Terminal segments (telomeres) of linear mitochondrial DNA (mtDNA) molecules of the yeast Candida parapsilosis consist of large sequence units repeated in tandem. The extreme ends of mtDNA terminate with a 5' single-stranded overhang of about 110 nucleotides. We identified and purified a mitochondrial telomere-binding protein (mtTBP) that specifically recognizes a synthetic oligonucleotide derived from the extreme end of this linear mtDNA. MtTBP is highly resistant to protease and heat treatments, and it protects the telomeric probe from degradation by various DNA-modifying enzymes. Resistance of the complex to bacterial alkaline phosphatase suggests that mtTBP binds the very end of the molecule. We purified mtTBP to near homogeneity using DNA affinity chromatography based on the telomeric oligonucleotide covalently bound to Sepharose. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the purified fractions revealed the presence of a protein with an apparent molecular mass of ~15 kDa. UV cross-linking and gel filtration chromatography experiments suggested that native mtTBP is probably a homo-oligomer. MtTBP of C. parapsilosis is the first identified protein that specifically binds to telomeres of linear mitochondrial DNA.

Although circularity is accepted as the most common form of mitochondrial DNA (mtDNA), there are numerous examples of organisms with linear mtDNA, raising questions about their evolutionary origin and mode of replication (1). Mitochondrial genomes of many yeast species are represented by linear DNA molecules with defined terminal structures (2–5). Two different types of yeast linear mtDNA have been described. Type 1 molecules with defined terminal structures (2–5). Twodifferent genomes of many yeast species are represented by linear DNA of evolutionary origin and mode of replication (1). Mitochondrial organisms with linear mtDNA, raising questions about their telomeres short term fellowship. To whom correspondence should be addressed: Dept. of Genetics, Faculty of Natural Sciences, Comenius University, Mlynská dolina B-1, 842 15 Bratislava, Slovakia. Tel.: 42-7-796-536; Fax: 42-7-729-064; E-mail: tomaska@fns.uniba.sk.

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The abbreviations used are: TBP(s), telomere-binding protein(s); mtTBP, mitochondrial telomere-binding protein; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by Genset (Paris, France) (their sequences and positions in C. parapsilosis mtDNA are shown in Fig. 1). DNase I and proteinase K were purchased from Boehringer (Mannheim, Germany). Cyanogen bromide-activated Sepharose 4B and RNase A were obtained from Sigma. Trypsin was
Oligonucleotides used in this study. Upper panel, schematic representation of mitochondrial telomere of C. parapsilosis (7) with oligonucleotide positions indicated. Arrows indicate the 5′ to 3′ direction of each oligonucleotide. Lower panel, sequences of corresponding oligonucleotides. Note that TEL31 and TEL51 represent the sequence of the 5′ overhang of the extreme end. Oligonucleotide 4 is complementary to TEL51.

Preparation of Mitochondrial Membrane Protein Extracts—Strains SR23 (CBS 7157) of C. parapsilosis is a stock from the Department of Biochemistry, Comenius University, Bratislava. Yeasts were grown in complete liquid medium (1% Bacto-yeast extract (Difco, Detroit, MI), 2% Bacto-tryptone, 2% glucose, 1% NaCl) at 30°C until about 90% of cells converted to spheroplasts (60–90 min). The reaction was stopped by the addition of an equal volume of ice-cold 1.2 M sorbitol, 0.1 M sodium citrate, 2,000 g for 10 min at 5°C, 15% SDS-PAGE according to the method of Laemmli (37) and stained with Coomassie Brilliant Blue R-250 or silver stain. Samples were treated with RNase A following deproteinization and dialysis against 0.1 M NaHCO3, 1 mM MgCl2 (pH 9.8) containing 0.15 mg/ml poly(dI-dC) for 10 min at 4°C. Spheroplasts were mechanically broken in Kenwood mixer (30 s at maximal speed), the pH was adjusted to 7.0 with several drops of 1 M Tris base, and cell debris was pelleted by centrifugation for 10 min at 2,000 × g. Mitochondria were pelleted from the supernatant by a 10-min centrifugation at 10,000 × g and washed three times with 0.5 M sorbitol, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA. Fractions from spheroplast crude lysate, postmitochondrial supernatant, and mitochondria were stored in small aliquots at −70°C.

For the extraction of mtTBP, mitochondria were resuspended in 10 volumes of buffer A (10 mM Tris-Cl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin A) and mixed with a magnetic stirrer for 10 min at 4°C. Spheroplasts were dialyzed against 2 volumes of buffer A using a Potter homogenizer. After a 10-min centrifugation at 10,000 × g and washed three times with 0.5 M sorbitol, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA. Fractions from spheroplast crude lysate, postmitochondrial supernatant, and mitochondria were stored at −70°C.

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UV Cross-linking of mtTBP to the Extreme End of mtDNA—To estimate the apparent molecular mass of a mtTBP:TEL51 complex, UV cross-linking experiments both in solution and in situ were performed (Fig. 5, A and B). Both of these assays gave rise to two prominent bands with apparent molecular masses of ~32 and 40 kDa, respectively (Fig. 5, panel A, lane 4, and panel B, lanes 2 and 4). The larger complex was more abundant in the sample after UV cross-linking in solution, whereas irradiation of mtTBP:TEL51 complex in mobility shift gel resulted in a higher proportion of the faster

drial RNA from C. parapsilosis were found to be ineffective competitors (data not shown). In addition to a major DNA-protein complex representing a specific interaction between the telomeric probe and a putative mtTBP, a slower migrating complex was also observed in some experiments (Fig. 2, lane 2). However, in this case all nonspecific competitors competed successfully with a probe (Fig. 2, lanes 3–17). Moreover, the formation of this complex was not observed when purified mtTBP was used for the gel retardation assay (see below), suggesting that the slower migrating complex probably resulted from nonspecific DNA-protein interaction.

MtTBP Activity Is Enriched in the Mitochondrial Fraction—Several assays were performed to assess the purity of mitochondria used for the extraction of mtTBP (Fig. 3). First, mtTBP activity was shown to be completely absent in the postmitochondrial supernatant (Fig. 3A). Second, the mitochondrial fraction was highly enriched for the mitochondrial immunological markers porin, malate dehydrogenase, and acinetase (Fig. 3B). Third, mitochondrial preparation was almost devoid of the cytosolic enzymatic marker glucose-6-phosphate dehydrogenase, which was in contrast to the distribution of cytochrome c oxidase activity (Fig. 3C). Finally, mtDNA isolated from the mitochondrial fraction was not substantially contaminated by nuclear DNA (Fig. 3D). All of these results strongly suggest that mtTBP is localized in mitochondria of C. parapsilosis.

MtTBP and mtTBP:mtDNA Telomere Complex Are Resistant to Heat and Several Protein- and Nucleic Acid-modifying Enzymes—C. parapsilosis is a petite negative yeast, and the stability of its mtDNA is probably essential for cell viability. Resistance of the mitochondrial telomeric structure to various damaging agents including proteases and DNA-modifying enzymes might reflect the need to maintain the integrity of the mitochondrial genome. The susceptibility of mtTBP to proteinase K and trypsin and the effect of heat on mtTBP activity were assayed by pretreatment of the extract followed by a gel retardation analysis using labeled TEL51 (Fig. 4A). Interestingly, mtTBP was highly resistant to proteinase K (Fig. 4A, lane 4) and partially resistant to both trypsin and to 15-min incubation at 62 °C (Fig. 4A, lanes 5 and 6). Moreover, neither an overnight incubation at 30 °C nor treatment with 0.1% SDS destroyed the mtTBP activity (data not shown).

Next, the susceptibility of the mtTBP:TEL51 complex to various DNA-modifying enzymes was evaluated (Fig. 4B). None of the tested enzymes completely destroyed the complex, whereas a free probe that served as an internal control was degraded completely in each case. To demonstrate that the enzymes are active in the mitochondrial extracts, reactions were also performed in the presence of the molar excess of homologous competitor (Fig. 4B, 3rd, 6th, 9th, 12th, 15th, and 18th lanes). Since the probe was terminally labeled by T4 polynucleotide kinase, resistance of mtTBP:TEL51 to bacterial alkaline phosphatase (Fig. 4B, 4th–6th lanes) suggests that mtTBP protects the very end of the mitochondrial telomere of C. parapsilosis. These results imply that mtTBP may play a similar protective role in vivo thus preventing mtDNA from degradation.

RESULTS

Mitochondria of C. parapsilosis Contain a Mitochondrial TBP—Preliminary experiments using the Southwestern assay indicated that most of the DNA binding activity is associated with the inner mitochondrial membrane (data not shown). The majority of membrane-associated DNA-binding proteins were extracted by treatment with 2 M NaCl. To assess the presence of a putative mtTBP in the extract, an oligonucleotide identical to the sequence of 51 nucleotides (TEL51) from the extreme 5’ end of the mitochondrial telomere (Fig. 1) was used in a gel retardation assay (Fig. 2). Mixing of TEL51 with mitochondrial membrane extract resulted in a DNA-protein complex that migrated slower than a free probe (Fig. 2, lanes 1 and 2). A 5–100-fold molar excess of unlabeled TEL51 competed quantitatively with the labeled probe for complex formation (Fig. 2, lanes 3–5). Similarly, an oligonucleotide representing the last 31 telomeric bases (TEL31) proved to be a competitor (Fig. 2, lanes 6–8). In contrast, oligonucleotides derived from other parts of mtDNA of C. parapsilosis did not compete with labeled TEL51 as effectively as homologous competitors (Fig. 2, lanes 9–17). Moreover, oligonucleotides complementary to TEL51 (oligonucleotide 4 in Fig. 1), yeast tRNA, and total mitochondrial DNA samples (250 ng) were digested with restriction enzymes HindIII and PvuII according to the manufacturer’s instructions and separated by agarose gel electrophoresis (42).

Detection of the Protein Component of DNA-Protein Complex by SDS-PAGE—A gel retardation assay was performed as described above except that 100 ng of the probe and a corresponding amount of protein were subjected to DNA-protein binding reaction. After separation of the DNA:mtTBP complex, the gel was exposed, and the shifted band and bands from the corresponding areas in control lanes (probe without mtTBP and mtTBP without a probe) were excised and denatured by boiling for 5 min in 3 ml of a modified SDS-PAGE sample buffer (1% SDS, 3 mM dithiothreitol, 125 mM Tris-Cl (pH 6.8)). The excised band was then placed between gel plates in the stacking gel area, proteins were separated by 15% SDS-PAGE by the method of Laemmli (37) and stained with silver using a silver stain kit.

Renaturation of mtTBP Isolated from SDS-PAGE Gel—Elution of mtTBP from the gel, SDS removal, guanidine hydrochloride treatment, and renaturation were performed essentially as described by Hager and Burgess (43) except that renaturation of the protein was achieved by overnight dialysis of the denatured sample against 50,000-fold excess of 10 mM Tris-Cl (pH 7.4), 1 mM EDTA at 4 °C.

 Determination of Molecular Mass of Native mtTBP—The apparent molecular mass of native mtTBP was determined using gel filtration FPLC on a Superose 12 column (Pharmacia) equilibrated with 10 mM Tris-Cl (pH 7.4), 0.1 mM EDTA, 150 mM NaCl. The column was calibrated with catalase (240 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsin (25 kDa) and cytochrome c (12.5 kDa).

Reproducibility of Data—All experiments were repeated at least twice with similar results.
migrating complex (Fig. 5B). The presence of a 100-fold excess of either TEL51 or TEL31 completely competed out a labeled probe from the complex (Fig. 5A, lanes 5 and 6). On the other hand, oligonucleotides derived from different regions of mtDNA of C. parapsilosis were not as effective as specific competitors (Fig. 5A, lanes 7–9), which is consistent with the results obtained from gel retardation assays.

Purification of mtTBP on DNA Affinity Chromatography—To purify mtTBP from a crude mitochondrial membrane extract, DNA affinity chromatography based on TEL51 covalently bound to Sepharose was employed. MtTBP activity was eluted from the column with relatively high salt concentrations as shown by both gel retardation (Fig. 6A) and UV cross-linking (Fig. 6B). Formation of a novel complex observed in a sample eluted by 0.5 M NaCl (Fig. 5A, lane 4) was probably due to the presence of a less specific TEL51-binding protein and the absence of mtTBP in this fraction. Silver staining of proteins after SDS–PAGE revealed the presence of a major protein band with an apparent molecular mass of about 15 kDa (Fig. 6C). Strong interaction between mtTBP and immobilized telomeric oligonucleotide caused most contaminants to elute with 0.3–0.5 M NaCl. Purified preparations of mtTBP from TEL51-Sepharose contained only minor impurities with a molecular mass above 40 kDa.

15-kDa Protein Represents mtTBP—Because of an apparent contradiction between the results of the UV cross-linking assay (which gave rise to two prominent bands with apparent molec-
ular masses of ~32 and 40 kDa, respectively) and the abundance of 15-kDa protein (p15) in the purified fractions, two experiments were performed to prove that p15 represents mtTBP. First, the mtTBP Tel51 complex was excised from the mobility shift gel, denatured, and separated by SDS-PAGE. As controls, gels slices were used from the same regions of the lanes with a probe and mtTBP activity containing fraction alone, respectively. A protein band comigrating with purified p15 was detected only in a sample containing both probe and mtTBP (Fig. 7A).

Second, affinity-purified p15 was isolated from the gel after SDS-PAGE, precipitated with acetone to remove SDS, resuspended in 6 M guanidine hydrochloride, renatured by dialysis, and tested for mtTBP activity (Fig. 7B, lane 3). As a control, the same protocol was performed on a gel slice in which no protein was present (Fig. 7B, lane 4). Although it lost some of the mtTBP activity, renatured p15 was able to bind the Tel51 probe in a gel mobility shift assay. Taken together these data demonstrate that p15 represents mtTBP and is responsible for the generation of the complexes in both gel retardation and UV cross-linking assays.

FPLC-Superose 12 Chromatography—It was shown previously that under standard SDS-PAGE conditions, covalently linked protein-DNA complexes usually migrate with the same electrophoretic mobility as the protein alone (38). However,
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Fig. 7. 15-kDa protein represents mtTBP. Panel A, extraction of mtTBP from the mtTBP-TEL51 complex after a mobility shift gel electrophoresis followed by SDS-PAGE analysis. Terminally labeled TEL51 probe alone (lane 1), with mitochondrial membrane protein extract (lane 2) or mitochondrial membrane protein extract alone (lane 3), was incubated for 10 min at room temperature and then subjected to mobility shift gel electrophoresis. The gel was autoradiographed, and slices corresponding to the mtTBP-TEL51 complex in lane 2 were excised from each lane. Slices were then denatured and loaded on SDS-PAGE as described under “Experimental Procedures.” TEL51-Sepharose-purified mtTBP was used as a control (lane 4). Protein size markers (lanes 5) and the position of mtTBP (arrow) are indicated in the margin. Proteins were detected by silver staining. Panel B, the protein fraction from TEL51-Sepharose was separated by SDS-PAGE, and the 15-kDa protein was isolated and renatured as described under “Experimental Procedures” (as a control, the same protocol was performed on a gel slice in which no protein was present) and analyzed for the ability to bind TEL51 probe in a gel mobility shift assay. Lane 1, TEL51 probe alone; lane 2, TEL51 probe + active fraction from TEL51-Sepharose; lane 3, renatured 15-kDa protein; lane 4, control.

purified mtTBP migrated in SDS gel as a 15-kDa protein band (Fig. 6C), whereas the UV cross-linked mtTBP-TEL51 complex exhibited an apparent molecular mass above 30 kDa (Fig. 5). The possibility that this discrepancy might be due to an oligomeric state of the native mtTBP was tested using FPLC gel filtration chromatography on Superose 12 (Fig. 8). TEL51-retarding activity was eluted between bovine serum albumin (68 kDa) and ovalbumin (45 kDa), suggesting that native mtTBP is either a trimer or tetramer. The absence of any major protein band other than p15 in purified mtTBP preparations suggests that mtTBP is a homo-oligomer.

DISCUSSION

Several lines of evidence indicate that nuclear telomeres form specific nucleoprotein structures (telosomes), consisting of telomeric DNA and various proteins involved in both replication and stabilization of the chromosome ends. Moreover, these structures are responsible for distinct interactions in the nucleus and influence the expression of subtelomeric genes (8, 44–47).

Specific nuclear TBPs from a variety of organisms were recently identified (33, 48). The first isolated and best characterized is TBP from Oxytricha nova which is a heterodimer of two different polypeptides α and β (12, 13). It binds specifically to the 3′ overhang both in vitro and in vivo, protects the telomeric DNA from chemical modification and nuclease digestion, and promotes the formation of a G4 structure typical for telomeric DNA (49). Another well characterized telomeric protein is RAP1 of S. cerevisiae which is a multifunctional polypeptide that binds to the duplex region of the telomeric sequence both in vitro and in vivo and plays not only a protective but also regulatory role at telomeres (20–22, 26, 50–53). The crystal structure of its DNA binding domain in complex with telomeric DNA was reported recently (54). Although several TBPs were isolated from vertebrates (29–32), only the protein described recently by Chong et al. (29) has been shown to be at telomeres in vivo.

Linear mtDNAs were described recently in more organisms than were expected previously. Their genomic organization and terminal structures differ greatly. Different types of mitochondrial telomeres may imply different strategies of their replication which do not resemble the replication of typical nuclear telomeres. For example, linear mtDNAs found in several species from yeast genera Pichia and Williopsis possess a terminal hairpin that may allow the formation of circular replication intermediates (2, 6). In contrast, termini of linear mtDNA of C. parapsilosis consist of tandem repeats that end with a 110-nucleotide-long 5′ single-stranded overhang that does not permit the formation of circles (7). It is not clear how the 5′ overhang is formed, how it is stabilized, and why DNA polymerase does not use this sequence as a template.

Although data about nuclear TBPs accumulate rapidly, no similar proteins were identified from mitochondria with linear genomes. We considered the possibility that specific mtTBPs are responsible for the stability and/or replication of mtDNA termini of C. parapsilosis. By both gel retardation and UV cross-linking experiments using a 51-base-long oligonucleotide derived from the 5′ overhang of the extreme end of linear mtDNA we detected a specific mtTBP in the salt extracts from mitochondrial membranes of this yeast. The interaction of mtTBP with the membrane was relatively strong since lower salt concentrations (~2 M NaCl) or nonionic detergents (1% Triton X-100) did not quantitatively extract mtTBP from the membrane environment. It is possible that the high salt is needed to remove mtTBP from endogenous DNA. However, treatment of the membranes with nuclease did not result in the extraction of mtTBP (data not shown). The mtTBP of C. parapsilosis may at least partly mediate the association of mtDNA with the inner mitochondrial membrane, which might be necessary for its replication and proper segregation into newly formed mitochondria. Association of mtDNA with the mitochondrial membrane was demonstrated for Physarum polycephalum and S. cerevisiae, and several protein components responsible for this interaction were detected (55–57).

One of the interesting characteristics of mtTBP was its resistance to protease and heat treatments. This resistance was higher in a crude fraction than in purified form. The crude fraction did not contain general protease inhibitors since more than 90% of other proteins were degraded by the two proteases tested (data not shown). It is possible that mtTBP in a crude
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fraction is in association with a partner that protects it from the action of proteases or which in a diluted state mtTBP is more susceptible to proteolytic cleavage. Moreover, mttBP activity was not affected by pretreatment of the sample with RNase A (data not shown), excluding the possibility that mttBP is a ribonucleoprotein complex similar to nuclear telomerase (58). The resistance of mttBP to heat (<7 min at 62°C) was helpful in removal of contaminating Mg2+-dependent nucleases. This allowed us to test the sensitivity of the mttBP-TEL51 complex to various DNA-modifying enzymes, some of which require magnesium ions (DNase I, exonuclease III). None of the used enzymes modified DNA in the complex. Surprisingly, free TEL51 was sensitive to exonuclease III (Fig. 4B, 16th lane) as this enzyme is specific for double-stranded DNA molecules with protruding 5′ ends. This suggests that TEL51 may adopt a double-stranded structure under the reaction conditions employed in this experiment. The relevance of such structures for in vivo function(s) of mitochondrial telomere remains to be determined. Since the probe was labeled terminally with polynucleotide kinase and the label was not lost by the action of bacterial alkaline phosphatase, mttBP probably protects the very end of the telomere. Tight interaction between mttBP and mitochondrial telomere may also play a role in preventing DNA polymerase from resuming synthesis of the 3′ end. In addition, mttBP may be involved in regulating the length of the mitochondrial telomere.

Preliminary experiments demonstrated that the formation of a complex between mttBP and a telomeric oligonucleotide is resistant to relatively high salt concentrations (data not shown). This allowed us to purify mttBP to near homogeneity in a single step using DNA affinity chromatography. Identification of a single protein with a molecular mass of about 15 kDa in the active fractions was in apparent contradiction with the data from UV cross-linking experiments, where the mttBP-TEL51 complex migrated as two bands with molecular masses of about 32 and 40 kDa, respectively. Three different chemical cross-linkers (3,3′-dithiodisulfosuccinimidylpro- pionate), bis(sulfosuccinimidyl)suberate, potassium permanganate) gave similar results when compared with cross-linking by UV light (data not shown). Since these two bands were formed also by UV cross-linking of a mobility shift gel in situ and since the 15-kDa protein was extracted from the retarded band after gel retardation assay we tested the possibility that native mttBP is a homo-oligomer. Data from FPLC gel filtration chromatography supported this hypothesis. The nature of two differently migrating bands after UV cross-linking of mttBP to a probe remains to be determined.

MttBP identified in this study is the first example of a protein specifically recognizing the terminal structures of linear mtDNA. It remains to be shown that the mttBP functions effectively in vivo as a termini-binding protein or fulfills some other tasks. To answer this question, the gene encoding mttBP will be cloned, and mutations will be created for functional analysis. Such studies are complicated by the absence of a genetic system and by the asexual mode of reproduction of C. parapsilosis which does not allow the use of powerful molecular and genetic tools available for S. cerevisiae. In spite of the lack of in vivo data the specificity of mttBP binding to a telomeric sequence in vitro suggests that it plays a specific role in mitochondria. Beside the protective role that is implied by the resistance of the mttBP-telomere complex to various DNA-modifying enzymes mttBP may play a part during the replication of mtDNA termini. Because of their unusual structure, mitochondrial telomeres of C. parapsilosis may reveal a novel strategy to solve the end replication problem associated with linear DNA genomes. In addition, mttBP might be involved in the recombination and segregation of mtDNA molecules in C. parapsilosis, thus serving as a guard of mtDNA integrity of this petite negative yeast. It will be also interesting to find the mttBP-encoding gene and its homologs in other organisms. At this stage we cannot rule out the possibility that mttBP is encoded by mtDNA since only one-third of the C. parapsilosis mtDNA sequence is known, and linear cytoplasmic genomes (viruses, plasmids) often code for their terminal proteins (59). All of these questions are addressed in experiments that are currently in progress in our laboratory.

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