Synergism of the Two Myb Domains of Tay1 Protein Results in High Affinity Binding to Telomeres*

Katarina Visacka1, Citrad Hofr5, Smaranda Willcox4, Ivona Necasova5, Jana Pavlouskova5, Regina Sepsiova1, Michaela Wimmerova5, Lucia Simoniova5, Jozef Nosek5, Jiri Fajkus5, Jack D. Griffith5, and Lubomir Tomaska1†,‡

From the 1Departments of Genetics and Biochemistry, Comenius University, Faculty of Natural Sciences, Mlynska dolina, 842 15 Bratislava, Slovakia, 5Faculty of Science and Central European Institute of Technology, Masaryk University, Kamenice 5, Brno, CZ-62500, Czech Republic, and 4Lineberger Comprehensive Cancer Center and Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599

Background: In contrast to mammalian TRF1 and TRF2, yeast telomeric protein YTTay1 possesses two Myb domains.

Results: Kinetic and thermodynamic analyses revealed binding properties of individual Myb domains of YTTay1p.

Conclusion: The combined presence of the two Myb domains synergistically increases the affinity of YTTay1p to telomeric DNA.

Significance: The study demonstrates evolutionary tinkering with telomere-associated proteins.

Double-stranded regions of the telomeres are recognized by proteins containing Myb-like domains conferring specificity toward telomeric repeats. Although biochemical and structural studies revealed basic molecular principles involved in DNA binding, relatively little is known about evolutionary pathways leading to various types of Myb domain-containing proteins in divergent species of eukaryotes. Recently we identified a novel type of telomere-binding protein YTTay1p from the yeast *Yarrowia lipolytica* containing two Myb domains (Myb1, Myb2) very similar to the Myb domain of mammalian TRF1 and TRF2. In this study we prepared mutant versions of YTTay1p lacking Myb1, Myb2, or both Myb domains and found that YTTay1p carrying either Myb domain exhibits preferential affinity to both *Y. lipolytica* (GGGTTAGTC)n and human (TTAGGG)n telomeric sequences. Quantitative measurements of the protein binding to telomeric DNA revealed that the presence of both Myb domains is required for a high affinity of YTTay1p to either telomeric repeat. Additionally, we performed detailed thermodynamic analysis of the YTTay1p interaction with its cognate telomeric DNA, which is to our knowledge the first energetic description of a full-length telomeric-protein binding to DNA. Interestingly, when compared with human TRF1 and TRF2 proteins, YTTay1p exhibited higher affinity not only for *Y. lipolytica* telomeres but also for human telomeric sequences. The duplication of the Myb domain region in YTTay1p thus produces a synergistic effect on its affinity toward the cognate telomeric sequence, alleviating the need for homodimerization observed in TRF-like proteins possessing a single Myb domain.

The ends of linear chromosomes are capped by nucleoprotein complexes (telomeres) providing a solution to the end-replication problem and shielding the chromosomal termini from recognition by exonucleases and DNA repair machinery (1, 2). The major players in mediating telomeric functions are proteins directly associated with chromosomal ends. These include (i) telomerase, a unique reverse transcriptase carrying its own RNA template representing the major molecular tool for maintaining telomere length (3), (ii) single-stranded (ss) DNA-binding proteins (e.g. human and fission yeast Pot1 or budding yeast Cdc13) tightly attached to 3’ single-stranded telomeric overhang through 1–3 OB-folds (4), and (iii) Myb/homeodomain-containing proteins (e.g. mammalian TRF1, TRF2, fission yeast Taz1, and budding yeast Rap1) binding to the double-stranded (ds) repetitive part of telomeres (1, 5). Other proteins (e.g. Rap1, Tin2, and Tpp1 in Metazoa, Rap1, Poz1, Tpz1, and Ccq1 in fission yeasts) associate with telomeres indirectly via protein-protein interactions, and together with DNA-binding proteins they form a protective complex called shelterin (6). Finally, some proteins (e.g. tankyrase, various nucleases, and DNA repair proteins) form transient contacts with telomeres, whose frequency and duration depend on the state of a particular telomere (1).

The sequences of telomere-associated proteins undergo relatively fast evolutionary diversification (7), thus hampering the identification of their counterparts in distantly related organisms needed to uncover general principles of telomere maintenance. These efforts are made easier by the fact that despite their dissimilarities at the level of amino acid sequences, telomere-associated proteins share common structural elements (8). All known ds telomeric DNA-binding proteins contain at least one conserved Myb/homeodomain, although the rest of the proteins have very different sequence and domain topology (1, 8). Thus, Myb domains of known telomeric proteins can be used effectively as queries for searches within whole genomic sequences of organisms distantly related to established models for telomere biology (9–11).
Yeasts, especially Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, and Candida albicans, have proved to be invaluable models for the studies of telomeres (12). The ascomycetous fungi are a highly heterogeneous group of microorganisms comprising more than 1,000 known species resulting from diverse evolutionary trajectories (13), making them ideal for comparative analysis of telomeres. With this concept we initiated studies of nuclear telomeres in nonconventional yeast species including Yarrowia lipolytica. We characterized Y. lipolytica mutants lacking telomerase and found that they rapidly lose telomeric repeats and survive due to structural changes at the chromosomal ends (14). Characterization of proteins associated with the telomeres of this yeast species led to identification of a protein Tay1 (telomere associated in Yarrowia lipolytica 1), which (with the exception of its two Myb domains) does not resemble any known dsDNA-binding telomeric protein (15). YTay1 protein and its homologs found in S. pombe (Mug152) and filamentous fungi thus represent a novel group of proteins protecting the ds portion of telomeres. It was shown that YTay1p exhibits a preference for telomeric DNA in vitro and seems to bind to the substrate DNA as a dimer (15). Interestingly, the sequences of both Myb domains (Myb1, Myb2) are more similar to the Myb domains of mammalian TRF1/TRF2 proteins than to Myb domains of yeast telomeric proteins Rap1 (S. cerevisiae, C. albicans, K. lactis) or Taz1 (S. pombe). However, the contribution of individual Myb domains to a high affinity binding to telomeric repeats was not examined, and the binding properties of YTay1 were not compared with those of its mammalian counterparts.

Here, using various biochemical and biophysical methods, we investigated the contribution of each Myb domain to the specificity of YTay1 toward telomeric DNA. We found that although the individual Myb domains exhibit preferential binding to telomeric sequences, their combined presence dramat-ically increases the affinity of the protein for telomeric repeats. Interestingly, YTay1 protein exhibits a substantially higher affinity for human telomeric repeats than either TRF1 or TRF2, although the individual Myb domains exhibit preferential binding to telomeric sequences, their combined presence dramatically increases the affinity of the protein for telomeric repeats.

EXPERIMENTAL PROCEDURES

Microbial Strains—S. cerevisiae strain W303–1A (MATa, ade2-1, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, can1-100). Yeast cultures were grown in YPD medium (1% (w/v) yeast extract (Difco), 2% (w/v) Bacto-peptone (Difco), 2% (w/v) glucose) at 28°C. Escherichia coli strain DH5α (Invitrogen; F− Φ80lacZΔM15 (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK− mK−) phoA supE44 Δ thi-1 gyrA96 relA1) was used for cloning of recombinant DNA, and strain BL21-DE3 (Invitrogen; F− ompT hsdSB (rB− mB− gal, dcm) (DE3)) was used for production of recombinant YTay1–6HN proteins.

Construction of Yeast and Bacterial Expression Vectors Carrying Versions of the YTay1 Gene—Recombinant DNA techniques were carried out by standard procedures (16). The oligonucleotides (supplemental Table 1) were synthesized by MWG Operon, Metabion, or Sigma. For expression in S. cerevisiae, YTay1 was amplified with primers YTay1_pYES_UP and YTay1_pYES_DOWN using pTay1–6HN plasmid DNA (15) as a template. The primers carried HindIII and XhoI restriction sites in regions flanking the start and the stop codons of the YTay1 open reading frame, respectively. To facilitate efficient translation initiation, the primer YTay1-pYES_UP contained the Kozak sequence (5′-AAAAAA-3′) immediately upstream of the initiation ATG codon. The resulting PCR product was gel-purified using the Zymoclean Gel Recovery kit (Zymo Research), digested with HindIII and XhoI, ligated into the pYES2/CT vector linearized with the same restriction enzymes, and transformed into E. coli DH5α grown on LB solid media with 100 μg/ml ampicillin. The resulting plasmid pYES-Tay1 carries YTay1 under the control of the GAL1 promoter.

The mutant versions of pYES-Tay1 lacking Myb1 (Δ1) and Myb2 (Δ2) were prepared by inverse PCR with primers ΔMyb1_UP and ΔMyb1_DOWN (for Δ1) or ΔMyb2_UP and ΔMyb2_DOWN (for Δ2) using pYES-Tay1 DNA as a template. The resulting PCR fragments were gel-purified, ligated using T4 DNA ligase, and transformed into E. coli DH5α grown on LB solid media with 100 μg/ml ampicillin. The mutant lacking both Myb domains (ΔΔ) was amplified by inverse PCR using ΔMyb2_UP and ΔMyb2_DOWN using pYES-Tay1-Δ1 as a template and cloned analogously as the single mutants. The mutant versions of pYES-Tay1 carrying substitutions at selected sites within either Myb1 or Myb2 domains were prepared by inversion PCR with the corresponding couple of primers (their names indicate the mutated amino acids; supplemental Table 1) using pYES-Tay1 DNA as a template. The bacterial expression vectors carrying mutant versions of YTay1 lacking Myb3 domains (pTay1–6HN-Δ1, -Δ2, and -ΔΔ) were prepared analogously as described for pYES-Tay1 plasmids with the exception of using pTay1–6HN DNA as a template.

All PCR reactions were performed using 1 unit of Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) and contained each dNTP at 200 μM, corresponding primers at 1 μM and 10 ng of template DNA. The conditions of PCR reactions were adjusted according to the sequences of the primers and lengths of the final PCR product.

Yeast Growth Conditions and Transformation—Plasmids carrying WT (pYES-Tay1) or mutant versions of YTay1 gene (pYES-Tay1-Δ1, -Δ2, and -ΔΔ) were transformed into S. cerevisiae strain W303–1A using the lithium acetate method (17), and transformants were selected for uracil prototrophy on solid SD media (0.17% (w/v) yeast nitrogen base, 0.5% (w/v) (NH4)2SO4, 2% (w/v) glucose) containing corresponding amino acids and bases with the exception of uracil. Cells from single colonies were grown on SD media, then inoculated into liquid SGal (the same composition as SD except that instead of glucose, 2% (w/v) galactose was used as a sole carbon source) and cultured for 6 h at 28°C. Three microliters were then spotted from 10-fold serial dilutions on either SD (repressible conditions) or SGal (inducible conditions) solid media, and the growth was inspected after a 3-day cultivation at 28°C.

Purification of Recombinant YTay1 Proteins—The expression and purification of various versions of YTay1 protein was performed essentially as described in Kramara et al. (15) with several modifications. Bacterial expression plasmids carrying wild-type (pTay1–6HN-WT) as well as mutant versions (pTay1–6HN-Δ1, -Δ2, and -ΔΔ) of YTay1 were transformed into One Shot BL21(DE3) cells, and the transformants were...
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grown on LB plates containing 100 μg/ml ampicillin. The cells were then inoculated into 30 ml of 2×YT media (1.6% (w/v) Bacto-Tryptone, 1% (w/v) Bacto-peptone, 1% (w/v) NaCl (pH 7.1)) containing 2% (w/v) glucose, 100 μg/ml ampicillin and cultivated overnight (15 h) at 37 °C and 225 rpm. The cells were centrifuged for 5 min at 3000 rpm (Sorvall RT 7 Plus) at 25 °C, washed once with 2×YT, inoculated into 1 liter of 2×YT containing 100 μg/ml ampicillin, and cultivated at 37 °C and 275 rpm until the A_{600} reached a value of 0.7–0.8. The culture was cooled to 28 °C followed by the addition of isopropyl β-D-thiogalactopyranoside (final concentration 1 mm) and cultivation for additional 3 h at 28 °C. The culture was then centrifuged for 15 min at 5000 rpm at 4 °C (F10–6, Sorvall RC 6+) and the culture was then incubated for 15 min on ice with occasional shaking. The insoluble material was pelleted by centrifugation for additional 15 min on ice. Triton X-100 was added to a final concentration of 1 mg/ml, and the suspension was stored on ice (30–45 min) and resuspended in a final volume of 30 ml of buffer A (20 mM HEPES-KOH (pH 7.3), 300 mM NaCl, 1 mM DTT) containing 1× Complete (EDTA-free) protease inhibitors (Roche Applied Science), 10 mM MgCl₂, 50 units of DNase I (Invitrogen), and 2 μg of PureLink RNase A (Invitrogen). Lysozyme (Sigma) was added to a final concentration of 1 mg/ml, and the suspension was incubated for 15 min on ice with occasional shaking. The cells were broken by sonication (5 × 20 s at a setting of 7 (Branson Sonifer 450)). Each cycle of sonication was followed by 40 s of incubation on ice. Triton X-100 was added to a final concentration of 0.1% (v/v), and the suspension was incubated for an additional 15 min on ice. The insoluble material was pelleted by centrifugation at 15,000 rpm at 4 °C (F21–8, Sorvall RC 6+) and the supernatant was mixed with a 0.5-ml bed volume of Mono Q—The double-stranded YTETL50 probe (carrying 5 Y lipolytica telomeric repeats) used for EMSA was prepared by digestion of 15 μg of pMH25 plasmid (15) in a final volume of 50 μl using 50 units of EcoRI (New England Biolabs) followed by dephosphorylation using 50 units of calf intestinal phosphatase. The resulting 50-bp fragment was gel-purified using the ZymoClean gel extraction kit (ZymoResearch). The gel-isolated YTETL50 DNA was labeled with 10 units of T4 polynucleotide kinase (Fermentas) and 50 μCi of [γ-32P]ATP (final concentration 0.5 μCi) for 60 min at 37 °C in a final volume of 20 μl. Reactions were diluted to 50 μl with water, and the labeled oligonucleotide was purified using G-50 Sephadex (GE Healthcare). The binding reaction and gel electrophoresis were performed as described (15).

Electrophoretic Mobility Shift Assay (EMSA)—The double-stranded YTETL50 probe (carrying 5 Y lipolytica telomeric repeats) used for EMSA was prepared by digestion of 15 μg of pMH25 plasmid (15) in a final volume of 50 μl using 50 units of EcoRI (New England Biolabs) followed by dephosphorylation using 50 units of calf intestinal phosphatase. The resulting 50-bp fragment was gel-purified using the ZymoClean gel extraction kit (ZymoResearch). The gel-isolated YTETL50 DNA was labeled with 10 units of T4 polynucleotide kinase (Fermentas) and 50 μCi of [γ-32P]ATP (final concentration 0.5 μCi) for 60 min at 37 °C in a final volume of 20 μl. Reactions were diluted to 50 μl with water, and the labeled oligonucleotide was purified using G-50 Sephadex (GE Healthcare). The binding reaction and gel electrophoresis were performed as described (15).

Electron Microscopy—The typical DNA binding reaction for electron microscopy was performed in 10 μl of 1× HN buffer containing 5 ng/μl substrate DNA and 7–10 ng/μl purified YTETL50. The reactions were carried out at room temperature for 15 min followed by the addition of 10 μl of 1.2% glutaraldehyde and incubation at room temperature for additional 6 min. To remove the unbound proteins and fixative, the samples were diluted to 50 μl in HN buffer and passed over 2-ml columns of 6% agarose beads (ABT Inc., Burgos, Spain) equilibrated with TE buffer (10 mM Tris-HCl, (pH 7.4), 0.1 mM EDTA-NaOH). Aliquots of the fractions containing the complexes were mixed with a buffer containing spermidine and adsorbed onto copper grids coated with a thin carbon film glow-charged shortly before sample application. After adsorption of the samples for 2–3 min, the grids were dehydrated through a graded ethanol series and rotary shadowcast with tungsten at 10–12 torr (18). Samples were examined in an FEI T12 TEM equipped with a Gatan 2kx2k SC200 CCD camera. Dimensions of particles in the images saved from the CCD cameras were analyzed using Digital Micrograph software (Gatan, Inc.). Adobe Photoshop software was used to arrange images into panels for publication.

Isothermal Titration Calorimetry (ITC)²—ITC measurements were performed on a VP-ITC instrument (Microcal; GE Healthcare) at 25 °C. Solutions of protein and DNA were prepared with the same batch of buffer (50 mM sodium phosphate, 50 mM NaCl (pH 7.0)) to minimize artifacts due to minor differences in buffer composition. The duplex portion of the telomeric DNA was prepared by hybridizing the complementary strands at a 1:1 molar ratio. The proper formation of the duplex was checked by ion-exchange chromatography using Mono Q HR 5/5 column (GE Healthcare) with a NaCl gradient from 0.1 to 2.0 M. The protein solution (11 μM) was degassed and placed in the cell (1.432 ml). The DNA solution (50 μM) in a syringe was gradually added to the protein solution in 25 injections of 10 μl at intervals of 5 min while stirring at 242 rpm. The experimental data were fitted in Origin 7.0 software supplied with the instrument using a one-site binding model. From the fit, the binding enthalpy change (ΔH), association constant (Kₘ), and binding stoichiometry (n) were obtained. Binding free energy (ΔG) and entropy (ΔS) contributions were determined from the standard equation ΔG = −RTlnKₘ = ΔH − TΔS. The uncertainties in the parameters obtained are given by standard deviation of three independent measurements and the minor inaccuracy in determination of protein and DNA concentrations (relative error contribution 10%).

Fluorescence Anisotropy—The equilibrium binding of YTETL50 protein variants to DNA oligonucleotide duplexes containing 1.5 telomeric repeat was analyzed by fluorescence anisotropy. The DNA oligonucleotide labeled with the Alexa Fluor 488 (Invitrogen) was used as a probe. The anisotropy of the labeled DNA duplex was measured using a spectrofluorometer (Perkin-Elmer, LS 50B) with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The anisotropy (r) was calculated using the following equation:

\[ r = \frac{I_v - I_h}{I_v + 2I_h} \]

where Iᵥ is the intensity of the vertical polarization and Iₕ is the intensity of the horizontal polarization. The anisotropy was measured at various concentrations of protein and DNA, and the binding constant (Kₘ) and association constant (Kₐ) were determined from a Scatchard plot.

²The abbreviations used are: ITC, isothermal titration calorimetry; FA, fluorescent anisotropy.
roogen) was allowed to hybridize with the complementary oligonucleotide at an equimolar ratio. The complete formation of the duplex was verified by PAGE. The measurements of fluorescence anisotropy were conducted on a FluoroMax-4 spectrofluorometer (Horiba Jobin-Yvon, Edison, NJ) equipped with a thermostable cell holder and magnetic stirrer. Samples were excited with vertically polarized light at 490 nm, and both vertical and horizontal emissions were recorded at 520 nm. The integration time was 3 s. All measurements were conducted at 25 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl. A fixed delay of 120 s was set between each protein aliquot addition and start of the measurement to allow the binding reaction to reach equilibrium. This delay was sufficient, as no further change in anisotropy was observed. Each data point is an average of three measurements. The experimental binding isotherms were analyzed by nonlinear least squares regression in SigmaPlot 11 software (Systat Software) using a single-site binding model according to Heyduk and Lee (19) and confirmed by numerical approach using DynaFit software (20).

RESULTS

Heterologous Expression in S. cerevisiae Is a Convenient System for Assessing the Role of Myb Domains on the Activity of YTay1p—In silico analysis of the amino acid sequence of YTay1 protein revealed that it contains two putative Myb domains (Ref. 15, Fig. 1A). To investigate their contribution to the biological effects of the protein, we took advantage of a serendipitous finding that the expression of the YITAY1 gene from a strong (ADH1) promoter inhibits growth of S. cerevisiae. To test the possibility that the inhibitory effect of YITAY1 expression is due to its DNA binding activity, we prepared an expression vector with the YITAY1 gene and its mutant versions under the inducible GAL1 promoter. As expected, whereas the growth of the transformants carrying the wild-type YITAY1 (WT) on glucose was similar to the control strain, their growth was dramatically reduced on media containing galactose (Fig. 1B). Next, we tested the mutant version of YITay1p lacking both putative Myb domains (ΔΔ) and found that their growth on galactose was restored, indicating that the DNA binding activity of YITay1p is responsible for the growth inhibition (Fig. 1B). Importantly, expression of the mutant versions of YITay1p lacking either Myb1 (Δ1) or Myb2 domain (Δ2) did not interfere with the growth of S. cerevisiae, demonstrating that both Myb domains are essential for inhibition of growth mediated by the expression of YITay1p on S. cerevisiae.

Although the molecular mechanism responsible for the inhibitory effect of YITay1p on S. cerevisiae is not clear (see “Discussion”), the fact that it is dependent on the two Myb domains enables studies assessing the importance of specific amino acid residues for DNA binding of YITay1p. With this aim we constructed a series of mutant versions of YITAY1 carrying mutations in the conserved positions of the Myb domains. The mutations were designed based on the phenotypes of the versions of hTRF2 mutated in different positions of its Myb domain (21, 22) (Table 1). The mutated genes were then expressed under the GAL1 promoter to assess their effect on growth of S. cerevisiae. We found that although two mutant versions of YITay1p (E159K and E246K) inhibited growth of S. cerevisiae cells on galactose, four versions (I178A, R189E, I264A, and R283E) lost this ability (Fig. 1C, Table 1). The same phenotype observed for deletion mutants and mutants carrying point mutations indicates that the deletion mutants (Δ1, Δ2, ΔΔ) can be used for a detailed biochemical analysis of the DNA binding properties exhibited by individual Myb domains. Furthermore, these results demonstrate that this heterologous experimental system has a potential for fine mapping of the

A

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B

C

FIGURE 1. YITay1p contains two TRF-like Myb domains, both necessary to confer growth inhibition in S. cerevisiae. A, shown is conserved features of Myb domains of human TRF1 and TRF2 proteins and two Myb domains of YTay1p. Amino acid alignment was produced by ClustalW. Indicated are the positions mutated in the corresponding mutant variants of YITay1p, chosen according to the results of Hanaoka et al. (21) and Konishi and de Lange (22). Closed circles, amino acids that interact with DNA bases; open triangles, amino acids that interact with the sugar-phosphate; closed triangles, amino acids that interact with both DNA bases and sugar-phosphate; solid lines, helical regions (positions of the residues in human TRF1 are adopted from Hanaoka et al. (21)). Arrow, see “Discussion.” B, the inhibitory effect of galactose-induced expression of YITAY1 gene from GAL1 promoter on growth of S. cerevisiae can be alleviated by deletion of Myb1, Myb2, or both Myb domains. The scheme on left illustrates the nomenclature of the deletion mutants lacking Myb1 and/or Myb2 used in this study. C, the inhibitory effect of expression of YITAY1 gene on growth of S. cerevisiae is alleviated by point mutations in some of the conserved amino acids.

3 A. Mihaliкова, S. Kinsky, and L. Tomaska, unpublished results.
**TABLE 1**

The positions mutated in \( YlTay1p \) and corresponding mutations in hTRF1/hTRF2

| Mutation in \( YlTay1p \) | Corresponding mutation in hTRF2/mTRF2 | Inhibition of growth in \( S. cerevisiae \) |
|---------------------------|---------------------------------------|----------------------------------------|
| WT                        |                                       | Yes                                    |
| \( \Delta 1 \)            |                                       | No                                     |
| \( \Delta 2 \)            |                                       | No                                     |
| \( \Delta \Delta \)       |                                       | No                                     |
| Myb1_E159K                | E454K/E449K                           | Yes                                    |
| Myb1_I778A                | I473A/I468A                           | No                                     |
| Myb1_R189E                | R482E/R477E                           | No                                     |
| Myb2_E246K                | E454K/E449K                           | Yes                                    |
| Myb2_I264A                | I473A/I468A                           | No                                     |
| Myb2_E246K                |                                       | Yes                                    |
| Myb2_R283E                | R490E/R485E                           | No                                     |

\( YlTay1p \) protein (for example by mutagenic PCR) because it provides a convenient read-out for mutations disabling DNA binding.

**Each Myb Domain of \( YlTay1p \) Mediates Preferential Binding to Telomeric Repeats**—The experiments in \( S. cerevisiae \) indicated that both Myb domains of \( YlTay1p \) are important for mediating the effect of the protein, but they did not reveal whether loss of a single Myb domain results in the inability of \( YlTay1p \) to bind DNA, or alternatively, if it is due to changes in its specificity toward telomeric sequences. To address this issue, we prepared a set of bacterial expression vectors carrying the three-deletion versions of \( YlTay1p \) (\( \Delta 1, \Delta 2, \Delta \Delta \)). The WT as well as mutant proteins were produced in \( E. coli \), purified by affinity chromatography (Fig. 2A), and assayed for their DNA binding activities in vitro using EMSAs. These experiments demonstrated that, similar to WT protein, both single mutants (\( \Delta 1, \Delta 2 \)) exhibit DNA binding (Fig. 2B), indicating that a single Myb domain is fully competent for DNA binding. The double mutant (\( \Delta \Delta \)) did not exhibit any observable binding, demonstrating that at least one Myb domain is necessary for DNA binding in vitro (Fig. 2B).

To assess the ability of various versions of \( YlTay1p \) to bind to telomeric repeats, the purified recombinant proteins were incubated with the plasmid pYLTEL81 digested with \( B_{fl}AI \), thus producing linear DNA molecules carrying 81 \( Y. lipolytica \) telomeric repeats at one end (15). Visualization of the DNA-protein complexes by electron microscopy clearly showed that protein complexes by electron microscopy clearly showed that \( YlTay1p \) and corresponding mutations in \( S. cerevisiae \) (21) and Konishi and de Lange (22).

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**FIGURE 2.** Both Myb domains of \( YlTay1p \) are able to bind DNA and exhibit preference for telomeric repeats. A, shown is SDS-PAGE analysis of purified 6xHN-\( YlTay1p \) protein and its mutated versions lacking Myb1 (\( \Delta 1 \)), Myb2 (\( \Delta 2 \)), and both (\( \Delta \Delta \)) Myb domains. B, electrophoretic-mobility shift assay demonstrates that both Myb domains of \( YlTay1p \) are able to bind telomeric DNA. \( YlTR5.0 \), double-stranded DNA probe carrying 5 \( Y. lipolytica \) telomeric repeats. C, shown is electron microscopic analysis of the binding of WT and mutant versions of \( YlTay1p \) to a model telomere containing 81 \( Y. lipolytica \) telomeric repeats. The total length of the linearized plasmid is 3500 bp; the length of the telomeric tract is 810 bp. The bound protein is represented by dark particles at the terminal parts of DNA molecules.

**FIGURE 3.** An ITC profile corresponding to the injection of labeled \( YlTR1.5 \) into \( YlTay1p \). A typical ITC profile of \( YlTay1p \) (15). A typical ITC profile obtained at 25 °C is shown in Fig. 3, top panel. Exothermic heat pulses were observed after injections of DNA into the protein solution. Each area of the exothermic peak was integrated and corresponded to actual concentration of the reacting molecules. The values of molar heat of binding for each injection were plotted as a function of DNA:protein molar ratio (Fig. 3, bottom panel). The resulting thermogram showed a best-fit according to a model for a single binding site by using a nonlinear least squares method. The thermodynamic parameters, indicated in Fig. 3, bottom panel, were determined from the fit. The experimental errors of the thermodynamic values shown comprise a standard deviation of the fit for three independent experiments and the accuracy in determination of reactant concentrations.

The ITC experiments revealed that \( YlTay1p \) binds to the double-stranded telomeric DNA with relatively high affinity (\( K_{DT}^{ITC} = 80 \text{ nM} \)) (Fig. 3). Furthermore, the position of the
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Inflection point of the thermogram indicates that one molecule of the DNA duplex YITR1.5 is bound by two molecules of YTTay1p (Fig. 3). The thermodynamic data show that the relatively high negative binding enthalpy ($\Delta H = -19 \text{ kcal/mol}$) is partly compensated by entropy contribution ($\Delta S = -30 \text{ cal/mol} \cdot K^{-1}$; $-T\Delta S = 9 \text{ kcal/mol}$). The negative binding enthalpy observed by isothermal titration calorimetry demonstrates the formation of hydrogen bonds and van der Waals interactions during protein-DNA complex formation. Additionally, the calculated negative entropy change corresponds to the constraint of the intramolecular vibrational flexibility and the reduction in the translational and rotational degrees of freedom after formation of the nucleoprotein complex (23). The enthalpy and entropy contributions result in a well pronounced free energy of binding ($\Delta G = \Delta H - T\Delta S = -10 \text{ kcal/mol}$), which corresponds to the observed high binding affinity. From the thermodynamic parameters obtained, it is obvious that the DNA binding of YTTay1p protein is enthalpy-driven. Overall, the thermodynamic analysis of YTTay1p binding to Y. lipolytica telomeric DNA disclosed the high affinity and dimeric protein binding stoichiometry.

**The High Affinity Binding of YTTay1p to Telomeric Repeats Is a Result of Synergic Effect of the Two Myb Domains**—If both Myb domains of YTTay1p are able to bind preferentially to telomeric repeats, what is their contribution to the overall binding characteristics of the protein? To address this question, we employed fluorescent anisotropy (FA) and compared the binding affinity of WT along with the mutant versions of YTTay1p lacking Myb domains. The binding affinity of YTTay1p variants to double-stranded DNA was monitored by FA measurement. If the solution contains only free fluorescently labeled DNA molecules, FA is relatively low, due to the fast rotational rearrangement of DNA molecules. If the protein aliquots are added to the solution of labeled DNA, a bulky slower rotating protein-DNA complex is formed, and the anisotropy value increases. We used YITR1.5 (labeled with Alexa Fluor 488) as a DNA substrate for YTTay1p binding assays (same substrate as the one used for ITC experiments). The anisotropy change described the extent of YTTay1p variants binding to telomeric DNA duplex. The equilibrium binding affinity was quantified by analysis of the recorded binding isotherms.

Importantly, both the association ($K_a$) and dissociation ($K_D$) constants obtained from the measurements of binding of WT YTTay1p to the DNA were in excellent agreement with these parameters calculated from the ITC data (Fig. 4, Table 2). The measurements of the binding of the mutant versions of YTTay1p to the telomeric oligonucleotide revealed that both single mutants ($\Delta 1$, $\Delta 2$) exhibit almost 4-fold lower affinity to the substrate than the WT protein (Fig. 4A; Table 2). The mutant version of YTTay1p lacking both Myb domains ($\Delta 3$), in agreement with the experiments described above, exhibited negligible binding to the DNA (Fig. 4A; Table 2). Thus, the equilibrium binding data support the cooperative contribution of both Myb domains to the overall high DNA binding affinity of YTTay1p.

**YTTay1p Protein Exhibits Higher Binding Affinity to Both Y. lipolytica and Human DNA Telomeric Repeats Than Human TRF1 and TRF2**—As the amino acid sequences of both Myb domains of YTTay1p are highly similar to the Myb domains of hTRF1 and hTRF2 (15), it was of interest to compare the binding affinity of all three proteins (YTTay1p, hTRF1, and hTRF2) to either Y. lipolytica (YITR1.5) or human (HsTR2.0) telomeric DNA. The binding of YTTay1p variants to double-stranded telomeric oligonucleotides was monitored by fluorescence anisotropy. As one would assume, YTTay1p exhibited much higher (13–23-fold) affinity for the *Y. lipolytica* telomeric DNA (YITR1.5) than either hTRF1 or hTRF2 (Fig. 4B, Table 2).

Surprisingly, the same result was observed when a human telomeric oligonucleotide was used for the binding reaction. Whereas the affinity of hTRF1 ($K_D = 114 \text{ nM}$) and hTRF2 ($K_D = 135 \text{ nM}$) for HsTR2.0 was similar to the affinity of YTTay1p for YITR1.5 ($K_D = 81 \text{ nM}$), WT YTTay1p bound human telomeric oligonucleotide with an ~8-fold higher affinity than either of the human telomeric proteins (Fig. 4B, Table 2). Further ITC analysis of WT YTTay1p binding to human telomeric DNA oligonucleotide HsTR2.0 has shown binding stoichiometry 2:1 (protein:DNA); i.e., two molecules of YTTay1p bind one dsDNA oligonucleotide. Moreover, the ITC measurement confirmed the higher binding affinity ($K_D^{\text{ITC}} = 18 \text{ nM}$) of WT YTTay1p to human telomeric dsDNA HsTR2.0 (data not shown). Even one of the single mutants lacking the Myb1 domain ($\Delta 1$) exhibited more than a 2-fold higher affinity for HsTR2.0 than hTRF1 or hTRF2; similarly, the affinity of $\Delta 2$ was 2-fold higher than in the
case of the human proteins (Fig. 4B, Table 2). Altogether, comparison of the binding affinity of YTay1p variants to Y. lipolytica and human telomeric DNA demonstrated that YTay1p binds both telomeric sequences significantly tighter than human telomeric proteins TRF1 and TRF2.

### DISCUSSION

Telomere binding factors associated with either ss- or dsDNA regions of the chromosomal ends represent an evolutionarily highly divergent group of proteins (7, 8). Their only common feature is the presence of a conserved sequence and/or structural domain (OB-fold or Myb domain) mediating specific binding to telomeric repeats. However, the topology and the number of these DNA binding modules on the corresponding proteins greatly vary. Comparative analysis of the representatives of various types of telomere-binding proteins is the only approach enabling extraction of both specific and general characteristics of telomere protection in eukaryotes (12).

YTay1p of Y. lipolytica together with its homologues in S. pombe (Mug152) and basidiomycetous fungi (15, 24) represent a unique group of telomeric proteins possessing two Myb domains that exhibit high similarity to the Myb domains of mammalian TRF1 and TRF2. The similarities between amino acid sequences of the Myb domains in some cases approaches 50% identity (Myb1 of YTay1p and Myb of TRF1; (15)), which is a much higher value than that of the Myb domain of SpTaz1, the most intensively studied fungal telomeric protein considered to be a functional orthologue of TRF1/TRF2. The main difference between YTay1p and SpTaz1p, TRF1, or TRF2 is the presence of two Myb domains within the single polypeptide of Tay1p. Although our previous data (gel filtration, electron microscopy) indicate that YTay1p forms oligomers in vitro, it is possible that in contrast to SpTaz1p, TRF1, and TRF2, where homo-oligomerization is a prerequisite for high affinity DNA binding, the tandem Myb domains of YTay1p would allow a single monomer of the protein to bind effectively its target sequences.

The data supporting this hypothesis are derived from an artificial, yet highly informative experimental system. When we tried to test the ability of YTay1p to bind Y. lipolytica telomeric repeats using the one-hybrid system, we were unable to transform S. cerevisiae with an expression plasmid carrying the YTAY1 gene under the control of a strong constitutive ADH1 promoter. When we placed YTAY1 under the inducible GAL1 promoter, we demonstrated that the expression of this gene inhibits growth of S. cerevisiae (Fig. 1). We currently do not understand the reason for this inhibitory effect. EMSA assays using Y. lipolytica, S. pombe, and S. cerevisiae telomeric tracts as probes revealed that YTay1p is able to bind to telomeres of distantly related yeast species in vitro (supplemental Fig. 1).
However, it is unlikely that the inhibition of growth of *S. cerevisiae* cells expressing the YITAY1 gene is caused by its binding to telomeres. First, we did not observe any differences in telomere length in the *S. cerevisiae*-expressing YITAY1 gene when the cells were transferred from glucose to galactose (supplemental Fig. 2). Second, YITAY1 seems to be an essential gene for *Y. lipolytica* (similarly to its *S. pombe* homologue MUG152 (15)), which is able to survive in the absence of telomeric repeats (14). It is more likely that in *S. cerevisiae* YITay1p competes for the binding sites with essential DNA-binding proteins, possibly ScRap1p and/or ScTbf1p, both telomere-associated proteins that are indispensable not due to their telomeric functions but due to their binding to nontelomeric sites within the *S. cerevisiae* genome (25, 26). Displacement of such proteins by YITay1p may thus interfere with their vital functions. Regardless of the reason, the ability of YITay1p to inhibit growth in *S. cerevisiae* is dependent on its DNA binding activity (Fig. 1). The fact that mutant versions lacking either Myb1 or Myb2 domain do not affect growth of *S. cerevisiae* indicates that the high affinity binding is achieved by the concerted action of both Myb domains.

Experiments reconstituting the binding of YITay1p and its mutant versions to telomeric sequences using EMSA and electron microscopy revealed that the individual Myb domains bind DNA (Fig. 2B) and exhibit preference for telomeric sequences (Fig. 2C). It is likely that the binding of the mutant proteins containing a single Myb domain results from oligomerization of the protein (see above). On one hand, this would suggest that the primary function of oligomerization for the wild-type protein is not to facilitate the specific binding to the DNA substrate but to mediate formation of telomere-telomere bridges as observed in our previous report (15). On the other hand, when the protein lacks a single Myb domain but retains its ability to form oligomers, it should still bind DNA with some specificity similar as in the case of the single hTRF1 Myb domain, which is able to specifically bind to human telomeres (27, 28). This line of argument together with data from *S. cerevisiae* (Fig. 1) predicts that even though single deletion mutants exhibit a preference for telomeric repeats, their affinity for the substrate should be substantially lower than in the case of wild-type protein.

The ITC and FA measurements point to exactly that direction (Figs. 3 and 4). Single deletion mutants lacking either Myb1 or Myb2 domain exhibited an almost 4-fold lower affinity for the *Y. lipolytica* telomeric sequence than the wild-type protein. This indicates that YITay1p Myb domains act synergistically to set the affinity of the protein to the values observed by both ITC and FA (*K_0D* = 70–80 nm). Similar *K_0D* values have been obtained for binding of human TRF1 and TRF2 to human telomeric repeats (Fig. 4B, Table 2). These values are in good agreement with data obtained by Hanaoka et al. (21), although these authors observed that the DNA binding domain of hTRF1 has an almost 4-fold higher affinity for human telomeric repeats (*K_0D* ~ 200 nm) compared with hTRF2 (*K_0D* ~ 750 nm). The *K_0D* values for hTRF1 DNA binding domain obtained by König et al. (27) were much lower (~3 nm). Furthermore, we compared the *K_0D* values obtained for YITay1p lacking either the Myb1 or Myb2 domain with those of plant proteins from the SMH family containing single Myb-like domains (29). We found that the affinities observed for YITay1p variants with only one Myb domain fall into the interval observed for the SMH proteins (*K_0D* ~ 100–400 nm).

The thermodynamic values determined by isothermal titration calorimetry are in good agreement with the values described in the single previous thermodynamic study showing equivalent c-Myb domain interaction with their cognate DNA sequence (30). In this study, Oda et al. (30) characterized the specific binding of c-Myb R2R3, containing two Myb domains, to its cognate DNA sequence using isothermal titration calorimetry. The value of binding enthalpy for c-Myb R2R3 at 25 °C (∆H = −15 kcal/mol) is in good agreement with the value −19 kcal/mol measured for the YITay1p interaction with DNA in our study. Similarly, values of free energy of binding ∆G are comparable: −10 kcal/mol for YITay1p and −12 kcal/mol for c-Myb R2R3; both values were measured at 25 °C. The comparable values of thermodynamic parameters for DNA binding of c-Myb R2R3 or YITay1p, both containing two DNA binding domains, allow us to speculate that the binding is mediated by the same number of Myb domains. So, even though two molecules of YITay1p containing a total of four DNA binding domains bind to the target sequence, only two DNA binding domains may take part in the interaction with DNA at any time. It is possible that the remaining two DNA binding domains may contribute to interactions leading to shaping of telomeric DNA into higher-order structures in vivo. The comparison of YITay1p binding affinity to its cognate *Y. lipolytica* and human telomeric DNA reveals unexpected results. Intriguingly, YITay1p exhibits about 8-fold higher affinity for human telomeric repeats than its mammalian counterparts (in fact, it binds to human telomeres with about 4-fold higher affinity than to its native *Y. lipolytica* telomeric substrate) (Fig. 4B, Table 2). Hanaoka et al. (21) observed that several substitutions at critical positions within the Myb domain substantially increased the affinity of binding of hTRF2, indicating that the natural selection does not necessarily lead to maximization of the binding affinity. In addition to differences in amino acid sequences, the higher affinity binding of YITay1p to human telomeric repeats compared with hTRF1/hTRF2 may be caused by the presence of tandem Myb domains on a single polypeptide, making the binding more efficient than in the case of a single Myb domain-carrying protein that provides the two Myb domains in the form of homodimer. The observation that the affinity of Δ1 version of YITay1p is within the same range as the TRF1/TRF2 goes in line with this argument (providing it still can form homo-oligomers observed for wild-type protein). Yet it is surprising that Δ1 has higher affinity for the human probe than Δ2, as Myb1 (retained in Δ2) is more similar to the Myb domain of TRF1/TRF2 than Myb2 (retained in Δ1), especially when both single mutants exhibit very similar affinity to *Y. lipolytica* telomeric repeats. At present we do not have an explanation for this discrepancy. Perhaps the presence of a lysine in Myb1, in the position in which serine or alanine mediate the contact with DNA (Fig. 1A, arrow; Ref 21), is responsible for a decreased binding of the Myb1 domain to the human telomeric sequence. As in the case of other telomere-binding proteins, like TRF1, TRF2, and ScRap1 (21, 27, 28, 31–33), more detailed structural
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analysis of YTay1p (or at least its Myb domains) would be highly instrumental in addressing these issues.

The observations that YTay1p exhibits higher affinity for human telomeres than to *Y. lipolytica* telomeres and that it shows lower dissociation constants for binding to human telomeres than human telomeric proteins TRF1/TRF2 underlie one important general evolutionary principle: natural selection does not necessarily lead to maximization of the affinity of a particular DNA-binding protein to its cognate DNA substrate. Perhaps the binding properties of a DNA-binding protein are tuned to inferior values, thus, enabling its dynamic association with the target DNA loci.

Comparative analyses, similar to that presented in our study, allow one to formulate hypotheses about evolutionary paths leading to contemporary telomeric binding factors. Even when limiting the comparison to fungi and mammals and disregarding proteins like Tbf1 able to bind telomeric repeats *in vitro* but binding to subtelomeric regions *in vivo* (25), the repertoire of Myb domain-containing proteins directly associated with double-stranded regions of telomeres is quite wide. Modern fungal or mammalian cells contain a diverse combination of three major players: (i) TRF-like proteins (SpTaz1, TRF1, TRF2) possessing a single Myb domain and binding to telomeres as homooligomers (although a Myb domain of hTRF1 can bind to telomeric sequence (27)), (ii) YTay1-like with two tandem Myb domains exhibiting high similarity to Myb domains of TRF-like proteins, and (iii) ScRap1-like proteins possessing two Myb domains exhibiting a weak similarity to the former two groups of proteins (32). The evolution of these three groups of proteins could have proceeded in two phases (supplemental Fig. 3). During the first phase, the ancestral genomes accumulated precursors of all three types of proteins possibly via the neutral evolutionary ratchet (34) involving gene and domain duplications. This might have generated a complex set of proteins that could have adapted to either general functions related to regulation of gene expression or more specialized functions at telomeres. In some cases the proteins started to play both telomere- and nontelomere-associated roles (ScRap1p, YTay1p) making them essential components of the cell. In other cases, all three types of proteins were adopted for a specialized function(s), leading to loss of DNA binding activity (SpRap1, hRap1) and/or gene loss (there does not seem to be a YTay1-like protein in mammalian cells and conventional yeast models like *S. cerevisiae, K. lactis, C. albicans*, or *S. pombe*). Are there any advantages of having two single Myb domain-containing proteins versus a protein containing two Myb domains within a single molecule? For example, the former is probably more dynamic in its effect (in response to its cellular level) as a certain minimum level of a protein is necessary to form homodimers with a reasonably high binding affinity. In addition, having two separate telomeric proteins forms a basis for higher levels of regulation that may be important for complex mammalian cells. On the other hand, the latter results in formation of more stable complexes independently of the instantaneous concentration of the protein and thus may be more tolerant to changes in environmental conditions as could be expected in single-cell organisms. Future studies on telomere-binding proteins as well as other components involved in telomere maintenance from various phylogenetic groups will fill the gaps in this incomplete picture.

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**REFERENCES**

1. de Lange, T. (2009) How telomeres solve the end-protection problem. Science 326, 948–952
2. McEachern, M. I., Krauskopf, A., and Blackburn, E. H. (2000) Telomeres and their control. Annu. Rev. Genet. 34, 331–358
3. Greider, C. W., and Blackburn, E. H. (1987) The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell 51, 887–898
4. Theobald, D. L., Mitton-Fry, R. M., and Wuttke, D. S. (2003) Nucleic acid recognition by OB-fold proteins. Annu. Rev. Biophys. Biomol. Struct. 32, 115–133
5. Palm, W., and de Lange, T. (2008) How shelterin protects mammalian telomeres. Annu. Rev. Genet. 42, 301–334
6. de Lange, T. (2005) Shelterin. The protein complex that shapes and safeguards human telomeres. Genes Dev. 19, 2100–2110
7. Linger, B. R., and Price, C. M. (2009) Conservation of telomere protein complexes. Shuffling through evolution. Crit. Rev. Biochem. Mol. Biol. 44, 434–446
8. Lewis, K. A., and Wuttke, D. S. (2012) Telomerase and telomere-associated proteins. Structural insights into mechanism and evolution. Structure 20, 28–39
9. Karamysheva, Z. N., Suurotsvea, Y. V., Vespa, L., Shakirov, E. V., and Shippen, D. E. (2004) A C-terminal Myb extension domain defines a novel family of double-strand telomeric DNA-binding proteins in Arabidopsis. J. Biol. Chem. 279, 47799–47807
10. Kuchar, M., and Fajkus, J. (2004) Interactions of putative telomere-binding proteins in Arabidopsis thaliana. Identification of functional TRF2 homolog in plants. FEBS Lett. 578, 311–315
11. Peška, V., Schrumpfová, P. F., and Fajkus, J. (2011) Using the telobox to search for plant telomere binding proteins. Curr. Protein Pept. Sci. 12, 75–83
12. Lue, N. F. (2010) Plasticity of telomere maintenance mechanisms in yeast. Trends Biochem. Sci. 35, 8–17
13. Dujon, B. (2010) Yeast evolutionary genomics. Nat. Rev. Genet. 11, 512–524
14. Kinsky, S., Mihalikova, A., Kramara, J., Nosek, J., and Tomaska, L. (2010) Lack of the catalytic subunit of telomerase leads to growth defects accompanied by structural changes at the chromosomal ends in *Yarrowia lipolytica*. Curr. Genet. 56, 413–425
15. Kramara, J., Willcox, S., Gunisova, S., Kinsky, S., Nosek, J., Griffith, J. D., and Tomaska, L. (2010) Tay1 protein, a novel telomere binding factor from *Yarrowia lipolytica*. J. Biol. Chem. 285, 38078–38092
16. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning, A Laboratory Manual, 3rd Ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
17. Giez, R. D., Schiestl, R. H., Willems, A. R., and Woods, R. A. (1995) Studies on the transformation of intact yeast cells by the LiAc/ss-DNA/PEG procedure. Yeast 11, 355–360
18. Griffith, J. D., and Christiansen, G. (1978) Electron microscope visualization of chromatin and other DNA-protein complexes. Annu. Rev. Biophys. Bioeng. 7, 19–35
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19. Heyduk, T., and Lee, J. C. (1990) Application of fluorescence energy transfer and polarization to monitor Escherichia coli cAMP receptor protein and lac promoter interaction. Proc. Natl. Acad. Sci. U.S.A. 87, 1744–1748
20. Kuzmic, P. (1996) Program DYNAFIT for the analysis of enzyme kinetic data. Application to HIV protease. Anal. Biochem. 237, 260–273
21. Hanaoka, S., Nagadoi, A., and Nishimura, Y. (2005) Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA binding activities. Protein Sci. 14, 119–130
22. Konishi, A., and de Lange, T. (2008) Cell cycle control of telomere protection and NHEJ revealed by a ts mutation in the DNA binding domain of TRF2. Genes Dev. 22, 1221–1230
23. Oda, M., and Nakamura, H. (2000) Thermodynamic and kinetic analyses for understanding sequence-specific DNA recognition. Genes Cells 5, 319–326
24. Sánchez-Alonso, P., and Guzman, P. (2008) Predicted elements of telomere organization and function in Ustilago maydis. Fungal Genet. Biol. 45, 554–562
25. Koering, C. E., Fourel, G., Binet-Brasselet, E., Laroche, T., Klein, F., and Gilson, E. (2000) Identification of high affinity Tbf1p-binding sites within the budding yeast genome. Nucleic Acids Res. 28, 2519–2526
26. Lieb, J. D., Liu, X., Botstein, D., and Brown, P. O. (2001) Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association. Nat. Genet. 28, 327–334
27. König, P., Fairall, L., and Rhodes, D. (1998) Sequence-specific DNA recognition by the myb-like domain of the human telomere binding protein TRF1. A model for the protein-DNA complex. Nucleic Acids Res. 26, 1731–1740
28. Nishikawa, T., Okamura, H., Nagadoi, A., König, P., Rhodes, D., and Nishimura, Y. (2001) Solution structure of a telomeric DNA complex of human TRF1. Structure 9, 1237–1251
29. Hofr, C., Sultesová, P., Zimmermann, M., Mozgová, I., Procházková Schrumpfová, P., Wimmerová, M., and Fajkus, J. (2009) Single-Myb-histone proteins from Arabidopsis thaliana. A quantitative study of telomere binding specificity and kinetics. Biochem. J. 419, 221–228
30. Oda, M., Furukawa, K., Ogata, K., Sarai, A., and Nakamura, H. (1998) Thermodynamics of specific and non-specific DNA binding by the c-Myb DNA binding domain. J. Mol. Biol. 276, 571–590
31. Court, R., Chapman, L., Fairall, L., and Rhodes, D. (2005) How the human telomeric proteins TRF1 and TRF2 recognize telomeric DNA. A view from high resolution crystal structures. EMBO Rep. 6, 39–45
32. König, P., Giraldo, R., Chapman, L., and Rhodes, D. (1996) The crystal structure of the DNA binding domain of yeast RAP1 in complex with telomeric DNA. Cell 85, 125–136
33. Nishikawa, T., Nagadoi, A., Yoshimura, S., Aimoto, S., and Nishimura, Y. (1998) Solution structure of the DNA binding domain of human telomeric protein, hTRF1. Structure 6, 1057–1065
34. Lukeš, J., Archibald, J. M., Keeling, P. J., Doolittle, W. F., and Gray, M. W. (2011) How a neutral evolutionary ratchet can build cellular complexity. IUBMB Life 63, 528–537