Mitochondrial Telomere-binding Protein from *Candida parapsilosis* Suggests an Evolutionary Adaptation of a Nonspecific Single-stranded DNA-binding Protein*

(Received for publication, July 20, 1998, and in revised form, December 24, 1998)

Jozef Nosek‡‡, L’ubomír Tomáška¶¶, Blanka Pagáčová††, and Hiroshi Fukuhara‡‡ |

From the Departments of ‡Biochemistry and ¶¶Genetics, Faculty of Natural Sciences, Comenius University, Mlynská dolina ¶¶CH-1 and VB-1, 842 15 Bratislava, Slovakia and ‡Institut Curie, Section de Recherche, Centre Universitaire Paris XI, 91405 Orsay, France

The mitochondrial genome in a number of organisms is represented by linear DNA molecules with defined terminal structures. The telomeres of linear mitochondrial DNA (mtDNA) of yeast *Candida parapsilosis* consist of tandem arrays of long repetitive units possessing single-stranded 5’ extension of about 110 nucleotides. Recently we identified the first mitochondrial telomere-binding protein (mtTBP) that specifically binds a sequence derived from the extreme end of *C. parapsilosis* linear mtDNA and protects it from attack by various DNA-modifying enzymes (Tomáška, L’, Nosek, J., and Fukuhara, H. (1997) J. Biol. Chem. 272, 3049–3059). Here we report the isolation of *MTPI*, the gene encoding mtTBP of *C. parapsilosis*. Sequence analysis revealed that mtTBP shares homology with several bacterial and mitochondrial single-stranded DNA-binding proteins that nonspecifically bind to single-stranded DNA with high affinity. Recombinant mtTBP displays a preference for the telomeric 5’ overhang of *C. parapsilosis* mtDNA. The heterologous expression of a mtTBP-GFP fusion protein resulted in its localization to the mitochondria but was unable to functionally substitute for the loss of the S. cerevisiae homologue Rimlp. Analysis of the *MTPI* gene and its translation product mtTBP may provide an insight into the evolutionary origin of linear mitochondrial genomes and the role it plays in their replication and maintenance.

Terminal structures (telomeres) of eukaryotic chromosomes and telomerases (specialized nucleoprotein enzymes that maintain the telomere length) are involved in several important cellular processes as senescence, immortalization, and carcinogenesis. Telomerase activation appears to be critical for cell immortalization and represents a promising target for cancer therapy. However, several studies have demonstrated that cells lacking functional telomerase utilize an alternative mechanism to elongate the chromosome ends, suggesting that some tumor cells may survive following treatment with telomerase inhibitors (1). Therefore a more detailed understanding of both telomerase-dependent and -independent replication mechanisms is crucial for cancer therapy and the design of therapeutic agents capable of specifically blocking telomere replication. Furthermore, the study of various linear genophores might shed some light on alternative solutions to the end-replication problem.

In contrast to the generally held belief that mitochondrial genomes are circular molecules, a large number of organisms contain linear mitochondrial DNA (mtDNA) molecules possessing a homogeneous terminal structure. Sequence analysis of mitochondrial telomeres from various organisms revealed that they do not conform to a single consensus sequence or structural motif (2). According to their terminal structures, two types of linear mtDNA have been identified in yeasts. Linear mtDNA of the yeast species in closely related genera Williopsis and *Pichia* terminates at both ends with an inverted terminal repeat possessing a covalently closed single-stranded hairpin loop resembling the structure of the vaccinia virus genome (3, 4). The terminal structures of type 2 linear mtDNA of a pathogenic yeast *Candida parapsilosis* are represented by inverted terminal repetitions consisting of tandem arrays of a 738-bp-long repetitive unit. This structure remotely resembles the organization of telomeres of nuclear chromosomes, although their repetitive unit is considerably shorter (5–8 bp). The variable number of tandem units generates a population of mtDNA molecules of heterogeneous size where the shortest molecules containing only incomplete repetitive unit predominate. A more detailed analysis of *C. parapsilosis* mitochondrial telomeres revealed that mtDNA molecules terminate at a defined position within a repetitive unit, thus generating a 5’ single-stranded extension of about 110 nucleotides (5). This unique telomeric structure has raised several important questions: (i) how the mitochondrial telomere is stabilized, (ii) how the 5’ single-stranded extension is generated, (iii) why DNA polymerase does not fill the protruding 5’ overhang, and (iv) how the shortest mtDNA molecules that do not possess a complete tandem unit restore the missing sequence.

Several proteins that specifically bind either double-stranded or single-stranded DNA of telomeres of nuclear chromosomes have been identified. These proteins mediate telomere functions such as capping the ends of chromosomes, preventing nucleolytic degradation and end-to-end fusions.

---

*This work was supported in part by Comenius University Grants 3856/98 and 3877/98, Slovak Grant Agency Grants 1/4164/97, 1/4159/97, and 1/6168/99, Howard Hughes Medical Institute Grant 57195–547301, and European Community Grant (BI04-CT96–0003). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by postdoctoral fellowship from the Ministry of Education of French government. To whom correspondence should be addressed: Dept. of Biochemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia. Tel.: 421-7-60296-536; Fax: 421-7-65429–064; E-mail: nosek@fns.uniba.sk.

§ Supported by postdoctoral fellowship from the Ministry of Education of French government. To whom correspondence should be addressed: Dept. of Biochemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia. Tel.: 421-7-60296-536; Fax: 421-7-65429–064; E-mail: nosek@fns.uniba.sk.

1 The abbreviations used are: bp, base pair(s); mtTBP, mitochondrial telomere-binding protein; SSB protein, single-stranded DNA-binding protein; PCR, polymerase chain reaction; GFP, green fluorescence protein; ssDNA, single-stranded DNA; PBS, phosphate-buffered saline.
Mitochondrial Telomere-binding Protein

promoting the formation of telomere chromatin structure and nuclear architecture, participating in the replication and regulation of telomere length, etc. (6). To decipher the role of mitochondrial telomeres, we have initiated a search for proteins interacting with terminal sequence of linear mtDNA.

Recently, we identified the first protein specifically recognizing the terminal structure of linear mtDNA from *C. parapsilosis*. Mitochondrial telomere-binding protein (mtTBP) is a heat- and protease-resistant protein that specifically recognizes the synthetic oligonucleotide identical to the terminal 51 nucleotides of the 5′ single-stranded overhang of *C. parapsilosis* mitochondrial telomere and protects it from various DNA modifying enzymes. Affinity-purified mtTBP exhibits a molecular mass of 15 kDa in its monomeric state under denaturing conditions but forms homo-oligomers under native conditions (7). The properties of mtTBP suggested that it may play an important role in the stabilization and/or replication of linear mtDNA of *C. parapsilosis*.

In this report we describe the isolation and characterization of a nuclear gene, *MTP1*, that encodes the mtTBP of the yeast *C. parapsilosis*. Surprisingly, the sequence analysis of mtTBP revealed a striking homology to a family of bacterial and mitochondrial single-stranded DNA binding (SSB) proteins. Although other members of the SSB protein family bind with high affinity to single-stranded DNA without apparent sequence specificity, mtTBP preferentially binds the terminal 5′ single-stranded overhang of the mitochondrial telomere. It has been proposed that the evolutionary appearance of linear mtDNA led to adaptation of the replication machinery to ensure complete replication of the terminal genophore. In mitochondria of *C. parapsilosis*, such adaptation might have forced the conversion of a sequence-non specific mitochondrial SSB protein to a telomere binding factor.

**EXPERIMENTAL PROCEDURES**

**Strains**—*C. parapsilosis* SR23 (CBS 7157) is a laboratory strain from the collection of the Department of Biochemistry (Comenius University, Bratislava, Slovakia). *Escherichia coli* DH5α (deorR, endA1, gyrA96, hsdR17 (rK–, mK+), recA1, supE44, thi-1, recA1, supE44, thi-1, supE44) was propagated in L-broth medium (Difco) to an OD600 of 0.5, and cells (0.1 ml of the culture) were lysed as described by Horváth and Riezman (12). Isolation of *MTP1* by Polymerase Chain Reaction (PCR)—The sequences of oligonucleotides (synthesized by Genset, France) used in this study are shown in Table I. Oligonucleotide I was used for priming the synthesis of cDNA on *C. parapsilosis* total RNA template using the First-strand cDNA synthesis kit (Amersham Pharmacia Biotech). According to the manufacturer instructions, the sequence of peptide HAEVIQWGBK was used to design *MTP1*-specific degenerate oligonucleotide II. The PCR reaction for amplification of the 3′ end of *MTP1* cDNA contained 50 mM KCl, 10 mM Tris-HCl, pH 9.0 (at 25 °C), 0.1% Triton X-100, 1.25 mM MgCl2, 0.2 mM dNTP, 2.5 units of Taq DNA polymerase, single-stranded cDNA prepared as described above as the template and 1 μM oligonucleotide I and 5 μM oligonucleotide II as the upstream and downstream primer, respectively. Amplification was performed in DNA Thermal Cycler 480 (Perkin-Elmer) with initial denaturation at 95 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min, and final polymerization at 72 °C for 5 min. The 125-bp PCR product was gel-purified and sequenced. The DNA sequence upstream of the stop codon of putative open reading frame was used to design reverse *MTP1*-specific primer (oligonucleotide III). The 5′ end of *MTP1* was amplified by reverse transcription-mediated PCR strategy (9). Briefly, total genomic DNA of *C. parapsilosis* was digested with *Ava*II endonuclease, denatured, annealed with *MTP1* specific downstream primer, then extended with Vent(exo-) DNA polymerase (New England Biolabs), followed by ligation of the primer extension products with a synthetic linker. The *MTP1* sequence was obtained using PCR generate downstream (oligonucleotide III) and 25-nucleotide-linker primers. The DNA sequence analysis of 600-bp PCR product revealed a putative 399-bp open reading frame containing the sequences corresponding to three known peptides. The integrity of *MTP1* gene was confirmed by sequencing of several independent PCR products obtained using *MTP1* oligonucleotides IV and V on *C. parapsilosis* genomic DNA and cDNA as the template, respectively.

**Construction of Plasmid Clones**—The plasmid pGFP-C-FUS-mtTBP containing the whole *MTP1* open reading frame (lacking stop codon) fused with green fluorescent protein was prepared by ligation of PCR product amplified using 5′-ATGGTGCGACAGCTCATAGATC-3′ and 5′-TCTTGAAGTCGGTCTATCTTC-3′ primers into *SmaI*-digested pGFP-C-FUS vector provided by J. Spirin (Department of Biochemistry, University of Cambridge, UK).

The *MTP1* sequence in the constructs was verified by DNA sequencing.

**Purification of Recombinant mtTBP and Rabbit Antisera Preparation**—Recombinant mtTBP was purified according to the instructions of the supplier of the expression vector (Amersham Pharmacia Biotech). Briefly, 20 ml of an overnight bacterial culture was inoculated into 1 l of LB medium supplemented with 50 mg/L of kanamycin, 20 μg/ml of ampicillin (Difco), 0.5% NaCl and grown at 37 °C to a final A600 = 0.7. The culture was then induced for 3 h at 30 °C with 1 mM isopropyl-1-thio-β-d-galactopyranoside (Sigma). Cells were washed once with ice-cold double-distilled water, resuspended in 20 ml of buffer G (20 mM HEPES-NaOH, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 0.1 mM EDTA, protease inhibitor mixture (Completeβ)).* Escherichia coli* DH5α strain was grown for 3 h to remove insoluble material. The supernatant was mixed with 0.3 ml of glutathione-agarose (Sigma) prewashed with buffer G and incubated for 60 min on ice with occasional mixing. Beads were loaded onto a 5-ml column, washed with 100 ml of buffer G (without protease inhibitor mixture) followed by 50 ml of ice-cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.6 mM KCl, 10 mM NaHPO4, 1.4 mM KH2PO4). The washed beads were resuspended in 0.45 ml of PBS containing thrombin (0.05 units/μl, Amersham Pharmacia Biotech) and incubated overnight at 4 °C. The cleaved mtTBP was eluted in 3 × 0.5 ml of PBS and stored at −70 °C. Rabbit polyclonal antisera was raised against 100 μg of purified recombinant thrombin-cleaved mtTBP and purified by Protein A-Sepharose (Eurogentec, Belgium).

**Immunoblotting**—*C. parapsilosis* SR23 was grown until the late logarithmic phase in VP medium (1% yeast extract, 1% peptone) supplemented with glucose (2% w/v), glycerol (3% w/v), or galactose (2% w/v), and cells (0.1 ml of the culture) were lysed as described by Horváth and Riezman (12). Proteins were separated by 13% SDS-polyacrylamide gel electrophoresis by a method of Laemmli (13). Resolved proteins were transferred to nitrocellulose filters in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) using a semidry electroblotter system (Owl Scientific) for 45 min at 250 mA. Filters were blocked for 2 h at room temperature with blocking solution (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% (w/v) skim milk (Difco)) and then incubated overnight at 4 °C in the blocking solution containing anti-mtTBP antibody (1:200). Membranes were washed four times with rinsing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), one time with...
rinsing buffer without Tween 20, and then incubated with blocking
solution containing goat anti-rabbit IgG alkaline phosphatase conju-
gate (Sigma, 1:3,000) for 2 h at room temperature. Blots were washed as
described above and developed by incubating with 0.3 mg/ml p-nitro
blue tetrazolium chloride (Sigma) and 0.15 mg/ml 5-bromo-4-chloro-3-
indolylphosphate toluidine salt (Sigma) in alkaline phosphatase buffer
(100 mM NaHCO3, 1 mM MgCl2, pH 9.8) for 5–20 min at room
temperature.

Binding of mtTPB to a Native Mitochondrial Telomere and Immu-
noprecipitation of the mtTPB-DNA Complex—Purified mitochondrial
DNA (3 μg) from C. parapsilosis was digested with BglII (30 units) in a
final volume of 250 μl, followed by heat-inactivation of the restriction
enzyme by incubation of the sample for 10 min at 65 °C. Digested
mtDNA (100 μl) was mixed with 5 μg of recombinant mtTPB in a final
volume of 250 μl of the DNA binding buffer (10 mM Tris-HCl, pH 7.4, 50
mM NaCl) and incubated for 30 min at room temperature.

To immunoprecipitate mtTPB-DNA complexes, the binding reaction
was combined with 50 μl of anti-mtTPB antibody for 2 h at 4 °C. Immune
complexes were immobilized by incubation with Protein A-
Sepharose 4B (Sigma) for an additional 2 h at 4 °C and centrifuged for
5 s in a microcentrifuge at maximal speed, and the supernatant was
removed and placed on ice. The pellet was washed 5 times with 1 ml of
PBS containing 200 mM NaCl. The immunoprecipitated complexes were
separated from free bands by boiling for 5 min in 300 μl of PBS contain-
ing 0.1% SDS. This step was repeated two more times, and the resulting
fractions were saved as eluates 1, 2, and 3, respectively.

Gel-mobility Shift Assay—Oligonucleotides were 5’ end-labeled us-
ing [γ32P]ATP by T4 polynucleotide kinase and separated from non-
corporated nucleotides by chromatography on a BioSpin-8 column (Bio-
Rad). 10 ng of purified recombinant mtTPB was incubated with 1 ng of
labeled oligonucleotide in 10 μl of DNA binding buffer (10 mM Tris-HCl,
pH 7.4, 50 mM NaCl) containing 1 mg/ml poly(dI:dC) for 10 min at room
temperature. DNA-protein complexes were resolved by electrophoresis
on 4% polyacrylamide gel in 0.5

In Vitro Oligomerization Assay—1 μg of recombinant mtTPB was
incubated with various concentrations of glutaraldehyde in 10 μl of PBS
for 10 min at room temperature. The cross-linking reaction was stopped
by boiling the samples for 5 min. The cross-linking reaction was stopped
by heating the samples in 1

DNA Manipulations and Analysis—C. parapsilosis genomic DNA
was isolated using a protocol described for S. cerevisiae (17). Restriction
and DNA modification enzymes were from New England Biolabs and
used according to manufacturer instructions. Southern and Northern
blotting, DNA hybridization, DNA cloning, and sequencing were per-
formed essentially as described in Sambrook et al. (18). Intact cells of S.
cerevisiae were transformed by standard lithium acetate/ssDNA/poly-
ethylene glycol protocol (19).
compete with labeled TEL31 as effectively as homologous competitor under identical conditions (Fig. 3, lanes 6–8). These data demonstrate that the preference of recombinant mtTBP for the telomeric sequence parallels the behavior of the natural mtTBP isolated from *C. parapsilosis* mitochondria (7).

Recombinant mtTBP was used to raise the rabbit polyclonal antiserum, which ultimately recognized a single 15-kDa protein under denaturing conditions in cell extracts prepared from *C. parapsilosis*. No similar size protein has been detected in several other yeast species from various genera including *Candida*, *Williopsis*, *Pichia*, *Saccharomyces*, and *Kluyveromyces*. It was shown previously that mtTBP forms homo-oligomeric complexes in its native state. Using two chemical cross-linkers, glutaraldehyde and bis(sulfosuccinimidyl)suberate, we now demonstrate that recombinant mtTBP also undergoes homo-oligomerization in vitro. Dimers were the predominant oligomeric form in the presence of either cross-linker (Fig. 4).

To compare the DNA binding properties of mtTBP with a homologous protein from a yeast with circular mitochondrial DNA, we performed a gel retardation experiment using mitochondrial protein extracts from *C. parapsilosis* and *S. cerevisiae*, respectively. Both extracts contained ssDNA binding activity. It was shown previously that the factor responsible for the ssDNA-protein complex formation is mtTBP in *C. parapsilosis* (7) and Rim1p in *S. cerevisiae* (20), respectively. However, in contrast to *C. parapsilosis*, 300-fold excess of OLI31 competed quantitatively with the labeled TEL31 probe for complex formation in mitochondrial lysates of *S. cerevisiae* (Fig. 5). This suggests that, in addition to general ssDNA binding activity, mtTBP gained a preference for a telomeric sequence.

Next we tested whether mtTBP was capable of binding natural mitochondrial telomeres in a reconstitution experiment. (Fig. 6). Recombinant mtTBP was incubated with *Bgl*II-digested mtDNA of *C. parapsilosis*, and the DNA-protein complexes were immunoprecipitated with anti-mtTBP and Protein A-Sepharose. DNA-mtTBP complexes were eluted from the beads, and the fractions were examined by Southern blot analysis following hybridization to either a specific 738-bp *EcoRI* fragment representing the telomere repeat unit or to a control 2.9-kilobase *Bgl*II fragment derived from an internal region of mtDNA. The results shown in Fig. 6 demonstrate that anti-mtTBP antiserum immunoprecipitated mtTBP complexed to terminal mtDNA fragments, producing a ladder pattern typical of telomeric DNA (5).

MTP1 Is Constitutively Expressed, and Its Protein Product Localizes to Mitochondria—Next we examined the expression of the MTP1 gene under different growth conditions. *C. parapsilosis* cells were grown to late logarithmic phase in YP medium.
Mitochondrial Telomere-binding Protein

(1% yeast extract, 1% peptone) supplemented with either glucose, glycerol, or galactose. Northern blot analysis indicated that MTP1 RNA was constitutively transcribed regardless of the carbon source present. Similar results were observed at the translational level when the mtTBP was assayed by immunoblotting in extracts from cells grown under identical conditions (Fig. 7).

Although cell fractionation experiments suggest that mtTBP is enriched in mitochondria (7), it was important to verify these results in intact cells. Because a system for the genetic transformation of C. parapsilosis is not available, we took advantage of S. cerevisiae as a heterologous expression system for these experiments. We constructed a plasmid expressing GFP fused to the C terminus of mtTBP (see "Experimental Procedures"). Visualization of cells harboring this plasmid by fluorescent microscopy showed that mtTBP-GFP was restricted to mitochondria and appeared to co-localize with mitochondrial DNA, as judged by 4',6-diamidino-2-phenyl-indol staining. In contrast, cells expressing GFP alone displayed a diffuse fluorescence throughout the cell. Together these data confirm the specific mitochondrial localization of mtTBP (Fig. 8).

**TABLE I**

| Competitor | TEL31 | OLI31 |
|------------|-------|-------|
| mtTBP      | 1     | 2     |
| 3x         | 3     | +     |
| 30x        | 4     | +     |
| 300x       | 5     | +     |

**Fig. 2.** MTP1 gene localizes on 2.2 Mbp chromosome. Chromosomal DNA of C. parapsilosis SR23 was separated using pulsed field gel electrophoresis (see "Experimental Procedures") and stained with ethidium bromide (left panel), then transferred onto a nylon membrane, subsequently hybridized with a radioactively labeled MTP1 probe, and subjected to autoradiography (right panel).

**Fig. 3.** Recombinant mtTBP specifically recognizes telomeric sequence in vitro. Gel retardation assay (see "Experimental Procedures") was performed without (lane 1) or with (lanes 2-8) 10 ng of recombinant mtTBP using terminally labeled TEL31 as a probe. The assay was done in the absence (lanes 1 and 2) or presence (lanes 3-8) of various molar excesses of the oligonucleotide competitors that are indicated above the lanes.

**Fig. 4.** mtTBP oligomerizes in vitro. Chemical cross-linking of mtTBP was performed as described under "Experimental Procedures." Concentrations of glutaraldehyde and bis(sulfosuccinimidyl)suberate (BS²), respectively, are indicated above each lane. The oligomeric forms of mtTBP were visualized either by immunoblot using anti-mtTBP antisera (A) or by autoradiography of mtTBP phosphorylated by the cAMP-dependent protein kinase before cross-linking (B).
is heterozygous for $RIM1$ and $S. cerevisiae$. Gel retardation assay (see “Experimental Procedures”) was performed without (lane 1) or with (lanes 2-15) $5 \mu g$ of mitochondrial protein extracts from $C. parapsilosis$ (lanes 2-8) and $S. cerevisiae$ (9-15) using terminally labeled TEL31 as a probe. The assay was done in the absence (lanes 1, 2, and 9) or presence (lanes 3-8 and 10-15) of various molar excesses of the oligonucleotide competitors that are indicated above the lanes.

**DISCUSSION**

DNA replication, recombination, and repair require an essential accessory protein that binds with high affinity to single-stranded DNA and protects the polynucleotide chain from refolding and nucleolytic degradation (21–23). Single-stranded DNA-binding proteins can be regarded as one big family of proteins sharing common functional, structural, and mechanistic features. However, the sequence comparison can distinguish several separate classes having little in common except their ability to bind the single-stranded nucleic acids (24). Although the replication of nuclear DNA in yeast and human requires heterotrimeric replication protein A protein, the nuclear genome encodes yet another SSB protein, which is imported into mitochondria. Mitochondrial SSB proteins isolated from several sources (human, rat, Xenopus, Drosophila, and bakers’ yeasts) are homologous to bacterial SSB proteins. The N-terminal domain of $E. coli$ SSB, which has been implicated in ssDNA binding, shares extensive homology with human mitochondrial SSB protein. The crystal structures of both proteins revealed that the polynucleotide chain wraps around the SSB homotetramer (25–27). These bacterial and mitochondrial proteins also share several common biochemical and physicochemical properties, which suggests a conserved mechanis(s) of binding to single-stranded DNA (27, 28). These data further support the hypothesis that mitochondria may have originated from a bacterial endosymbiont. Interestingly, the genome of the archean Methanococcus jannaschii apparently does not encode a protein homologous to bacterial and mitochondrial...
Mitochondrial Telomere-binding Protein

Citing of mitochondrial DNA (mtTBP-GFP fusion), S. cerevisiae, correspond to Trp-61 and Phe-67, respectively. In the ssDNA-binding (31), are conserved in mtTBP and correspondingly present, albeit SSB 1 is weakly corresponding to both motifs are present, albeit SSB 1 is weakly conserved (Fig. 1B). In addition, amino acids Trp-54 and Phe-60 in E. coli SSB protein, histidine in position 55 has been found to play a central role in tetramerization, and its replacement to tyrosine in E. coli ssb-1 mutant displays temperature-sensitive DNA replication defect and causes the dissociation of tetramers to dimers and monomers. The mutant protein is still minimally capable of forming tetramers at high protein concentration, and the effects of ssb-1 mutation can be suppressed by the overexpression of ssb-1 gene product (32, 33). His-55 of E. coli SSB is equivalent to His-69 in human mitochondrial SSB (24), but it is apparently replaced by tyrosine in both S. cerevisiae (Tyr-61 in Rim1p) and C. parapsilosis homologues (Tyr-62 in mtTBP).

Because the mitochondrial SSB protein from bakers’ yeast forms tetramers in solution (20), it was of interest to determine whether mtTBP behaves similarly. Previous results from UV cross-linking and gel filtration experiments suggest that mtTBP forms homo-oligomers in its native state (7). We now show that recombinant mtTBP also forms tetrameric complexes in vitro, although dimers were the predominant oligomeric form under the conditions used in this study.

In contrast to other SSB proteins that bind essentially any single-stranded DNA, mtTBP was identified by virtue of its selective interaction with the synthetic oligonucleotide derived from the single-stranded overhang of the mitochondrial telomere using both gel-retardation and UV cross-linking assays (7). We have now expanded our observation to show that recombinant mtTBP binds not only synthetic telomeric oligonucleotide with a specificity similar to the endogenous protein, but more importantly, binds the natural mitochondrial telomere from C. parapsilosis in a reconstitution experiment.

Subcellular fractionation experiments have shown that mitochondria from C. parapsilosis were enriched in mtTBP and DNA binding activity selective for the terminal sequences of mitochondrial telomeres (7). In vivo analysis of MTP1 is complicated by the lack of tools necessary for the genetic manipulation of C. parapsilosis (i.e. absence of sexual state, diploid or aneuploid cells, no suitable mutant strains, no system for genetic transformation, etc.). Therefore we exploited the S. cerevisiae system in an attempt to partially circumvent this problem. Because the heterologous expression of the mtTBP-GFP fusion protein resulted in its localization to mitochondria of S. cerevisiae, we were tempted to test if the expression of MTP1 can prevent the loss of mtDNA in Δrim1 cells. The inability of mtTBP to replace Rim1p implies that these proteins differ in their DNA binding properties and/or their capacity to interact with other components of mtDNA replication machinery.

The results of Northern and Western analysis of C. parapsilosis grown in media containing either glucose, glycerol, or galactose as the sole carbon source suggested that MTP1 gene is constitutively expressed. Previous studies indicate that DNA binding activity, but not oligomerization, is affected by the phosphorylation of mtTBP by the CAMP-dependent protein kinase, thereby suggesting a potential mechanism for the regulation of mitochondrial telomere replication (15). Although mtTBP contains several putative protein kinase A phosphorylation sites, their physiological relevance also awaits the generation of an appropriate genetic transformation system for C. parapsilosis.

The study of linear mitochondrial genomes evokes several questions with regard to the evolutionary origin of linear and circular mitochondrial DNAs and the mechanisms that lead to the generation of the linear form. Several lines of evidence suggest that the linear and circular forms of mitochondrial DNA neither have an independent origin nor represent a radical difference in their life styles. Rather, the conversion from one form to another may have occurred accidentally through a relatively simple mechanism during evolution. This means that the cell with linearized mitochondrial genome may mobilize a pre-existing set of proteins to ensure its replication (2). The nature of mtTBP from C. parapsilosis supports this idea and has prompted us to speculate that it might be derived from an SSB protein that lacks sequence specificity. Although mtTBP had retained some features common to other mitochondrial and bacterial SSB proteins, it has gained a preference for binding the terminal 5’ single-stranded overhang of the mitochondrial telomere. This suggests that evolutionary emergence of linear mtDNA has been accompanied by the adaptation of the component(s) of the replication machinery to complete the replica-

**Fig. 8.** A mtTBP-GFP fusion protein localizes to mitochondria. Fluorescence micrographs of S. cerevisiae cells expressing GFP (A) and mtTBP-GFP fusion (B), respectively. Visualization of nuclear and mitochondrial DNAs by 4,6-diamidino-2-phenyl-indol staining showed that fusion protein colocalizes with 4,6-diamidino-2-phenyl-indol staining of mitochondrial DNA (C).
Mitochondrial Telomere-binding Protein

8857

1. Autexier, C., and Greider, C. W. (1996) Trends Biochem. Sci. 21, 387–391
2. Nosek, J., Tomáška, L., Fukuhara, H., Suyama, Y., and Kováč, L. (1998) Trends Genet. 14, 184–188
3. Fukuhara, H., Sor, F., Drisli, R., Dinouel, N., Miyakawa, I., Rousset, S., and Viola, A. M. (1995) Mol. Cell. Biol. 15, 2309–2314
4. Dinouel, N., Drisli, R., Miyakawa, I., Sor, F., Rousset, S., and Fukuhara, H. (1995) Mol. Cell. Biol. 13, 2315–2323
5. Nosek, J., Dinouel, N., Kováč, L., and Fukuhara, H. (1995) Mol. Gen. Genet. 247, 61–72
6. Fang, G., and Cech, T. R. (1995) in Telomeres (Blackburn, E. H., and Greider, C. W., eds) pp.69–105, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
7. Tomáška, L., Nosek, J., and Fukuhara, H. (1997) J. Biol. Chem. 272, 3049–3056
8. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
9. Garrity, P. A., Wold, B., and Mueller, P. R. (1995) in PCR 2: A Practical Approach (McPherson, M. J., Hames, B. D., and Taylor, G. R., eds) pp. 309–322, IRL Press at Oxford University Press, Oxford
10. Niedenthal, R. K., Riles, L., Johnston, M., and Hegemann, J. H. (1996) Yeast 12, 773–786
11. Gietz, R. D., and Sugino, A. (1988) Gene 74, 527–534
12. Horváth, Á., and Riezman, H. (1994) Yeast 10, 1395–1310
13. Laemmli, U. K. (1970) Nature 277, 680–685
14. Resnick, R. J., and Racker, E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2474–2478
15. Tomáška, L. (1998) Biochem. Biophys. Res. Commun. 242, 457–460
16. Nosek, J., and Fukuhara, H. (1994) J. Bacteriol. 176, 5622–5630
17. Phillippsen, P., Stotz, A., and Scherf, C. (1991) Methods Enzymol. 194, 169–182
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989 Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Gietz, R. D., and Schiestl, R. H. (1995) Methods Mol. Cell. Biol. 5, 255–269
20. Van Dyck, E., Foury, F., Stillman, B., and Brill, S. J. (1992) EMBO J. 11, 3421–3430
21. Chase, J. W., and Williams, K. R. (1986) Annu. Rev. Biochem. 55, 103–136
22. Meyer, R. R., and Laine, P. S. (1990) Microbiol. Rev. 54, 342–380
23. Lohman, T. M., and Ferrari, M. E. (1994) Ann. Rev. Biochem. 63, 527–570
24. Suck, D. (1997) Nat. Struct. Biol. 4, 161–165
25. Yang, C., Curth, U., Urbanke, C., and Kang, C. H. (1997) Nat. Struct. Biol. 4, 153–157
26. Raghunathan, S., Ricard, C. S., Lohman, T. M., and Waksman, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6652–6657
27. Webster, G., Genschel, J., Curth, U., Urbanke, C., Kang, C. H., and Hägenfeld, R. (1997) FEBS Lett. 411, 313–316
28. Curth, U., Urbanke, C., Greipel, J., Gerberding, H., Tiranti, V., and Zeviani, M. (1994) Eur. J. Biochem. 221, 435–443
29. Edgell, D. R., and Dolittle, W. F. (1997) Cell 89, 995–998
30. Appel, R. D., Bairoch, A., and Hochstrasser, D. F. (1994) Trends Biochem. Sci. 19, 258–260
31. Casas-Finet, J. R., Khamsi, M. I., Maki, A. W., and Chase, J. W. (1987) FEBS Lett. 220, 347–352
32. Curth, U., Bayer, I., Greipel, J., Mayer, F., Urbanke, C., and Maass, G. (1991) Eur. J. Biochem. 196, 87–93
33. Bujalowski, W., and Lohman, T. M. (1991) J. Biol. Chem. 266, 1616–1626
34. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
35. Tiranti, V., Rocchi, M., DiDonato, S., and Zeviani, M. (1993) Gene 126, 219–225

Acknowledgments—We thank L.Kováč (Comenius University, Bratislava, Slovakia) for continuous support, helpful discussions, and comments, J.Kolarov (Comenius University, Bratislava) for technical assistance. We also thank R. Resnick (Cornell University, Ithaca, NY) for reading the manuscript and for valuable editorial advice.

REFERENCES