTERF started to elute from the column at 0.5 M KCl. On the contrary, when the HMW pool was loaded in the heparin column, all the mTERF was present in the flowthrough, and none was detected in the eluates, even at KCl concentrations able to partially disrupt the

interaction between DNA and the protein(s) responsible for the upper shifted band, as determined previously by band-shift assay (fig. 25a). This fact denoted that the HMW form did not bind to heparin.

From the combined results of the band-shift, super-shift, SDS-PAGE/Western blotting and heparin chromatography, it can be concluded that only the LMW form of mTERF shows DNA-binding activity, and whatever protein is responsible for the upper-shifted band, it is probably unrelated to mTERF.

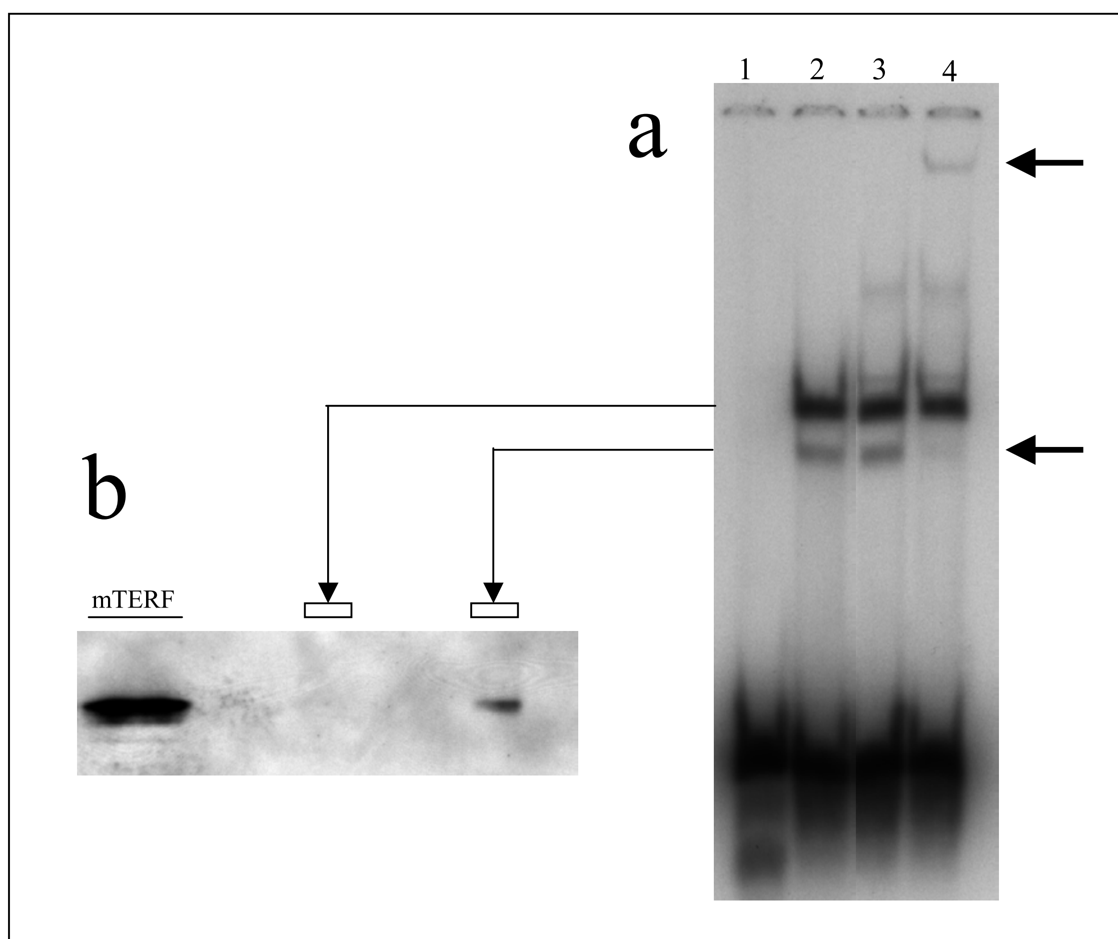


Fig. 24. (a) super-shift assay. 1, probe only. 2, fraction 27 from the gel filtration experiment depicted in figure 23. 3, fraction 27 in the presence of preimmune serum (A3-1). 4, fraction 27 in the presence of anti-mTERF antiserum (A3-6). The thick black arrows signify the disappearance of the lower shifted band and the appearance of a new, super-shifted band when the anti mTERF antibody is present in the band-shift assay. (b) Western blot of the excised bands run on a SDS-GAGE using anti mTERF antiserum. Only the lower shifted band shows the presence of mTERF.

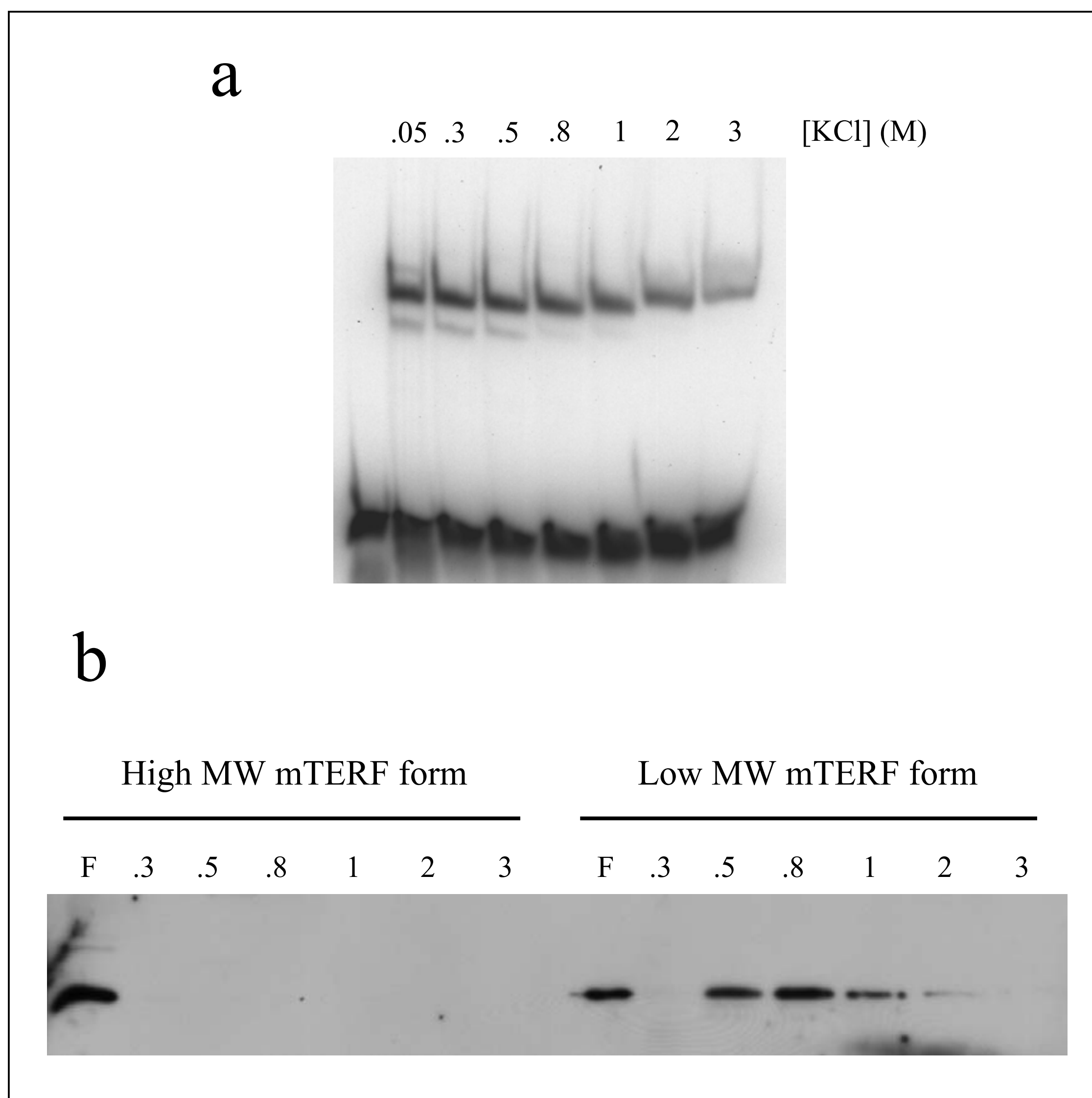


Fig. 25. (a) KCl titration of a band-shift assay using fraction 26 of the gel filtration chromatography showed on figure 23. (b) Western blot of the flowthrough (F) and eluates (.3 through 3) run on a SDS-PAGE, using anti-mTERF antiserum. High and low MW mTERF forms refer to Pools A and B described on figure 21.

6) The HMW form of mTERF is a reversible structure

In order to test the reversibility of the HMW form, two fractions from a gel filtration chromatography containing this form were pooled and re-loaded in the same

column and the fractions obtained in this second gel filtration were run on a SDS-PAGE gel and submitted to immunoblotting with the anti-mTERF antiserum. Both gel filtration chromatographies were carried out on a HiPrep^R Sephacryl S-200 column (Pharmacia), with a KCl concentration of 0.5 M. Surprisingly, all the mTERF in the second run eluted as the LMW form (see fig. 26). Using a different column (Superdex 200 HR 10/30 column, from Amersham Pharmacia) and/or lower KCl concentration (0.15 M) gave the same results (data not shown). Although it is not clear why a second gel filtration chromatography dissociated the HMW form of mTERF, the results obtained reveal that this form is highly reversible. This fact has important functional implications, as will be discussed later on.

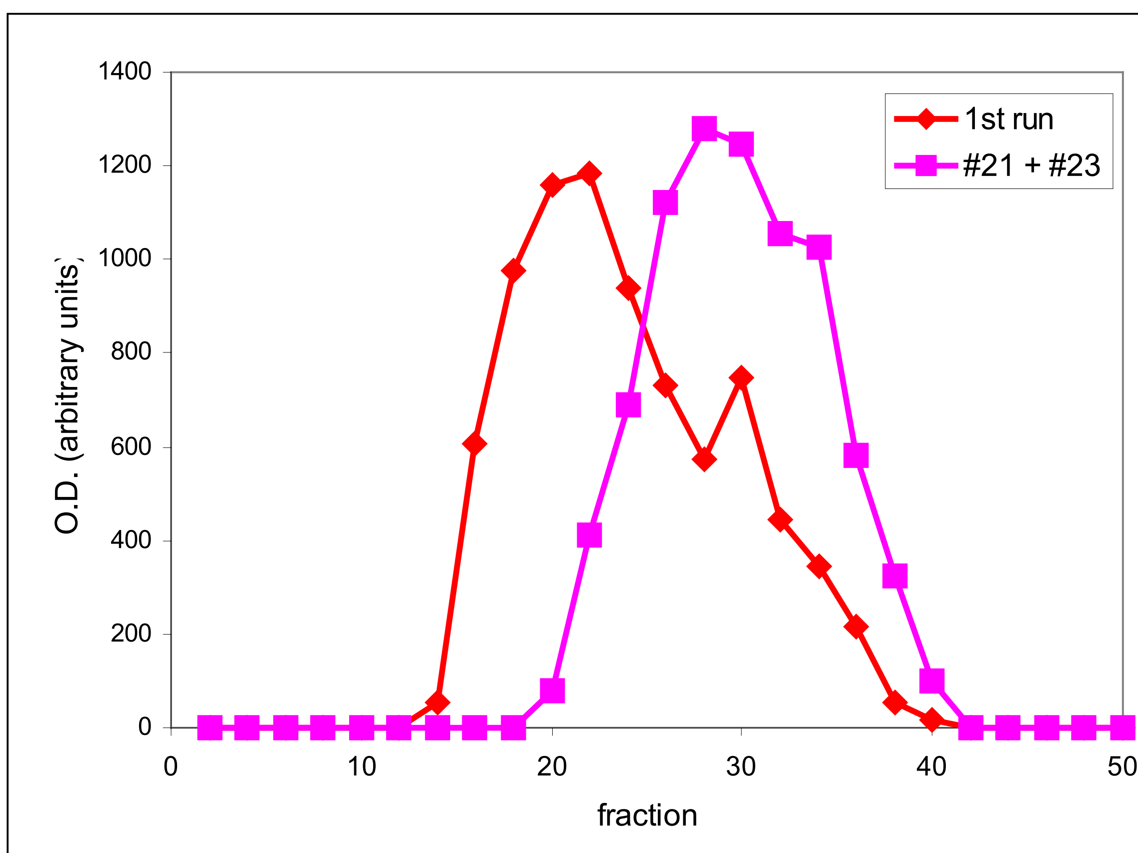


Fig. 26. In red, elution profile of mTERF from gel filtration chromatography of a S-100, assessed by Western blot. In blue, elution profile of mTERf from fractions 21 and 23 from the first chromatography. Those fractions correspond to the HMW form of mTERF, and are clearly shifted towards the LMW form after the second gel filtration.

7) Homopolymer vs. heteropolymer

The exact nature of the polymer of which mTERF is a component has not yet been determined. Attempts to purify it by immunoprecipitation and immunoaffinity chromatography were unsuccessful (data not shown). Nevertheless, two sets of experiments brought some light to this matter, allowing us to formulate an hypothesis as to the nature of this protein complex.

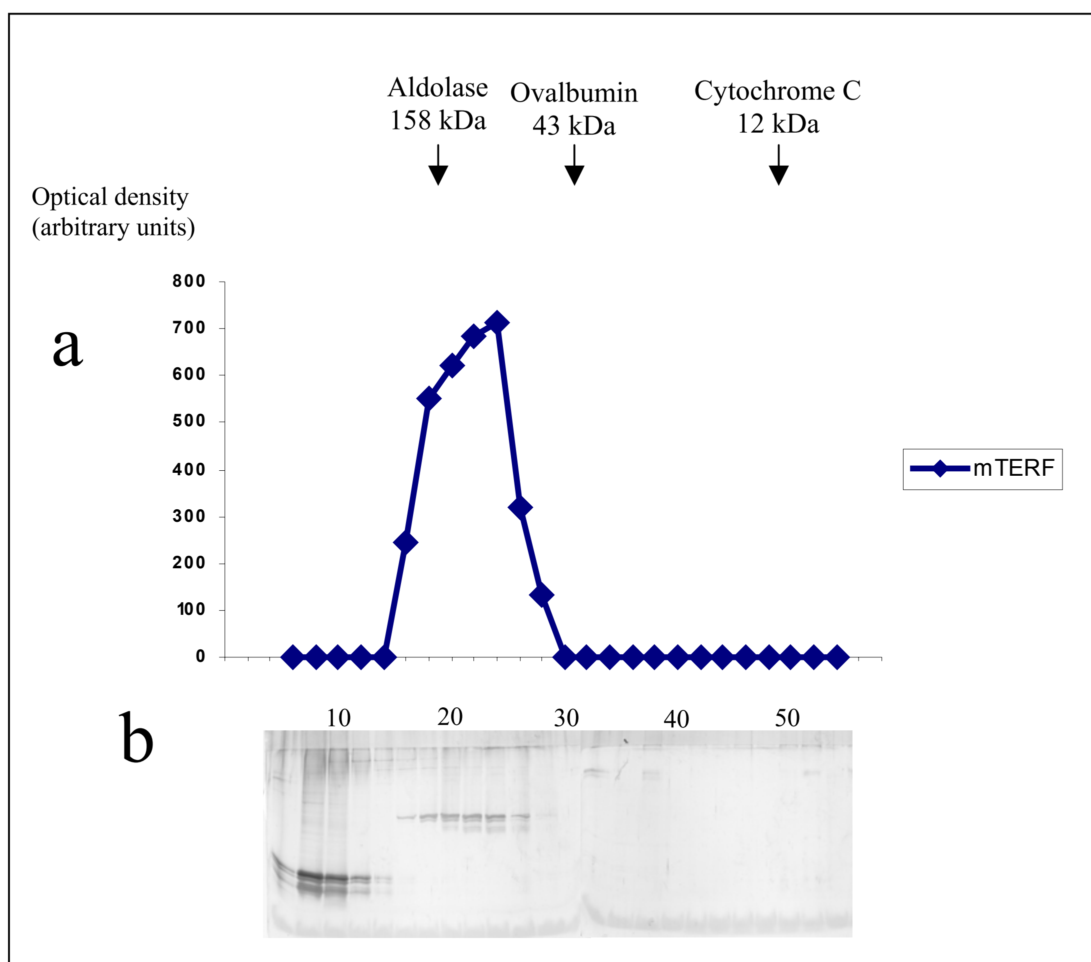
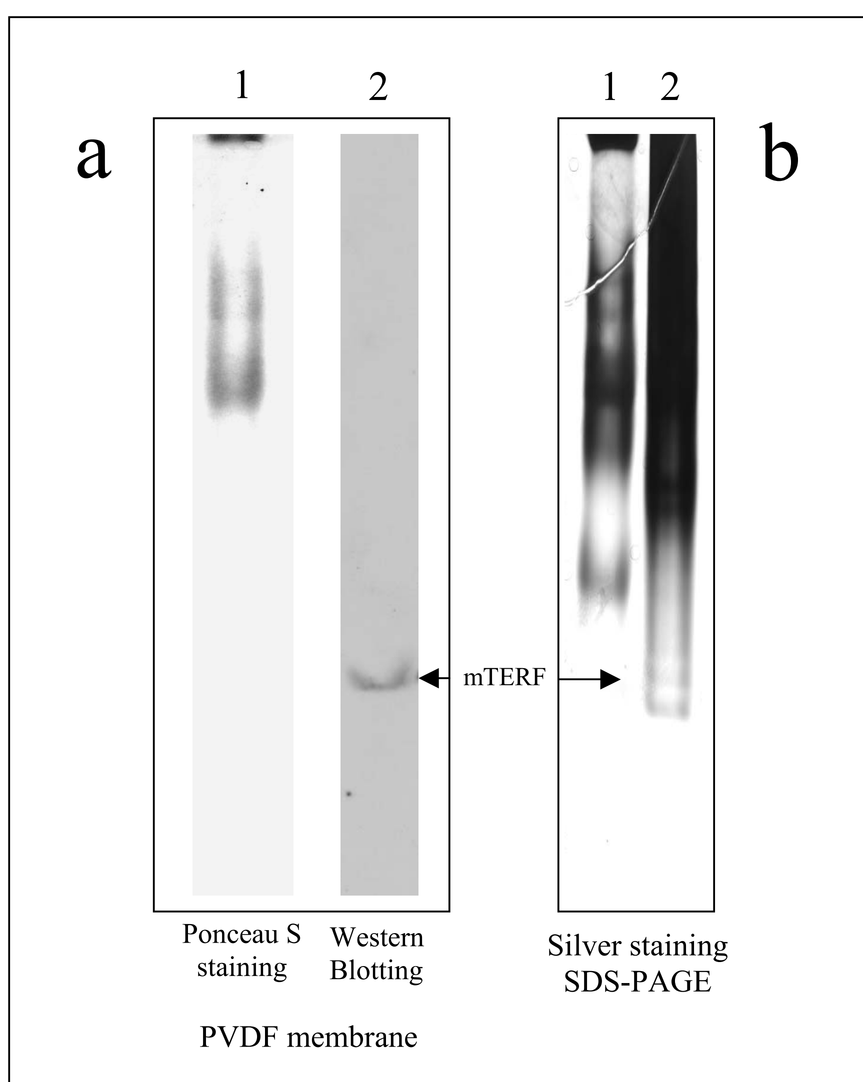


Fig. 27. (a) elution profile of purified mTERF from HeLa cells after gel filtration chromatography. (b) Actual silver-stained SDS-PAGE gel of the gel filtration fractions.

Fig. 28. Precise identification of the HMW form of mTERF on a native PAGE. A sample from Pool A was run in duplicate on a native-PAGE, together with a set of native protein markers, also in duplicate. Half of the gel, containing one of the samples and one set of markers was transferred onto a PVDF membrane (a). The lane corresponding to the native markers (1) was stained with Ponceau S, while the lane containing the sample (2) was submitted to Western blot using anti-mTERF antiserum. The other half of the gel was silver-stained (b). The native markers allow a precise alignment between the Western blot and the silver stained gel, and that permits to precisely locate the HMW form of mTERF in the silver-stained gel.



The first set of experiments consisted in submitting DNA-affinity purified mTERF from HeLa cells to gel filtration chromatography under exactly the same conditions described above for S-100. As shown in figure 15, DNA-affinity chromatography yielded a highly purified mTERF, migrating in a SDS-PAGE gel as a typical ~34 kDa. doublet. The only evident contaminants in this purification, apart from a band at 31 kDa that might correspond to a degradation product of mTERF often observed in mTERF preparations (Daga *et al*, 1993), were two bands migrating at ~16 kDa. Gel filtration chromatography of the 0.5 M KCl fraction from DNA-affinity chromatography, followed by silver staining of SDS-PAGE of the fractions revealed two interesting results. On the one hand, the low molecular weight contaminants eluted in the first fractions, consistent with the fact that these peptides aggregate or form high molecular weight complexes. But more importantly, they eluted separately from mTERF, clearly indicating that these contaminants are not components of any mTERF-containing polymer, and therefore can be disregarded for the purpose of characterizing the polymeric form of mTERF. On the other hand, mTERF eluted entirely as the polymeric form (fig. 27). Interestingly, previous experiments revealed that those same purified mTERF fractions failed to show any termination activity (data not shown), which is in accordance with the lack of activity observed for the polymer. These results strongly support the hypothesis that mTERF is capable of organizing itself as a homopolymer. In fact, the estimated molecular weight for the polymeric form (107-113 kDa.) is very close to the expected molecular weight of a mTERF trimer, that is, 117 kDa.

A second set of experiments aimed at testing this hypothesis consisted in analyzing the polymer by 2D-electrophoresis. As a first step, the mTERF-containing polymer was precisely identified in the first dimension, this being a native-PAGE (fig. 28) performed under the same conditions as described earlier. For this purpose, gel filtration chromatography fractions containing the HMW form of mTERF were pooled, 10x concentrated, and a sample run on a native PAGE in duplicate, together with two sets of native protein markers. After the run, half of the gel, containing one of the samples and one of the sets of protein markers, was transferred onto a PVDF membrane. Ponceau S staining of the membrane allowed visualization of the protein markers, and further Western blotting located mTERF. The other half of the gel was silver-stained. After

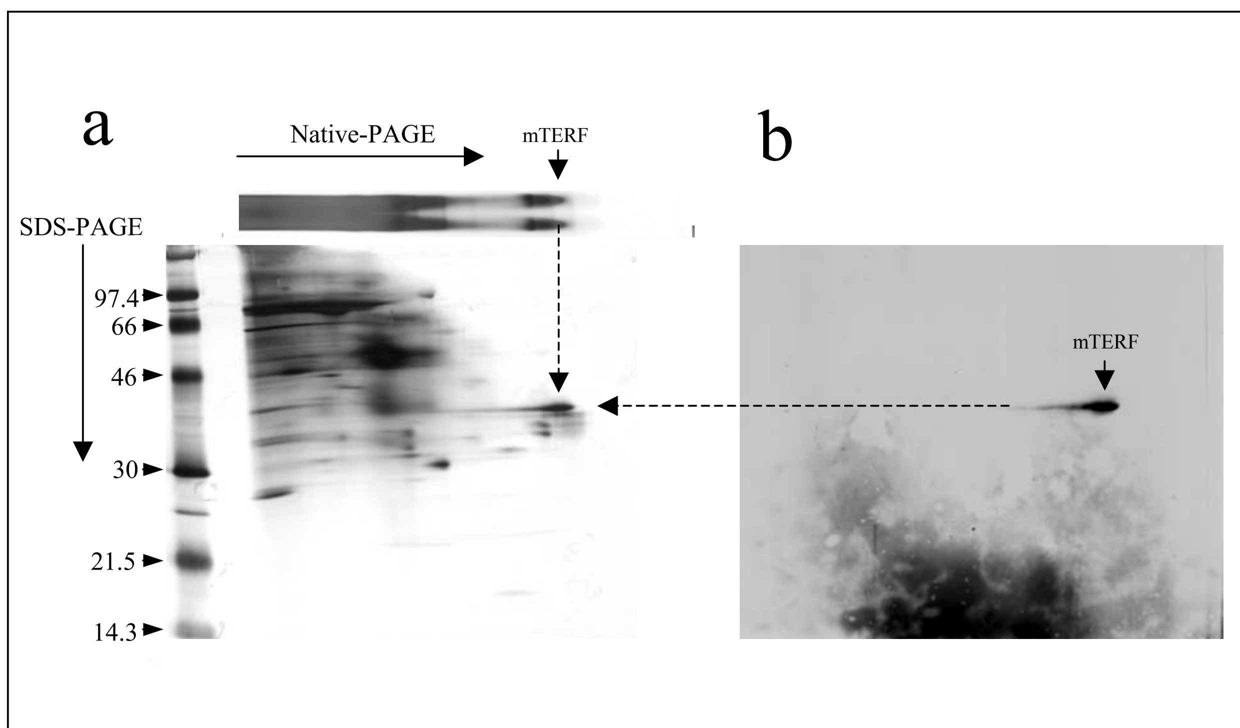


Fig. 29. (a) Silver-staining of 2D electrophoresis of Pool A. First dimension: native-PAGE. Second dimension: SDS-PAGE. (b) Western blot of a 2D electrophoresis using anti-mTERF antiserum.

carefully aligning the markers in both the membrane and the silver-stained gel, mTERF could be unequivocally identified in the first dimension. In further experiments (fig. 29), the same sample was run on a native-PAGE, this time in triplicate. One lane was silver-stained, while the other two were each run on a SDS-PAGE gel. Of these, one was silver-stained, and the other one was transferred to a PVDF membrane for Western blotting. Alignment of the molecular weight markers on the silver-stained gel and on the membrane allowed the precise location of mTERF in the second dimension. A careful observation of the silver-stained SDS-PAGE (fig.29) reveals that there is only one visible candidate to form a complex with mTERF. This peptide is migrating immediately below mTERF, and might perfectly correspond to the lower component of the typical 34 kDa. doublet. If that were true, this data would add to the evidence in support of the hypothesis of mTERF being able to establish intermolecular interactions with other mTERF molecules to form a homotrimer.

In summary, the data produced have allowed us to identify two forms of mTERF, an active monomer and an inactive polymer. Although the nature of the polymer is still not well established, gel filtration of purified mTERF, together with 2D electrophoresis has brought some light into the issue, leading us to formulate an hypothesis that will be developed in detail in the Discussion.

DISCUSSION

Fernandez-Silva and co-workers contributed very significantly to the characterization of mTERF with the publication of its sequence, and proof that mTERF binds to DNA as a monomer (Fernandez-Silva *et al*, 1997). The presence in its sequence of three leucine zipper domains, typical protein-protein interaction motifs, led to the authors to propose a tertiary structure for this protein in which the leucine zipper domains form intramolecular interactions among themselves, bringing the two DNA-binding domains together, thus conferring DNA-binding activity.

Still, most leucine-zipper proteins described in the literature use their leucine-zipper domains to establish interactions with other peptides, in the form of homo- or heterodimers, as described in the Introduction. A considerable number of those are DNA-binding proteins, like mTERF. It was in this context that we decided to explore the possibility of mTERF interacting with other proteins. Even tough it is well established that mTERF interacts with DNA as a monomer, still there might be other polymeric forms of mTERF. That possibility would have important regulatory connotations.

A preliminary requirement to face the study of the interaction of mTERF with other peptides was the preparation of an anti-mTERF antibody. That would allow us to carry out Western blot, immunoprecipitation and immunoaffinity chromatography, *a priori* essential techniques for the task we planned to undertake.

Once the anti-mTERF antiserum was obtained, the first approach to the study of the interaction of mTERF with other peptides was gel filtration chromatography of S-100 followed by Western blot of the fractions. This technique is fast, easy to perform and highly reproducible, and certainly gave us the first strong evidence that, apart from the monomeric form of mTERF, there was a second, bigger form in the S-100 of HeLa cells. The first chromatographies were performed with a concentration of KCl of 0.5 M (fig.19). This concentration of salt is the one classically used to obtain mTERF from HeLa mitochondria, since these conditions guarantee that mTERF is essentially detached from DNA. High salt, though, can disrupt the interaction between leucine zipper domains, and therefore there was a possibility that in those conditions we were missing other structures. Nevertheless, when gel filtration was repeated at low ionic strength (0.15 M; see fig. 20), no differences were observed. The stability of the HMW form of mTERF

at high ionic strength might be indicative of the fact that electrostatic interactions are not essential for the formation of such structure. In fact, the interaction between leucine-zipper domains is basically maintained by the interaction of the hydrophobic residues at positions **a** and **d**, and the stabilizing role of the electrostatic interactions between residues in positions **e** and **g** described in some cases, is far from universal (Lovejoy *et al,* 1993; Skolnick and Holtzer, 1985). This data is in accordance with the low presence of charged residues in the leucine-zipper domains of mTERF (6 polar residues out of 15 at position **e**, and only 2 out of 15 at position **g**).

One of the peaks obtained by gel filtration chromatography was compatible with the monomeric form of mTERF, according to the calculated molecular weight, but the other one clearly corresponded to a bigger structure. The estimated molecular weight of the latter was around 110 kDa, roughly three times bigger than the monomeric form. Few post-translational modifications, like glycosylation, can account for such a big increase in molecular weight. But the fact that both forms of mTERF migrated equally in SDS-PAGE gels (see figs. 19 and 20, panels b) indicates that the difference in elution volume observed in the gel filtration chromatography was not due to a covalent post-translational modification, but rather to the interaction with other polypeptides that dissociated from mTERF during the SDS-PAGE analysis. There was also the possibility that the higher molecular weight peak was the result of the interaction of several molecules of mTERF to form a homopolymer.

Although the elution profiles depicted in figures 19 and 20 show very similar HMW/LMW ratios, these changed considerably from experiment to experiment. Several factors may account for this variability. The preparation of S-100 involves lysis steps which may expose mTERF to different levels of proteases or other agents capable of affecting its stability. Also, freezing and thawing of the S-100 or of the gel filtration fractions can affect both forms differently. Cell culture conditions might also play a role. Although much care was taken to carry out all cell cultures in the same conditions, changes in the batch of culture media, or different cell densities at the time of collection (although cells were always collected while growing in exponential mode) could affect the metabolic status of the cells (Stacey, 1997; Dodson and Schaeffer, 2000), and that

might plausibly be responsible for changes in the relative concentration of both forms of mTERF in different preparations.

The results obtained by gel filtration chromatography were further corroborated by native-PAGE (fig. 21). It is interesting to note that the heavier form of mTERF migrates faster than the lighter, contrary to what might be expected. However, this phenomenon is by no means extraordinary, considering that mass is not the only factor contributing to the migration of a protein on a native gel, where charge plays an equally important role. This 'paradoxal' behaviour is an indication that the charge/mass ratio of the HMW form is higher than that for the LMW form under the conditions used for the native-PAGE. In a first lecture, this would go in favour of a HMW form being a heteropolymer, since a homopolymer should, in principle, conserve a similar charge/mass ratio to that of the monomeric form of mTERF. Still it is plausible that the charged residues of mTERF are buried in the tertiary structure of the monomeric form, while they are exposed in the polymeric form. That would confer the homopolymer a higher charge/mass ratio.

Once the existence of two forms of mTERF was established, the next step was to determine the activity of each form. *In vitro* transcription experiments clearly showed transcription-termination activity associated to the LMW form but, surprisingly, transcription of both strands was inhibited in the fractions corresponding to the HMW form. To our knowledge, no inhibitors of mtDNA transcription have been reported to this date, and further investigation in this direction might render interesting information for the understanding of the control of mtDNA transcription. Of course, the possibility of mTERF itself, in its polymeric form, being responsible for the arrest of transcription initiation cannot be ruled out. Further studies need to be carried out, in which the *in vitro* transcription experiments are performed in the presence of the purified polymeric form of mTERF. Only then a role of the HMW form of mTERF as inhibitor of transcription can be proposed.

Another surprising result derived from the *in vitro* transcription experiments was that the increase in termination activity was accompanied by a concomitant increase in H-strand transcription initiation. If it is confirmed that the monomeric form of mTERF is

responsible for both activities, the concept that mTERF exerts control of rRNA transcription solely by terminating transcription at the 3'-end of the transcription unit will have to be revised, and a broader role for mTERF, also involving stimulation of transcription initiation, should be considered. The hypothesis that mTERF-mediated transcription initiation 'earmarks' the resulting transcript to terminate at the 3'-end of 16S rRNA, and that both initiation and termination are part of the same regulatory mechanism is very attractive, and in view of the evidences, worth pursuing. This mechanism reminds that described for TTF-I. TTF-I-mediated termination is accompanied by an increase in initiation, and two models were proposed to explain this phenomenon. One postulated that the transcription unit forms a protein-mediated loop that brings promoter and termination elements in close contact, so the RNA polymerase is 'handed over' from the termination to the initiation sites (add reference). The other model, which happened to be the correct one for TTF-I, proposed that the increase in initiation observed after termination was due to facilitation of reinitiation after RNA polymerase release from the termination site (Jansa et al, 2001). Although there are significant differences between the mitochondrial and the Pol I systems, like the fact that TTF-I-induced termination is strictly orientation dependent and Pol I-specific (Kuhn *et al*, 1990), while mTERF-mediated termination shows a biased polarity *in vitro* and is not strictly mitochondrial RNA polymerase specific (Shang and Clayton, 1994), both models can still be applied to mTERF, and more work needs to be done to clarify which one is correct.

Unfortunately, the *in vitro* transcription experiments did not give any direct information about the transcription-termination activity of the HMW form. But from the results obtained from the combination of band-shift experiments, Western blot of the excised shifted bands and heparin chromatography, it was fairly assumed that the LMW form, but not the HMW form of mTERF, had DNA-binding activity. Therefore, if the HMW form of mTERF is incapable of binding to DNA, it is fair to assume that this form does not have transcription termination activity, since DNA-binding is essential to promote termination. Band-shift experiments show that the HMW form of mTERF does not bind to the mTERF DNA-binding site, but the heparin chromatography goes a step further, extending its inability to bind DNA not just to the mTERF DNA-binding site, but

to any DNA sequence. This fact is important to keep in mind, especially in relation to the putative role of the HMW form of mTERF in the inhibition of H-strand transcription initiation (see above). In other words, if the HMW form of mTERF plays a role in transcription-initiation, it will have to be through interaction with the transcription machinery, and not through interaction with the H-strand promoter.

The data obtained so far clearly identified the LMW form as the monomeric form of mTERF. Its estimated molecular weight by gel filtration chromatography and its DNA-binding and termination activities, matched what would be expected from mTERF as a monomer. The HMW form, though, represented a novel form, and by its lack of DNA-binding activity it could be plausibly assumed that it was an inactive polymeric form of mTERF. At this point, the hypothesis that the activity of mTERF was regulated by polymerization began to take shape. A first approach to this hypothesis consisted in testing the reversibility of the two forms of mTERF by running them a second time through a gel filtration column. To our surprise, the polymeric form shifted completely to the monomeric form after the second chromatography. Dissociation of the complex during storage of the fractions is unlikely, since we were able to detect differences in migration on native PAGE between LMW and HMW forms (see fig.21), and the HMW fractions from the first chromatography lacked DNA-binding activity. Therefore, it is fair to assume that dissociation occurred during the second gel filtration. One possibility is that the formation of the HMW form is concentration dependent. The mTERF contained in two 1ml-fractions from the first gel filtration corresponded roughly to 10% of the total mTERF present in 1.5-2 ml of S-100 loaded into the column. That represents a ~10x dilution from the original material. When the second gel filtration was carried out, the mTERF from those two fractions was further distributed along 20 fractions, thus diluting mTERF even more. If the first gel filtration brought the mTERF concentration close to a putative ‘dissociation point’, further dilution occurring during the second might have triggered the complete dissociation of the polymeric form of mTERF. Other possible explanation to this phenomenon might be the sequential elimination of a factor necessary to keep mTERF in the polymeric form. If such factor had a different molecular weight than HMW, the first chromatography would separate most of it from HMW, but some of

it might still be present in the HMW fractions, enough to keep mTERF as a polymer, although the ratio between HMW and this factor in these fractions would be higher than in the S-100. A second gel filtration chromatography of the HMW fractions would increase that ratio to a point in which HMW can no longer stay as a polymer, and dissociation would occur.

But whatever the mechanism responsible for this observation, three conclusions can be derived from it. On the one hand, the polymeric form of mTERF can shift to the monomeric form, and the interaction between the different components of the polymeric form is reversible. On the other hand, the ratio HMW/LMW found after gel filtration chromatograpy might not reflect the actual ratio in intact cells, and perhaps some or most of the monomeric form observed is due to artifactual dissociation during the experimental procedure.

The exact composition of the polymeric form is not known, despite all the effort invested so far. However, gel filtration of pure mTERF (fig. 27) and 2D electrophoresis (fig. 28) seem to point out towards the homopolymer hypothesis. As mentioned in the introduction, there are two well documented examples of leucine-zipper proteins that control their activity by homotrimerization. These are the heat shock transcription factor (HSF) and the influenza virus haemagglutinin. In both cases, the interaction between the three peptides is established through their leucine zippers, forming a triple coiled-coil structure. mTERF, though, would be unique in the fact that its activity resides in the monomeric form, since for the two cited proteins trimerization is required for activity.

The hypothesis that the leucine zippers of mTERF form a triple coiled-coil structure is not new. Fernandez-Silva and colleages already postulated that this sort of structure was adopted among the three leucine zippers contained in the mTERF molecule to bring the two basic domains in close register with its target DNA sequence (Fernandez-Silva *et al*, 1997). Some examples of leucine zippers establishing intramolecular interactions had by then been described, like spectrin (Yan *et al*, 1993) and seryl tRNA synthetase (Cusack *et al*, 1990). In view of the data discussed so far, 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 polymer, in the form of a homotrimer, would depend on intermolecular interactions. This hypothesis is illustrated in figure 30, and it should be emphasized that the nature of the interactions depicted in it is purely conjectural, and other patterns of interaction between leucine zippers cannot be excluded. The structure of the monomer shown in the figure is that proposed for mTERF by Fernandez-Silva and colleages (Fernandez-Silva *et al*, 1997), and the parallel triple coiled-coil structure chosen for the homotrimer is inspired by that proposed for the human heat shock

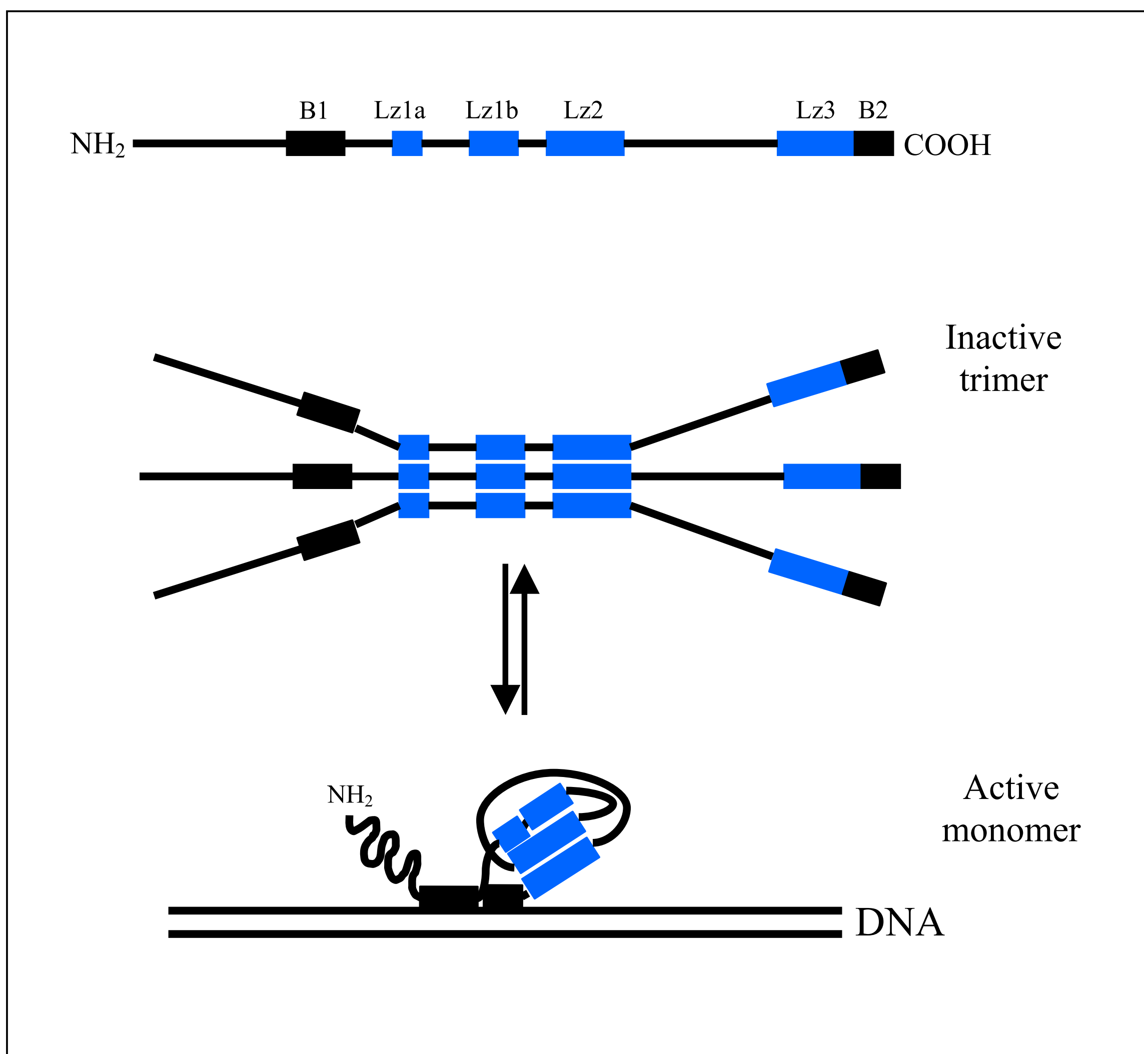


Fig. 30. Proposed model for the control of the activity of mTERF.

transcription factor (HSF, see Zuo *et al*, 1994), a protein with a similar distribution of leucine zippers and DNA binding domains to that present in mTERF.

An aspect generated by this hypothesis that deserves special attention is the mechanism responsible for the transition between the monomer and the polymer. Once again inspired by HSF, experiments were designed to study the possible role of phosphorylation. Gel filtration fractions from S-100 obtained from HeLa cells containing either form were incubated with a battery of serine/threonine and tyrosine protein phosphatases and subsequently analyzed by native-PAGE followed by Western blotting with anti-mTERF antiserum. This approach failed to show any sensitivity of neither form to dephosphorylation (data not shown), suggesting that phosphorylation is not involved in the transition between both forms of mTERF

It is obvious that understanding the control of mTERF activity is still an open field, and much work needs to be done. Confirmation of the homotrimeric nature of the polymer will require identification of the peptide migrating immediately below mTERF in the 2D electrophoresis by mass spectrometry, as well as two-hybrid analysis. The final proof about the role of leucine-zippper interactions in the adoption of one form or the other, as well as the identification of the exact nature of such interactions, will be provided by X-ray christallography. As concerns the mechanism responsible for the transition between the two forms, very little is known, and the role of co-factors and post-translational modifications, including phosphorylation, will have to be considered. Our contribution to this particular point has been essentially methodological, through the optimization of a native-PAGE in conditions that allow to distinctly separate and identify each form.

CONCLUSIONS

1. The immunization of a rabbit with pure His-mTERF rendered anti-mTERF antiserum that has been successfully used for Western blot analysis throughout this project.
2. mTERF from HeLa mitochondrial lysate S-100 elutes in two forms from a gel filtration column. The estimated sizes for each form are 41+/-1.73 kDa and 111.3 +/-4.62 kDa.
3. Band-shift assays, Western blot of the shifted bands and heparin chromatography show that the low molecular weight form but not the high molecular weight form binds to the mTERF DNA binding site.
4. *In vitro* transcription experiments show that the low molecular weight form has transcription termination activity at the mTERF binding site.
5. From the previous data it is reasonable to conclude that mTERF exists in two forms, a monomer with DNA-binding and transcription-termination activities, and an inactive polymeric form.
6. The optimization of native-PAGE followed by analysis by Western blotting will be a very useful tool to study the mechanism responsible for the transition between the monomeric and the polymeric forms of mTERF.

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