The human mitochondrial transcription termination factor (mTERF) is a multizipper protein but binds to DNA as a monomer, with evidence pointing to intramolecular leucine zipper interactions

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The human mitochondrial transcription termination factor (mTERF) cDNA has been cloned and expressed *in vitro*, and two alternative precursors of the protein have been imported into isolated mitochondria and processed to the mature protein. The precursors contain a mitochondrial targeting sequence, and the mature mTERF (342 residues) exhibits three leucine zippers, of which one is bipartite, and two widely spaced basic domains. The *in vitro* synthesized mature protein has the expected specific binding capacity for a double-stranded oligonucleotide containing the tridecamer sequence required for directing termination, and produces a DNase I footprint very similar to that produced by the natural protein. However, in contrast to the latter, it lacks transcription termination-promoting activity in an *in vitro* system, pointing to another component(s) being required for making mTERF termination-competent. A detailed structure–function analysis of the recombinant protein and mutagenized versions of it by band shift assays has demonstrated that both basic domains and the three leucine zipper motifs are necessary for DNA binding. Furthermore, a variety of tests have shown that both the recombinant and the natural mTERF bind to DNA as a monomer, arguing against a dimerization role for the leucine zippers, and rather pointing, together with the results of mutagenesis experiments, to intramolecular leucine zipper interactions being required to bring the two basic domains in close register with the mTERF target DNA sequence.

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Introduction

A large amount of evidence from *in vivo* and *in organello* studies (Cantatore and Attardi, 1980; Montoya *et al.*, 1982, 1983; Yoza and Bogenhagen, 1984; Gaines and Attardi, 1984a,b; Gaines *et al.*, 1987) has indicated that the mechanism underlying the 20- to 50-fold higher

expression of the rRNA gene region relative to the downstream genes transcribed from the heavy (H) strand of mitochondrial DNA (mtDNA) (Gelfand and Attardi, 1981) involves the differential activity of two independently controlled overlapping transcription units starting at two closely located initiation sites in the D-loop region (Montoya *et al.*, 1983). Besides being regulated at the level of transcription initiation, this differential expression of the two transcription units involves an attenuation phenomenon at the border between the 16S rRNA and tRNA^{Leu(UUR)} genes (Christianson and Clayton, 1988; Kruse *et al.*, 1989). A central role in this attenuation is played by the mitochondrial transcription termination factor (mTERF), a DNA-binding protein that protects a 28 bp region within the tRNA^{Leu(UUR)} gene at a position immediately adjacent to and downstream of the 16S rRNA gene (Kruse *et al.*, 1989); this region comprises a tridecamer sequence critical for directing accurate termination (Christianson and Clayton, 1988). In an *in vitro* transcription system utilizing a mitochondrial lysate, mTERF has been found to promote termination at the 16S rRNA–tRNA^{Leu(UUR)} gene boundary of the H-strand transcripts starting at the rRNA-specific initiation site (Kruse *et al.*, 1989).

A molecular characterization of mTERF recently has shown that its specific footprinting activity is associated with three sequence-related polypeptides, i.e. two polypeptides of ~34 kDa and a polypeptide of ~31 kDa, whereas the termination-promoting activity appears to reside only in the 34 kDa components (Daga *et al.*, 1993). Understanding of the functional role of mTERF has acquired special significance after the demonstration that an A→G transition in the middle of the mTERF-protected mtDNA segment is associated with the MELAS encephalomyopathy (Goto *et al.*, 1990; Kobayashi *et al.*, 1990), with progressive external ophthalmoplegia (PEO) (Johns and Hurko, 1991) and with some forms of adult onset diabetes (van den Ouweland *et al.*, 1992), and that this mutation dramatically reduces the binding affinity of mTERF for its target sequence (Hess *et al.*, 1991; Chomyn *et al.*, 1992).

In the present work, the mTERF cDNA has been cloned and sequenced, and the NH₂-termini of the precursors and of the mature protein have been identified. The mature recombinant protein, while exhibiting the expected specific DNA-binding capacity, is unable to promote transcription termination in an *in vitro* system, pointing to another component(s) being required for termination activity. Furthermore, a detailed structure–function analysis of the *in vitro* synthesized mTERF has provided evidence for a novel DNA-binding motif, in which three leucine zippers form an intramolecular three-stranded coiled-coil that brings two widely separated basic domains in close register with the mTERF target DNA sequence.

Results

Cloning and sequencing of mTERF cDNA

Preliminary sequence analyses carried out on peptides obtained by trypsin digestion and reverse-phase HPLC fractionation from the two ~34 kDa polypeptides (34 Ka and 34 Kb) identified in affinity-purified mTERF yielded only short peptide sequences (R.Aebersold, unpublished data), which proved to be unsuitable for cloning the mTERF cDNA. Therefore, another series of sequence analyses was carried out on peptides obtained by endopeptidase Lys-C digestion and reverse-phase HPLC fractionation from the 34 Ka component. These analyses yielded three peptide sequences of 17, 23 and 33 amino acids (solid underlines in Figure 1b).

As detailed in Materials and methods, a particular combination of two degenerate oligodeoxynucleotide mixtures derived from the peptide sequences mentioned above, when used to amplify cDNA obtained from HeLa cell poly(A)⁺ RNA, yielded a 125 bp specific product (Figure 1a). Primers designed from this product were then used to apply the rapid amplification of cDNA ends (RACE) protocols (Apte and Siebert, 1993). Primers derived from the 5' end and 3' end RACE products thus obtained finally allowed the production by PCR from HeLa cell poly(A)⁺ RNA of a single fragment containing most of the mTERF cDNA (S52xAS1) (Figure 1a). In other experiments, the 5' end and 3' end RACE fragments were utilized to screen ~2×10⁶ plaques from a λgt11 HeLa cell cDNA library. This screening yielded five putative positive clones with two different types of overlapping inserts (Figure 1a). The products of the RACE protocols and the PCR-amplified inserts from the positive λgt11 clones were cloned in the TA vector, and then sequenced on both strands, following the strategy shown in Figure 1a.

The concordant sequences obtained from the RACE products and λ gt11 library-derived clones revealed a cDNA of ~2 kb, containing an open reading frame (ORF) of 399 amino acids, with a 5'-untranslated region (UTR) of 77 bp and a 3'-UTR of 712 bp (Figure 1b). The latter contains a poly(A) addition signal, and is followed by a poly(A) tail 25 bp downstream from the signal. The ORF includes not only the three long peptide sequences obtained from the endopeptidase Lys-C digestion products of the purified 34 Ka component, but also three of the short peptide sequences previously obtained from the trypsin digestion products of the 34 Ka and 34 Kb components (dashed underlines in Figure 1b).

Identification of the NH₂-termini of the mTERF precursors and of the mature protein

The ORF was expected to encode the precursor(s) of mTERF, including the mitochondrial targeting sequence. The first 90 amino acids encoded in the ORF contain six methionine residues, one or more of which could be the initiator amino acid(s) of the mTERF precursor(s) (Figure 1b). In order to identify this precursor(s) and the NH₂-terminus of the mature protein, *in vitro* transcription-translation assays in a coupled reticulocyte lysate system, and import/processing reactions utilizing isolated HeLa cell mitochondria were carried out, as described in Materials and methods

Fig. 1. mTERF cDNA cloning and sequence. **(a)** Scheme of the cDNA clones isolated by RT-PCR of HeLa cell poly(A)⁺ RNA and by screening a λ gt11 cDNA library. In the upper part of the figure, the entire mTERF cDNA is represented. The black bar represents the protein-coding region, the open bars, the 5'- and 3'-untranslated regions; A_n: poly(A) tail. The arrows represent the direction and extent of sequencing from the indicated sites; the open blocks at the origins of the arrows indicate the positions of hybridization of the synthetic oligodeoxynucleotide primers used for sequencing. **(b)** mTERF cDNA nucleotide sequence and derived protein sequence. The sequences of the peptides obtained by digestion of the purified protein are shown by a single continuous underline (endopeptidase Lys-C) or a dashed underline (trypsin). The positions of the putative leucine zippers are shown by a double underline, with the residues at the d positions boxed. The two possible initiator methionine residues for the precursors are circled, and the processing site is shown by an arrow preceding the boxed first amino acid of the mature protein. The asterisk represents the first stop codon. The polyadenylation signal in the DNA sequence is boxed.

As shown in Figure 2a, right panel, when a construct containing the entire ORF for the 399 amino acid protein without any modifications (P1) was used in a coupled

transcription/translation system, two main products were obtained. It seemed very likely that the slower moving polypeptide (p1) started at the first AUG codon, and that its lower abundance was due to the fact that this codon is located in a suboptimal consensus sequence context (Kozak, 1986). A similar argument suggested that the more abundant, faster moving polypeptide (p2) started at the second AUG, which is situated in an optimal sequence context. This tentative identification of the initiation codons of the two main polypeptides synthesized in the coupled transcription-translation system was confirmed by radioactive sequencing from the NH₂-terminus of the faster moving polypeptide labeled with [³⁵S]methionine (data not shown). Further evidence supporting the above

identification was obtained by using two new templates for *in vitro* protein synthesis, one, in which the sequence surrounding the first AUG was modified, as described in Materials and methods, to optimize it for translation initiation (construct P1m), and the second one, in which the sequence upstream of the second AUG codon was removed (construct P2) (Figure 2a, left panel). As shown in Figure 2a, right panel, construct P1m yielded again, in the *in vitro* system, the two polypeptides previously obtained from the P1 template; however, in this case, the slower moving polypeptide (p1m) had become the strongly predominant one. On the other hand, construct P2 yielded as the major product the slower moving polypeptide (p2).

In order to identify the processing site of the mTERF precursor(s), import/processing reactions were carried out in which each of the two putative precursors was labeled *in vitro* with an amino acid, and then incubated with isolated HeLa cell mitochondria. As shown in Figure 2b, both [³⁵S]methionine-labeled precursors synthesized from the P1m construct (p1m and p2, lane 1) and that synthesized from the P2 construct (p2, lane 3) yielded, after incubation with the organelles, two polypeptides (lanes 2 and 4, respectively) with the same electrophoretic mobilities as observed for the purified *in vivo* synthesized 34 Ka and 34 Kb components (not shown). The amount of mature protein imported into mitochondria from the p2 precursor was about twice that from the p1m precursor. The import/processing reaction was ATP dependent (lane 5); furthermore, it was inhibited by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (lane 6), as expected from the requirement for a membrane potential for mitochondrial import. The processed products, in contrast to the precursors, were resistant to trypsin treatment of the mitochondria (lanes 7 and 8), but became protease sensitive after solubilization of the membranes with Triton X-100 (lane 9). The above properties are characteristic of a

Fig. 2. Import into mitochondria and processing of mTERF precursors, and analysis of the mature products. (a) Left: schematic representation of the different mTERF precursor and mature protein constructs; the precursor presequences are shown by shaded boxes. The numbers represent the amino acid positions, according to the numbering system used in Figure 1b. The asterisk indicates the substitution of the first amino acid in mTERF (phenylalanine) by a methionine residue (mTERF_m). Right: SDS-PAGE analysis of the translation products synthesized, in the presence of [³⁵S]methionine, using as templates the constructs represented in the left panel. The positions of molecular mass markers of 46 and 30 kDa are indicated. (b) SDS-PAGE analysis of the translation products from constructs P1m (lanes 1 and 2) and P2 (lanes 3–9), analyzed directly (lanes 1 and 3, respectively) or after incubation with isolated HeLa cell mitochondria for 30 min at 37°C under different conditions (lanes 2 and 4–9). Trypsin was used at 5 (+) or 10 µg/ml (++). (c) Radioactive sequencing from the NH₂-terminus of the mature 34 Ka mTERF product isolated after import into mitochondria. The [³H]lysine- or [³⁵S]methionine-labeled mature products obtained in large-scale import experiments were transferred onto PVDF membranes after SDS-PAGE. The band corresponding to the slower moving mature product (34 Ka) was excised and sequenced. The radioactivity eluted at each cycle of Edman degradation is represented (□: [³H]lysine c.p.m.; ♦: [³⁵S]methionine c.p.m.). The arrows indicate the cycles corresponding to the positions of the lysine (K) and methionine (M) residues. The deduced position of the processing site in the precursor sequence is shown by a thick arrowhead in the upper part of the panel. (d) Alkylation experiment. SDS-PAGE analysis of samples of mitochondrial lysates obtained after import experiments carried out with the products of construct P1 or of *in vitro* synthesized mTERF_m, untreated (C) or after treatment with DTT and iodoacetamide (Alkyl.).

typical mitochondrial import reaction (Schatz, 1987; Hartl *et al.*, 1989). The finding that both precursors yielded mature products with the same electrophoretic mobilities strongly suggested that they were processed at the same site.

To determine the precise position of the processing site, radioactive sequencing was performed from the NH₂-terminus of the processed products obtained in large-scale import experiments, in which the precursor had been labeled with different amino acids. As shown in Figure 2c, in an experiment in which the product of construct P1m labeled with [³H]lysine was used as a precursor, and the 34 Ka component sequenced, the first radioactivity peak was obtained at cycle 4, corresponding precisely to the position of the third lysine in the precursor, with two other peaks at cycles 15 and 21, i.e. one cycle after the positions of the fourth and fifth lysine (14 and 20, respectively). The appearance of radioactivity at cycle 3 (anticipation) suggests a certain NH₂-terminal heterogeneity of the sequenced product, due possibly to processing imprecision. On the other hand, the displacement by one fraction of the radioactivity peaks at cycles 15 and 21 relative to the positions of the corresponding lysine residues reflects the well known background due to incomplete Edman degradation (Brown *et al.*, 1991). When the precursor was labeled with [³⁵S]methionine, a peak was obtained at position 27, with the first jump in counts over the background occurring at cycle 25. These results, together with others obtained in identical experiments using a [³H]leucine-labeled precursor (not shown), strongly support the conclusion that the processing site is located between residues -1 (leucine) and +1 (phenylalanine) of the precursor, according to the numbering system used in Figure 1b. Independent sequencing experiments using the 34 Kb processed product gave similar results, indicating that the 34 Ka and 34 Kb polypeptides share the same NH₂-terminus.

In conclusion, the presequence of the *in vitro* synthesized mTERF is either 57 or 37 amino acids long, and it contains a large proportion of hydrophobic, hydroxylated and positively charged residues (with only one negatively charged residue) and an arginine at position -2. These features are characteristic of most presequences found in yeast and mammalian mitochondrial proteins (Hartl *et al.*, 1989). The fact that about twice as much mature product appears to be imported into mitochondria from the p2 precursor as from the p1m precursor may reflect a greater efficiency in promoting import of the p2 presequence relative to the p1m presequence, in agreement with its higher relative content of basic residues (6/37 versus 7/57). Further work will be necessary to determine whether the two precursors identified *in vitro* are also synthesized *in vivo*, and whether p2 is also the major precursor *in vivo*. The mature mTERF is 342 amino acid long, and has a calculated M_r of ~39 kDa. The discrepancy from the apparent molecular mass (~34 kDa), estimated from the migration in SDS-polyacrylamide gels, is due either to an aberrant electrophoretic mobility, exhibited also by the precursors (Figure 2a and b), or to an unidentified secondary modification(s) of the polypeptides, that also occurs in the reticulocyte lysate.

The two 34 kDa electrophoretic components of mTERF are differentially denatured forms of the same polypeptide

The observation described above that both the 34 Ka and 34 Kb components were produced by *in vitro* processing of the same precursor confirmed their relatedness in primary sequence previously indicated by the spectroscopic analysis of their tryptic peptides (Daga *et al.*, 1993). In the work cited above, considerable attention was devoted to the question of the relationship between the two ~34 kDa polypeptides, and the conclusion was reached that a different degree of phosphorylation was not responsible for their difference in electrophoretic mobility. The finding that the relative proportions of the two components varied between different mTERF preparations, and even in the same preparation, depending upon the conditions of gel electrophoresis, strongly suggested the possibility that secondary or tertiary structure differences, due to incomplete denaturation of the sample, could be the basis for the difference in electrophoretic mobility of the two ~34 kDa polypeptides. In order to obtain evidence concerning this possibility, alkylation experiments were performed. Samples of mitochondrial lysate obtained after the import/processing reaction or samples of an *in vitro* synthesized mature version of mTERF [mTERFm, constructed by substituting the NH₂-terminal phenylalanine with a methionine residue (Figure 2a)] were treated first with dithiothreitol (DTT) as a reducing agent, and then with iodoacetamide as an alkylating agent, and subsequently were analyzed by SDS-PAGE in parallel with untreated controls. As shown in Figure 2d, the alkylation treatment produced in both kinds of samples a clear shift of material from the slower moving to the faster moving band, indicating that the first represents the less denatured form, and the second, the more denatured one. This result suggests that the protein tends to have a very strong tertiary structure, probably stabilized by internal S-S bonds, that the DTT and alkylation treatments are able to disrupt to a great extent (Chomyn *et al.*, 1988).

Functional analysis of mTERFm

In order to investigate the functional capacity of the recombinant mTERF (mTERFm) synthesized in the coupled transcription-translation system, its DNA-binding properties and its transcription termination-promoting activity in an *in vitro* system were analyzed. The binding capacity of mTERFm was first determined by gel shift assays using a 3'-³²P-labeled double-stranded 44mer DNA probe that contains the mTERF-binding site (Daga *et al.*, 1993). The recombinant wild-type version of the protein (mTERFm) was able to bind the probe, producing a single retarded band (Figure 3a) with the same electrophoretic mobility as the one produced by the *in vivo* protein purified by affinity chromatography (Figure 3b). By contrast, no complex formation was detected using a probe containing a 4 bp deletion comprised within the tridecamer sequence necessary for directing accurate transcription termination (Christianson and Clayton, 1988), indicating the specificity of the interaction (Figure 3a). Increasing the amount of mTERFm over a >30-fold range produced an increase in the intensity of the retarded band obtained with the wild-type probe, in parallel with a progressive shift of up to ~60% of the probe, without the appearance of any more

Fig. 3. Functional properties of mTERFm. (a) Mobility shift assays using the *in vitro* synthesized wild-type version of mTERFm and a wild-type 32 P-labeled double-stranded 44mer oligodeoxynucleotide probe (WT) or a probe with a 4 bp deletion within the binding site (Del). C, protein-DNA complex; F, free probe. (b) Mobility shift assays using *in vivo* synthesized mTERF or increasing amounts of *in vitro* synthesized mTERFm (0.5–16 μ l of reticulocyte lysate) and the wild-type DNA probe, with a minus protein (–) and a non-programmed reticulocyte lysate control (Retic.lys.). (c) DNase I footprinting of the H-strand of the mtDNA termination region, carried out using purified natural mTERF (13 ng) and purified *in vitro* synthesized mTERFm (13 and 26 ng), with 'DNA only' as a control. (d) Assays of transcription termination activity using different amounts of purified natural mTERF and purified *in vitro* synthesized mTERFm. R, runoff transcripts; T, terminated transcripts; Term %, percentage of termination activity.

slowly moving secondary band (Figure 3b). This result argued against the formation of different complexes of mTERFm with the probe.

More conclusive evidence concerning the specificity of interaction of mTERFm with DNA was obtained by a DNase I footprinting analysis. As shown in Figure 3c, the pattern of DNase I protection of the H-strand obtained with the *in vitro* synthesized recombinant protein is very similar to that produced by the *in vivo* synthesized mTERF, differing from it for some non-protected or hypersensitive bands in the segments bordering the critical tridecamer sequence (positions 3237–3249) and in the region 3260–3270.

Figure 3d shows the results of termination assays carried out with equivalent amounts of recombinant and natural mTERF proteins. It is clear that the recombinant protein lacks transcription termination-promoting activity almost completely. The significance of this observation will be discussed below.

Structure–function analysis of mTERFm by DNA-binding assays

The hydropathy profile failed to show the presence of a possible membrane-spanning segment of 20 or more hydrophobic residues. A database search of the amino acid sequence of mTERF did not reveal any significant

similarity with known sequences. The most evident feature of the primary sequence of this protein is the presence of multiple hydrophobic heptad repeats or leucine zipper motifs (doubly underlined in Figure 1b). The most typical one is Lz3, situated near the COOH-terminus between residues 292 and 326, in which the sequence X_3LX_3 is repeated five times, with the expected preponderant hydrophobic residues at the a (3/5) and, especially, at the d position (5/5) of the heptad repeats [the residues of the heptad being designated as a–g (Landschulz *et al.*, 1988; Hurst, 1995)] (Figure 1b). Another potential leucine zipper motif (Lz2), with five heptad repeats, a preponderance of hydrophobic residues at the a (3/5) and the d position (4/5), and one valine and one asparagine substitution for leucine at the d position of the second and fourth heptad, respectively, occurs between residues 185 and 219. Furthermore, in the segment between positions 116 and 171, extensive mutagenesis experiments, to be discussed below, have revealed that the two-heptad repeat between residues 116 and 129 (Lz1a) and the three-heptad repeat between residues 151 and 171 (Lz1b), which also have a preponderance of hydrophobic residues at the a and d positions (4/5), and which are separated by a 21 residue loop, play an essential role in DNA binding of mTERF.

There is no basic domain sequence in the region adjacent on the NH_2 -end side to any of the three leucine zipper

Fig. 4. DNA-binding properties of deleted mTERFm versions. **(a)** Schematic representation of the different wild-type (mTERFm) and deleted constructs used, and observed DNA binding values. The leucine zipper domains are indicated by hatched boxes, the two basic regions B1 and B2, by black boxes. The numbers represent the amino acid positions, according to the numbering system used in Figure 1b. The binding activity values are the averages of two independent experiments, and are expressed as percentages of the value observed for mTERFm, after normalization for differences in efficiency of translation (see Materials and methods). **(b)** Mobility shift assays using two different amounts of lysate (1 and 2 μ l), programmed with equivalent amounts of template corresponding to the different mTERFm deleted versions represented in (a), and the wild-type DNA probe. The DNA-protein complexes were analyzed on 5% native polyacrylamide gels. C, protein-DNA complex; F, free probe.

motifs, as in the typical bZip (basic leucine zipper) proteins (Hurst, 1995). However, the COOH-terminal 16 amino acid stretch adjacent on the COOH-end side to Lz3 (B2) contains seven basic amino acids, with only one acidic residue. Another region of the mTERF sequence rich in basic amino acids is the 22 amino acid stretch between residues 70 and 91 in the NH₂-terminus-proximal third of the protein, that contains seven basic and two acidic residues (B1). Apart from the two basic amino acid-rich domains mentioned above, no clear putative DNA-binding motif was detected in the mTERF sequence.

In order to gain insights into the role of the different regions of mTERF in its DNA-binding activity, a series of deletion mutants were derived from mTERFm (Figure 4a), since this has been shown to have the same binding properties as the natural mTERF. Two deletion constructs were designed to produce NH₂-terminal and COOH-terminal truncated versions of the protein (Δ N and Δ C), lacking 30 and 34 amino acids, respectively. The latter deletion at the same time disrupts the COOH-terminus-proximal leucine zipper and removes the COOH-terminal

basic region. Construct Δ B1 contains a deletion from amino acids 77 to 104 that removes five out of the seven positively charged residues present in the basic region B1, and construct Δ B2 lacks the 15 COOH-terminal residues, including all seven positively charged ones. Another construct, Δ Lz3 (lacking amino acids 301–317), was made in order to test the effect of specifically disrupting Lz3 on the binding activity of the protein (Figure 4a).

Among the mutated versions of mTERFm described above, only Δ N retained some binding activity (~20%, as estimated from densitometric measurements of the autoradiogram, after normalization for differences in translation efficiency), while all the other deleted recombinant proteins had completely lost their binding activity (Figure 4a and b). In the case of Δ C, Δ B1 and Δ B2, the loss of binding activity could be explained by the removal of a domain that presumably interacts with DNA. On the contrary, the observation that Δ Lz3 did not bind to DNA was more difficult to interpret.

In order to explore further the role of the basic regions B1 and B2 and to have some insights into the function of

Fig. 5. DNA-binding properties of point mutants of mTERFm. (a) The top line illustrates the organization in mTERFm of identified domains required for DNA binding. Below are shown a schematic representation of the different mTERFm point mutants with amino acid changes in single domains and the observed values for their binding activity. The Lz1a and Lz1b putative heptad repeats are underlined. The amino acid substitutions in each mutant are indicated. The binding activity was determined and is expressed as described in the legend of Figure 4a. (b) Shown are mTERFm point mutant versions with simultaneous changes in more than one leucine zipper domain and the observed values for their DNA-binding activity.

the three leucine zipper motifs in the DNA-binding activity of mTERF, a series of mutated versions of mTERFm containing single, double or triple residue substitutions in these domains were carried out by site-directed mutagenesis. A substitution of the lysine and arginine residues at positions 84 and 85 in the basic region B1 by a glutamic acid and a glycine residue, respectively (construct B1 KR→EG), and a substitution of the two lysine residues at positions 336 and 337 in the basic region B2 by an asparagine and a glutamic acid residue (construct B2 KK→NE) dramatically reduced the DNA-binding activity of mTERFm (Figures 5a and 6a), pointing to a direct role for B1 and B2 in DNA binding. Mutant L97,103VV, which contains two valine residues at positions 97 and 103 substituting for two leucine residues, outside any identified structural domain, had a normal DNA-binding capacity (Figures 5a and 6a).

In the region of potential adjacent leucine zippers between positions 116 and 171, a substitution of the

Fig. 6. Mobility shift assays utilizing the *in vitro* synthesized wild-type version of mTERFm or the different point mutant versions represented in Figure 5. The DNA-protein complexes were analyzed on 5% native polyacrylamide gels. Only the band corresponding to the protein-DNA complex observed in each lane is shown.

leucine residues 119 (L1) and 126 (L2) by two valine residues (construct Lz1aL1,2VV), or of leucine 126 (L2) by a proline residue (construct Lz1aL2P), of the asparagine and leucine residues at positions 154 (N1) and 161 (L2) by an alanine and, respectively, a valine residue (construct Lz1bN1L2AV), or of the leucine residues 161 (L2) and 168 (L3) by two valine residues (construct Lz1bL2,3VV) dramatically reduced or completely abolished the DNA-binding activity of mTERFm (Figures 5a and 6b). By contrast, a substitution of leucine 161 (L2) by a valine residue (construct Lz1bL2V), or a substitution of arginine 133 and leucine 140 by an alanine and, respectively, a valine residue (construct R133L140AV), or of leucine residues 140 and 147 by two valine residues (construct L140L147VV) left the DNA-binding capacity of mTERFm unaffected or nearly so (Figures 5a and 6a and b). A substitution of the leucine residues 147 and 161 (L2) by two valine residues (construct L147VLz1bL2V) reduced mTERFm binding to DNA only moderately (Figures 5a and 6b). These results, as well as the presence at position 144 of a proline residue (Figure 1b), which is expected to disrupt the α -helix of a leucine repeat (Chou and Fasman, 1973; Turner and Tijan, 1989), strongly suggested that the region 116–171 contains two potential short heptad repeats: a two-heptad repeat, Lz1a, at the NH₂ end and a three-heptad repeat, Lz1b, at the COOH

end, that could be two portions of a bipartite leucine zipper, separated by a 21 residue loop (see below).

In the potential leucine zipper at positions 185–219 (Lz2), a substitution of the leucine at position 202 (L3) by valine (construct Lz2L3V) did not have any significant effect on DNA binding of mTERFm, whereas substitution of the same leucine by proline (construct Lz2L3P) completely abolished the DNA-binding capacity of the protein. A substitution of L3 by valine and of either leucine 188 (L1) by alanine (construct Lz2L1,3AV) or leucine 216 (L5) by alanine (construct Lz2L3,5VA) reduced to ~10 or ~25%, respectively, the DNA-binding activity of mTERFm, while substitution of L1, L3 and L5 (construct Lz2L1,3,5AVA) produced an almost complete loss of DNA binding (Figures 5a and 6c). These results strongly supported a leucine zipper role for Lz2.

In the leucine zipper Lz3, a substitution of leucine residue 316 (L4) by an alanine (construct Lz3L4A) left the binding capacity of mTERFm unchanged relative to that of the wild-type protein. By contrast, a substitution of leucine residue 302 (L2) by a proline (construct Lz3L2P) completely abolished DNA binding. Constructs Lz3L2,3VA and Lz3L2,4VA, which have L2 substituted by a valine and leucine residue 309 (L3) or 316 (L4), respectively, substituted by an alanine, retained only ~8 and ~15%, respectively, of the binding capacity of mTERFm, and construct Lz3L2,3,4VAA, which has L2, L3 and L4 substituted by a valine and two alanine residues, has almost completely lost it (Figures 5a and 6d).

In the DNA-binding assays described above, no gross structural alteration was expected to be produced by the valine and alanine substitutions for leucine residues in mTERFm, as opposed to the substitution of a leucine by the α -helix disrupter proline. Therefore, the greatly reduced DNA-binding activity of the mutant constructs containing two or three leucine substitutions by valine or alanine residues strongly suggested that some specific interaction of the leucine zippers is necessary for sequence-specific binding of mTERFm to DNA. In particular, the results pointed to the possibility that either intramolecular interactions between the leucine zippers are required for maintaining the appropriate tertiary configuration of the protein to bring its basic domains in register with the DNA recognition sequence, or, alternatively, that a di- or oligomerization of the protein is necessary for DNA binding.

Evidence for intramolecular interaction of the leucine zippers

In order to obtain evidence concerning possible intramolecular interactions between two or three of the leucine zippers identified in the mTERF sequence, single leucine substitutions by a valine or alanine residue in two or three of the heptad repeats, which by themselves did not have any effect on DNA binding of the protein, were combined in the same molecule (Figure 5b). As shown in Figure 6e, substitution of L2 in Lz1b and L3 in Lz2 by valine residues strongly decreased (by ~80%) the DNA binding of mTERFm. Similarly, substitution of L3 in Lz2 by a valine and of L2 in Lz3 by a valine or L4 in Lz3 by an alanine reduced by ~84 and 66%, respectively, the mTERFm binding to DNA. Substitution of L2 in Lz1b and L4 in Lz3 by a valine and, respectively, an alanine

residue decreased the DNA binding of mTERFm by ~73%. Finally, substitution of L2 in Lz1b and L3 in Lz2 by valine residues and of L4 in Lz3 by an alanine residue dramatically decreased (by ~92%) the DNA binding of mTERFm. By contrast, substitution of L147 and of L3 in Lz2 by valine residues caused only a moderate reduction (by ~30%) in the DNA binding of the protein. These results are fully consistent with the idea that intramolecular interactions of Lz1b, and presumably Lz1a, with Lz2 and Lz3 are required for mTERFm to take the proper conformation that allows binding of the B1 and B2 domains to the target DNA sequence.

mTERFm binds to mtDNA as a monomer

Although the results discussed in the previous section strongly suggested an intramolecular interaction of the leucine zippers of mTERF as being required for DNA binding, they did not exclude the possibility that mTERF binds to DNA as a homodimer or homo-oligomer. As described in a previous section, band shift assays using mTERFm and a DNA probe containing the specific binding site had revealed a single retarded band, even when large amounts of the protein were used which shifted up to 60% of the probe. Whether this band was produced by a monomer or a dimer (or higher oligomer) of the protein was investigated by three different approaches. First, direct measurements of the molar ratio of mTERFm to DNA were made in mobility shift experiments in which the [³⁵S]methionine-labeled protein was incubated with the ³²P-labeled DNA probe. The band corresponding to the complex was excised from the dried gel, and the ³⁵S and ³²P radioactivity determined. From the specific activities of the protein and DNA, their molar ratio in the complex could thus be calculated. The value obtained in six separate runs, utilizing different ratios, over a 4-fold range, of mTERFm to probe, was 0.94 ± 0.05 (mean $\pm$ SE). As a second approach, advantage was taken of the fact that Δ N binds to the DNA probe, and produces a retarded complex moving slightly faster than the wild-type protein-containing complex (Figure 4b). Thus, a mixture of mTERFm and Δ N (synthesized either separately or in the same transcription–translation reaction) was used to perform mobility shift experiments with the 3'-end-labeled probe. As shown in Figure 7a, using different proportions of the two proteins, only two retarded bands, with the same electrophoretic mobility as the ones produced by the two proteins alone, were obtained, and no third intermediate band was observed, as would be expected in the case of a dimer (Murre *et al.*, 1989). In a similar kind of experiment, a 4-fold excess of the product of mutant Δ B2, which lacks only the 15 COOH-terminal residues of mTERFm and has no DNA-binding activity, failed to compete for the binding of the wild-type mTERFm (data not shown), arguing also against the binding of the protein as a homodimer.

To test the homodimer or homo-oligomer hypothesis, as well as the possibility of formation of heterodimers or hetero-oligomers, cross-linking assays were carried out. For these purposes, the *in vitro* synthesized mTERFm was treated with different concentrations of the chemical cross-linking agents glutaraldehyde (GD) and disuccinimidyl suberate (DSS) in the absence or, to detect the possible formation of heterodimers or hetero-oligomers, in the

Fig. 7. mTERFm binds to mtDNA as a monomer. **(a)** Mobility shift assays using mixtures, in different proportions, of the wild-type and the NH₂-terminus truncated (ΔN) version of mTERFm, and the specific DNA probe. The protein–DNA complexes were analyzed on 10% native polyacrylamide gels. **(b)** SDS–PAGE analysis of *in vitro* synthesized mTERFm, after incubation in the absence (C) or presence of 0.01% glutaraldehyde (GD) or 1 mM disuccinimidyl suberate (DSS). **(c)** SDS–PAGE analysis of *in vitro* synthesized mTERFm, after incubation with the specific DNA probe (50 fmol), treatment with GD or DSS and separation of the protein–DNA complexes on a 1.5% native agarose gel. C, sample not treated with the cross-linker after incubation with the DNA probe. **(d)** Mobility shift assays using purified natural mTERF and purified *in vitro* synthesized mTERFm. C, protein–DNA complex; F, free probe.

presence of a mitochondrial S-100 fraction. In spite of the different conditions tested, no evidence of formation of dimer (or higher oligomer) was obtained with this approach (see, for example, Figure 7b), although the presence of high molecular weight aggregates was observed (the doublet in the DSS-treated sample presumably results from intramolecular cross-linking in a portion of mTERFm). Other cross-linking experiments were carried out in the presence of the oligodeoxynucleotide probe. One such experiment is illustrated in Figure 7c. In this experiment, the [³⁵S]methionine-labeled mTERFm was first incubated with the unlabeled probe under the conditions used in the band shift experiments, in order to allow the formation of protein–DNA complexes, and then treated with either GD or DSS under conditions which previously had been shown to produce cross-linking of the proteins (Figure 7b). Following the cross-linking reaction, the protein–DNA complexes were separated on a 1% native agarose gel, in parallel with complexes formed between mTERFm and 3′-³²P-labeled probe. After the run, the portion of the gel containing the ³⁵S-labeled protein–DNA complexes, identified from the position of the ³²P band, was excised, melted and the complexes run on a denaturing SDS–polyacrylamide gel to identify the protein forms present in the complex, as detailed in Materials and methods. As shown in Figure 7c, no trace of a band that

could correspond to a cross-linked dimer was observed. Taken together, the three kinds of approaches discussed above provided convincing evidence that the retarded band obtained with mTERFm was produced by a monomer of the protein bound to the probe.

To test whether the conclusion that mTERFm binds to DNA as a monomer also applies to the natural mTERF, different amounts of the latter and of the recombinant mTERFm were subjected to band shift assays using a 3′-³²P-labeled probe. As shown in Figure 7d, the natural protein produced a single retarded band with the same mobility as that produced by the recombinant protein. However, the most significant observation was that the natural protein exhibited only a monomer band when tested at a concentration that supported transcription termination *in vitro* (Figure 3d). The *K_d* for the binding to DNA of the natural mTERF and the recombinant mTERFm was estimated from the data of Figure 7d to be in the range of 50–100 nM.

Discussion

In the present work, the cloning, sequencing and *in vitro* expression of mTERF has provided significant insights into the structure and function of this factor. The recombinant mature protein was found to have the expected specific

DNA-binding capacity, but, as synthesized in the coupled transcription–translation system, or by itself, was not able to support transcription termination. This observation indicates that either a secondary modification of the primary translation product or another component present as a contaminant in the affinity-purified natural product is necessary to confer upon the latter the terminating activity. In agreement with this conclusion is the finding that the recombinant protein produced a footprint very similar, but not identical, to that produced by the natural protein. This conclusion is also supported by previous results obtained during the optimization of the *in vitro* transcription system, showing that KCl concentrations which did not affect transcription initiation or mTERF binding to DNA strongly reduced transcription termination (P.Fernandez-Silva, unpublished results). These observations indicate that the termination process is more complex than previously thought, requiring the participation of at least another component besides mTERF. The nature of this other component(s) is presently under investigation in our laboratory.

As concerns the structure of mTERF, the central observation made in the present work is its modular organization. Two widely separated basic regions and three leucine zipper motifs have been shown to be necessary for mTERFm binding to DNA. The three leucine zippers Lz1a/Lz1b, Lz2 and Lz3 have the typical features described for the hydrophobic heptad repeats, with a strong predominance of hydrophobic residues in the a and d positions (Landschulz *et al.*, 1988; Hurst, 1995). A deletion and point mutation analysis has clearly indicated that the B1 and B2 domains are involved in directly binding the protein to its target sequence. As to the essential role of the leucine zipper motifs in DNA binding of mTERF, strong evidence has been obtained indicating that both the recombinant and the natural protein bind to DNA as a monomer. This conclusion, which is consistent with the observation that the mTERF-binding site lacks a dyad symmetry (Kruse *et al.*, 1989), argues strongly against the three leucine zippers being required for the dimerization or oligomerization of the protein, as in typical bZip proteins (Hurst, 1995). Therefore, the most plausible interpretation is that the three leucine zippers of mTERF form an intramolecular three-stranded coiled-coil structure; this would allow mTERF to acquire the compact conformation required to bring the B1 and B2 basic domains in close register with the mTERF target DNA sequence (Figure 8).

Support for the model proposed above has come from the observation of a strongly decreased binding of the protein to its target sequence in assays utilizing mTERFm carrying simultaneously, in two or three separate zippers, single point mutations, each of which by itself had no effect on DNA binding. This 'cooperative' effect of substitutions of residues in the d position of the heptad repeats appears to be characteristic of double-stranded or triple-stranded coiled-coils, the stability and oligomerization state of which depends on multiple interhelical interactions involving residues at the a and d core positions of the repeats, as well as residues at the e and g positions (Harbury *et al.*, 1993; Krylov *et al.*, 1994; Nautiyal *et al.*, 1995; Woolfson and Alber, 1995). In the three leucine zippers of mTERF, the relative abundance of hydrophobic

Fig. 8. Proposed model of the role of the leucine zippers in DNA binding of mTERF. (a) Linear representation of mTERF structural features. (b) Proposed mechanism of DNA binding of mTERF. The model assumes that intramolecular interactions of Lz1a + Lz1b with Lz2 and Lz3 are required to bring the B1 and B2 basic domains in close register with the mTERF target DNA sequence.

residues as compared with hydrophilic residues at the e and g positions is consistent with a trimeric, rather than dimeric, coiled-coil structure (Harbury *et al.*, 1994; Woolfson and Alber, 1995). Of the three α -helices in the model shown in Figure 8, two, i.e. Lz2 and Lz3, have a parallel orientation, whereas Lz1a/Lz1b and Lz2, because of the short connection between them, have an antiparallel orientation. A structure similar to the latter has been described previously in seryl-tRNA synthetase (Cusack *et al.*, 1990) and in spectrins (Yan *et al.*, 1993), in which the antiparallel helices are adjacent in the primary sequence. It should be mentioned that the packing spaces of the core positions are very similar in parallel and antiparallel coiled-coils (Woolfson and Alber, 1995). Although final proof of the proposed model for mTERF will require X-ray crystallographic analysis, the evidence obtained in the present work concerning the unusual structure and DNA-binding mechanism of mTERF will open up new perspectives in the investigation and understanding of transcription termination in mammalian mitochondria.

The model proposed here of an mTERF conformation which brings together the B1 and B2 domains for DNA binding is reminiscent of that suggested for the RNA polymerase I (PolI)-specific transcription termination factor of yeast (Reb-1p), in which folding of the protein is assumed to produce two separate DNA-binding domains adjacent to each other and able to interact with a 15–20 nucleotide stretch (Morrow *et al.*, 1993). As to the detailed features of the proposed interactions of the leucine zippers in mTERF, Lz2 and Lz3 cannot make simultaneous contact with the two short heptad repeats (Lz1a and Lz1b) which have been shown to be essential for DNA binding. A plausible structure (shown in Figure 8) is one in which the 21 amino acid stretch between the two repeats forms a small loop. Although this proposed structure raises the question of the stability of the interaction of Lz1a and Lz1b with Lz2 and Lz3, it is quite possible that some features of the upstream sequence may help in stabilizing this interaction. The strong decrease in DNA-binding capacity of the NH₂-terminus truncated version of mTERFm (Δ N) (Figure 4b) is consistent with this possibility. On the other hand, it is conceivable that, *in vivo*, the contact between Lz1a/Lz1b and Lz2 and Lz3 may be influenced by the binding of mTERF to other proteins or

by the mitochondrial microenvironment. In a more general sense, the modular organization of mTERF may reflect the capacity of this protein to change its conformation and, therefore, its capacity to bind to DNA or to other proteins depending on the physiological conditions. It should be noted that the function of mTERF in mammalian mitochondria must be modulated in relation to the cellular needs for rRNA and mRNA synthesis, since mRNA synthesis demands an escape from termination of the H-strand transcripts at the 16S rRNA-tRNA^{Leu(UUR)} boundary (Montoya *et al.*, 1983). It is, therefore, an attractive idea that changes in the mTERF leucine zipper interactions may play a central role in this mTERF functional modulation. There is a precedent for a role of intramolecular interactions of leucine zippers in regulating DNA binding. In fact, in the heat shock factor from human and *Drosophila* cells, interactions between the COOH-terminal leucine zipper and the NH₂-terminal zippers suppress the ability of the factor to form trimers, a step which is required for nuclear localization and DNA binding (Rabindran *et al.*, 1993; Sheldon and Kingston, 1993; Westwood and Wu, 1993; Zuo *et al.*, 1994).

It is interesting to compare the mitochondrial transcription termination system with that involved in termination of transcription of the nuclear rRNA genes by PolII in mammalian cells and yeast. It has been shown previously that the latter system also involves the interaction of a DNA-binding protein [TTF1 in mouse cells (Grummt *et al.*, 1986; Evers *et al.*, 1995) and Reb-1p in yeast (Morrow *et al.*, 1993; Lang *et al.*, 1994)] with a terminator element downstream of the nascent transcripts. Structural and functional similarity has been demonstrated between TTF1 and Reb-1p (Evers *et al.*, 1995). Both mTERF and TTF1 induce DNA bending (Smid *et al.*, 1992; Shang and Clayton, 1994). However, there are significant functional differences between the mitochondrial and PolII systems. Thus, while TTF1-induced termination is strictly orientation dependent and appears to be PolII specific (Kuhn *et al.*, 1990), termination promoted by mTERF shows a biased polarity *in vitro* and does not appear to be strictly RNA polymerase specific (Shang and Clayton, 1994). Furthermore, a comparison of the mTERF sequence determined in the present work with the sequences of TTF1 and Reb-1p has failed to show any structural homology.

Materials and methods

Purification of mTERF and cDNA cloning

mTERF was purified from ~10 batches (~3×10¹⁰ cells each) of HeLa cells essentially as described previously (Daga *et al.*, 1993), except for the substitution of the detergent Nonidet P-40 by Tween-20 in the mitochondrial lysis step (Fernandez-Silva *et al.*, 1996). After the affinity chromatography purification, the eluates were concentrated 10× and dialyzed using Centricon-30 microconcentrators (Amicon) (Kruse *et al.*, 1989). For cloning purposes, the proteins were gel fractionated and electrotransferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes and stained with 1% amido black. The bands corresponding to the two sequence-related 34 kDa polypeptides which have been associated with specific DNA binding and transcription termination activities (Daga *et al.*, 1993) were excised, washed with HPLC-grade water and digested *in situ* with trypsin (Aebersold *et al.*, 1987) or endoproteinase Lys-C (W.Lane, Harvard Microchemistry Facility). The peptides were fractionated by reverse-phase HPLC and subjected to microsequencing. In preliminary experiments, in which the two 34 kDa components were digested separately with trypsin (Daga *et al.*, 1993), several short peptides were sequenced, yielding five continuous stretches

of 5–7 amino acids, that exhibited considerable codon degeneracy (R.Aebersold, unpublished data). Use of different combinations of degenerate oligodeoxynucleotide mixtures derived from these sequences for RT-PCR of poly(A)⁺ RNA from HeLa cells failed, however, to yield any specific product. In subsequent experiments, digestion with endopeptidase Lys-C of the upper 34 kDa component yielded longer polypeptides, from which three peptide sequences of 17, 23 and 33 amino acids could be determined (Figure 1b). A particular combination of two degenerate oligodeoxynucleotide mixtures, one derived from the 23 amino acid peptide sequence KEVIASII (forward primer), and the other from the 33 amino acid peptide sequence KIVTSDLEIVNI (reverse primer), when used to amplify cDNA obtained from HeLa cell poly(A)⁺ RNA, produced a PCR product of 125 bp (Figure 1a). This product was shown to be specific by Southern blot hybridization with a third oligodeoxynucleotide mixture derived from the 23 amino acid peptide sequence YPRAIT. Using the sequence of this PCR product, primers were designed to perform the RACE protocols (Apte and Sievert, 1993), in order to obtain the entire cDNA sequence from HeLa cell poly(A)⁺ RNA. From repeated experiments using this approach and a Clontech kit, a product of ~1500 bp for the 3' end-containing cDNA fragment, that included the polyadenylation signal and a portion of the poly(A) tail, and a product of ~500 bp for the 5' end-containing fragment were obtained (Figure 1a). The use of primers derived from the 5' end-proximal and 3' end-proximal extremes of the RACE products made it possible to obtain, by PCR, a fragment containing most of the cDNA (S52xA31).

The 5' end- and 3' end-containing PCR products from the RACE protocols were then used to screen a λgt11 cDNA library from HeLa cells (Clontech). From ~2×10⁶ plaques five positive clones with two different types of overlapping inserts were isolated (Figure 1a). The products from the RACE protocols and the PCR-amplified inserts from the λgt11 positive clones were cloned in the TA vector (Invitrogen), and subsequently sequenced on both strands by the dideoxy chain termination method using a total of 12 different primers, the Applied Biosystems Taq DyeDeoxy™ Terminator Cycle sequencing kit and an automated sequencer (Applied Biosystems, model 373A). Analysis of the amino acid and nucleotide sequences was performed using the GCG software package from the University of Wisconsin Genetics Computer Group, run on a VAX computer.

Plasmid constructs and *in vitro* translation of wild-type and mutated versions of mTERF

To produce different versions of mTERF by *in vitro* translation, the appropriate fragments were amplified by PCR from the clone λ-5, which contains the entire ORF for the longer mTERF precursor (see below), and inserted into the pSPUTK vector (Stratagene). Constructs lacking a segment at the 5' end of the longer precursor ORF (P2), or at the 5' end (ΔN) or 3' end (ΔB2, ΔC) of the mature protein ORF were PCR amplified from clone λ-5 by using, as forward primer, an oligodeoxynucleotide matching the sequence with the desired 5' end, and, as a reverse primer, an oligodeoxynucleotide matching the sequence with the desired 3' end. Other constructs, either carrying a sequence modification upstream of the initiation codon and in the second codon of the longer precursor ORF (GGGATGCAG to ACCATGGCG, producing a substitution of alanine for glutamine) (P1m), or completely lacking the mitochondrial targeting sequence and carrying a change of the first codon of the mature protein ORF from a phenylalanine to a methionine codon (mTERFm), were PCR amplified using the appropriately modified forward primers. Two constructs carrying an internal deletion in the reading frame (ΔLz3 and ΔB1) were obtained directly from the construct mTERFm by digestion with the restriction enzymes *ScaI* or *MaeII*, respectively, and subsequent ligation, after removal of the unwanted segment. Finally, another series of constructs, carrying two substitutions of basic residues in B1 or B2, or one, two or three leucine substitutions in one or the other of the leucine zipper domains, were obtained from mTERFm by site-directed mutagenesis (Deng and Nickoloff, 1992). The correct nucleotide sequence of all the constructs was verified, and the corresponding encoded proteins were expressed in the TNT-coupled transcription–translation system (Promega), following the manufacturer's protocols. When necessary, the recombinant mTERFm was purified by sequential chromatography through a heparin–agarose column and DNA affinity chromatography (Daga *et al.*, 1993).

Mitochondrial import/processing assays

The HeLa cell mitochondrial fraction was isolated as described (Gaines and Attardi, 1984b), washed twice in incubation medium [10 mM Tris–HCl, 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH₂PO₄,

0.05 mM EDTA and 5 mM MgCl₂ (pH 7.4 at 25°C) (Enriquez *et al.*, 1991)], resuspended in the same medium at ~2 mg/ml, and immediately used for the import/processing assays. The mTERF precursors (P1, P1m or P2) were synthesized *in vitro* in the presence of a radioactive amino acid, and the reticulocyte lysate was then added, in the proportion of 10% (v/v) to samples of the mitochondrial suspension. One mg/ml bovine serum albumin (BSA), 2 mM Na succinate, 1 mM ATP and 1 mM of the amino acid used to label the precursor (included to avoid labeling of newly synthesized proteins) were added to the reaction mixtures (usually 50–200 µl), and these were then incubated at 37°C for 30 min. The mitochondria were washed twice with ice-chilled incubation medium, and the final pellets were lysed with 1 vol of twice-concentrated gel sample buffer. In some experiments, CCCP was included in the reaction mixture to 1.7 µg/ml; in other assays, trypsin was added to the incubation mixture at 5 or 10 µg/ml after the import/processing reaction, and the samples were incubated at room temperature for 10 min before the washing and lysis of the mitochondria. In a control experiment in which, besides trypsin (10 µg/ml), Triton X-100 was added to the reaction mixture to 1% (v/v), the washing of the mitochondria was omitted. The mitochondrial lysates were analyzed by SDS–PAGE, as previously described (Daga *et al.*, 1993). The gels were fixed, treated with Amplify™ (Amersham), dried and exposed.

Determination of the processing site in mTERF precursors

Large import reactions (200 µl), using either ³⁵S or ³H-labeled precursors, were set up as described above, and the products were separated by SDS–PAGE and electrotransferred onto PVDF membranes. After exposure of the membranes, the processed mTERF products were located directly (³⁵S-labeling) or by comparison with the migration of the ³⁵S-labeled ones (in the case of ³H-labeling), and excised. Edman degradation was performed on an Applied Biosystems sequenator (model 470A), and the amino acid residues released at each cycle of degradation were analyzed by liquid scintillation counting (Matsudaira, 1987).

Alkylation experiments

Alkylation was performed on samples of *in vitro* synthesized mTERFm in gel sample buffer or on mitochondrial lysates obtained after import/processing assays, as described above. The reactions were carried out by adding dithiothreitol (DTT) to 30 mM, incubating the samples at room temperature for 30 min, and then adding iodoacetamide to 50 mM and incubating at 37°C for 60 min (Chomyn *et al.*, 1988). The treated samples were then analyzed by SDS–PAGE in parallel with untreated samples.

DNA-binding assays

The DNA-binding activity of the different *in vitro* synthesized versions of mTERFm was determined by mobility shift assays using the double-stranded, 44mer oligodeoxynucleotide probe previously described (Kruse *et al.*, 1989), or a mutated version of it (Δ3) (Daga *et al.*, 1993). Different amounts of the *in vitro* translated products were incubated with 50 fmol of probe at 25°C for 20 min, in 20 µl of reaction mixture containing 25 mM HEPES–KOH, pH 7.8, 50 mM KCl, 12.5 mM MgCl₂, 0.05% Tween-20, 1 mM DTT, 20% glycerol, 1 µg of poly(dI–dC) (added as non-specific competitor DNA) and 5 µg of BSA. Either [³⁵S]methionine-labeled protein products and unlabeled oligodeoxynucleotide probe or unlabeled protein and 3′-³²P-labeled probe (Daga *et al.*, 1993) were used, as specified in the text. After incubation, the reaction mixtures were loaded on a native 5 or 10% polyacrylamide gel or on a 1.5% agarose gel in Tris–glycine buffer, and run at 4°C, as described (Fernandez-Silva *et al.*, 1996). The DNA-binding activity of purified natural mTERF or recombinant mTERFm was tested under the conditions described above, using a ³²P-labeled probe, and the products analyzed by electrophoresis on a native 5% polyacrylamide gel. For quantifying the efficiency of DNA binding of the mutant versions of mTERFm relative to the wild-type protein, in each experiment, a constant amount of 3′-³²P-labeled probe (~50 fmol) was incubated with two different amounts (1 and 2 µl) of reticulocyte lysate which had been programmed with either wild-type or mutant mTERFm constructs and contained 50–100 fmol of recombinant protein products per µl. The DNA-binding activities of the mutant proteins relative to the wild-type protein were then corrected for differences in the efficiency of translation of different lysates, determined by SDS–polyacrylamide gel analysis of the ³⁵S methionine-labeled products obtained in parallel experiments.

DNase I footprinting

DNase I footprinting reactions were carried out as described previously (Kruse *et al.*, 1989). Briefly, 20 fmol of the *Stu*I (position 3147)–*Nco*I

(position 3311) mtDNA fragment, 5′-³²P-labeled in the H-strand, were incubated for 1 min at room temperature with the amounts of purified natural mTERF or *in vitro* synthesized mTERFm specified in Figure 3 and 0.1 U of DNase I (Sigma DN-25) in buffer containing 50 mM KCl. The samples were loaded on a 6% polyacrylamide–7.7 M urea sequencing gel in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA.

Transcription termination and S1 protection assays

Transcription termination and S1 protection analysis were performed as described previously (Daga *et al.*, 1993; Fernandez-Silva *et al.*, 1996). The transcription reactions were carried out for 30 min at 30°C in a 25 µl volume, using *Eco*RI- and *Hind*III-digested pTER plasmid at 20 µg/ml, 10 µCi [α-³²P]UTP, 2.5 µl of the S-100 of a Tween-20 mitochondrial lysate and different amounts of purified natural mTERF or purified *in vitro* synthesized mTERFm, in transcription buffer [10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 1 mM DTT, 100 µg/ml BSA, 10% (v/v) glycerol, 1 mM ATP, 0.1 mM GTP and CTP, and 0.01 mM UTP]. The S1 protection assays were performed using, as specific probe, the unlabeled RNA synthesized utilizing *Bam*HI-linearized BSAND plasmid and T3 RNA polymerase, as described in the aforementioned references. The conditions of hybridization, S1 nuclease digestion and PAGE have been described previously (Gaines and Attardi, 1984b; Fernandez-Silva *et al.*, 1996). The termination activity was calculated as the percentage of terminated transcripts relative to total transcripts, after correction for the different content of uridine (U) (108 U in runoff transcripts and 64 U in terminated transcripts).

Quantitative analysis

The radioactivity in the gels was quantified by PhosphorImager analysis (Molecular Dynamics) using Image-Quant software (Molecular Dynamics). The purified unlabeled proteins were fractionated on a 12.5% SDS–polyacrylamide gel and visualized by silver staining (Silver Stain Plus Kit, Bio-Rad), and the mTERF or mTERFm content was determined by comparison with marker proteins by laser densitometry.

Cross-linking assays

To detect the possible presence of dimers or higher oligomers, preliminary tests of chemical cross-linking of *in vitro* synthesized mTERFm were carried out, using a wide range of concentrations of GD (0.001–0.1%) or DSS (0.01–10 mM), with the aim of finding the conditions which produced a substantial degree of cross-linking of the protein without the disappearance of the monomer band, as judged by electrophoretic analysis on a 12.5% SDS–polyacrylamide gel. The optimum conditions thus established (0.01% GD, 30 min at room temperature; 1 mM DSS, 30 min at 37°C) were tested on mTERFm alone or, to detect the possible occurrence of heterodimers, in the presence of S-100 of the mitochondrial lysate (Daga *et al.*, 1993), added in the proportion of 5–25%. The reactions were stopped by addition of lysine to 20 mM and one volume of twice-concentrated gel sample buffer, and the samples were analyzed by SDS–PAGE. Other cross-linking experiments were carried out in the presence of the oligodeoxynucleotide probe. For this purpose, the [³⁵S]methionine-labeled protein was incubated, under the conditions described for the DNA-binding assays, with unlabeled oligodeoxynucleotide probe, and then treated with the cross-linking reagent, as detailed above. The cross-linking reactions were stopped by the addition of 20 mM lysine, and the samples were loaded immediately on a native 1.5% agarose gel in Tris–glycine buffer (50 mM Tris base, pH 8.5, 380 mM glycine, 2 mM EDTA), and run in parallel with a position marker represented by unlabeled mTERFm which had been incubated with the 3′-³²P-labeled probe. The gel was exposed wet, and the area corresponding to the retarded labeled protein–unlabeled oligodeoxynucleotide complex (identified from the position of the ³²P band) was excised, melted at 100°C, after addition of 1/4 vol of 5-fold concentrated gel sample buffer, and run on an SDS–polyacrylamide gel.

Accession number

The cDNA sequence of the *H.sapiens* mTERF gene has been submitted to the DDBJ/EMBL/GenBank data library under accession number Y09615.

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