The yeast telomere length regulator TEL2 encodes a protein that binds to telomeric DNA

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ABSTRACT

TEL2 is required for telomere length regulation and viability in Saccharomyces cerevisiae. To investigate the mechanism by which Tel2p regulates telomere length, the majority (65%) of the TEL2 ORF was fused to the 3′-end of the gene for maltose binding protein, expressed in bacteria and the purified protein used in DNA binding studies. Rap1p, the major yeast telomere binding protein, recognizes a 13 bp duplex site 5′-GGTGTGGGTGTTG-3′ in yeast telomeric DNA with high affinity. Gel shift experiments revealed that the MBP–Tel2p fusion binds the double-stranded yeast telomeric Rap1p site in a sequence-specific manner. Analysis of mutated sites showed that MBP–Tel2p could bind 5′-GTGTGTGGG-3′ within this 13 bp site. Methylation interference analysis revealed that Tel2p contacts the 5′-terminal guanine in the major groove. MBP–Tel2p did not bind duplex telomeric DNA repeats from vertebrates, Tetrahymena or Oxytricha. These results suggest that Tel2p is a DNA binding protein that recognizes yeast telomeric DNA.

INTRODUCTION

Telomere length regulation, regulation of the number of repeated DNA sequences that make up the chromosome ends in many organisms, has recently been recognized as a critical function in human cells. Telomere DNA repeats are lost from somatic cell telomerases as cells divide (1), leading to the proposal that telomeres both activate cellular senescence when telomeres become too short and eliminate many transformed cells by causing chromosome destruction by loss of telomere function. The telomeric DNA repeats are the only sequences required for telomere function in yeast and humans (2–6), so loss of these repeated sequences that are elongated by telomerase, a reverse transcriptase containing its own RNA template (12,16), and both contain telomere binding proteins whose cellular concentration alters telomere length (14,17). Yeast molecular genetics has allowed identification of a number of genes that alter telomere length and identification of two yeast telomere length regulators, TEL1 and TEL2 (18–20). TEL1 shows strong sequence similarity to human ATM (ataxia telangiectasia mutated) and DNA-PKcs (the catalytic subunit of the DNA-dependent serine/threonine kinase) (19–22) and inactivating mutations of TEL1 yield viable cells with short telomeres. In contrast, TEL2 has no strong sequence homology to any known genes and is required for cell viability (20).

Yeast telomeres consist of 325 ± 75 bp of irregular TG1–3 repeats in many laboratory strains (reviewed in 23). Telomere length is probably maintained by balancing lengthening (mediated by telomerase) and shortening (due to incomplete replication, rapid deletion and nuclease action) processes (23–25). Wild-type yeast cells respond to different perturbations that cause telomeres to be maintained at different equilibrium lengths. Cells double the length of their telomeres if a large excess of yeast telomeric sequences are introduced on a high copy plasmid (26). A similar increase occurs when the C-terminus of the yeast telomere binding protein Rap1p is overproduced in cells (17). Unlike wild-type cells, cells bearing viable tel1-1 and tel2-1 mutations maintain short telomeres (~50 and 100 bp respectively) (18) and these telomeres do not increase in length when cells are subjected to the above perturbations (20). These results have classified the TEL1 and TEL2 gene products as part of the telomere length regulatory system because these mutations disrupt the feedback loops that cause telomere lengthening. In addition, the double

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tell1-1 tel2-1 mutant maintains telomeres at the same length as tell1-1 cells (18), indicating that both TEL1 and TEL2 act in the same genetic pathway.

Mutations in a number of other genes encoding proteins that bind telomeric DNA in vitro also alter telomere length. RAP1 encodes the major yeast telomere binding protein and different mutations can cause an increase (27,28) or decrease (29) in telomere length. Mutations in the genes encoding two proteins that associate with the Rap1p C-terminus, rif1 (30) and rif2 (31), cause telomeres to be maintained at longer equilibrium lengths. Mutations in either of two single-stranded yeast telomere binding proteins encoded by EST1 (32) and CDC13/EST4 (33) can cause telomeres to gradually shorten until cells die. Thus, yeast telomere binding proteins play a crucial role in regulating yeast telomere length. How the actions of these proteins are coordinated to maintain telomeres within a defined range yet to be determined.

The DNA binding and cellular functions of Rap1p have been characterized both genetically and biochemically. In vitro binding studies have shown that Rap1p binds a 13 bp telomeric site 5′-GGTGTGTTGGGTGTT-3′ (35,36). Besides telomeres, Rap1p also binds to various upstream activating sequences and silencer elements with a minimal consensus binding site 5′-RRT/ GGNNTGGGTGY-3′ (35,37). The Rap1p site from the yeast telomere is one of the highest affinity Rap1p sites known (35).

As described above, TEL2 plays an unknown role in yeast telomere length regulation and cell viability. TEL2 has no significant sequence similarity to any known gene in the database and the deduced amino acid sequence of Tel2p has no known functional motifs. To understand the role of Tel2p in telomere length regulation, we constructed an MBP–Tel2p fusion and tested its ability to bind telomeric dsDNA. The MBP–Tel2p fusion binds to duplex yeast telomeric DNA in a sequence-specific manner, requiring only 8 bp of the 13 bp Rap1p site. Methylation interference and competition experiments indicate that this binding is specific. These data suggest that Tel2p function involves interactions with DNA.

**MATERIALS and METHODS**

**Construction of RAPI and TEL2 fusions**

A 446 amino acid portion of the 688 amino acid TEL2 ORF (amino acids 181–626) was fused to the 3′-end of the maltose binding protein (MBP) gene. The MBP gene is present in plasmid pMAL-c2 under control of the pTac promoter (New England Biolabs). Expression of full-length Tel2p is toxic to bacteria and repeated attempts to generate a full-length fusion resulted in no bacterial transformants or transformants containing rearranged plasmids. The TEL2 (Genbank accession no. U38538) NheI fragment from nt 1159 to 2499 was cloned into pMal-c2 at the Xhol site. The Nhel fragment was brought in-frame with the MBP ORF by sequential filling in of the upstream BamHI and EcoRI sites with T4 DNA polymerase. This fusion could be propagated and produced in bacteria. This fusion contains 65% of the entire TEL2 ORF, where only nt 1441–2683 are required for cell viability (20). This plasmid construct is designated pMT-1. A MBP–Rap1p fusion was also constructed (857–3245, as numbered in 38; Genbank accession no. M18068) as a positive control, since Rap1p is known to bind yeast duplex telomeric DNA with high affinity (35). Plasmid D123 (RAP1 in YCp50; 38), which contained the RAP1 gene, was first digested with EcoRI and the ends rendered blunt with the Klenow fragment of DNA polymerase I and then digested with PstI. Plasmid pMAL-c2 was cut with HindIII, the ends rendered blunt with the Klenow fragment of DNA polymerase I and then digested with PstI. The cleaved plasmid was gel purified. The PstI–blunt RAP1 fragment was also gel purified and ligated to the PstI–blunt pMAL-c2 vector. The RAP1 ORF was then brought in-frame with the MBP ORF by filling in the upstream SacI site with T4 DNA polymerase. This plasmid construct is designated pMR-1. All constructs were verified by DNA sequencing of the MBP–TEL2 and MBP–RAP1 fusion junctions.

**Expression and purification of Tel2p and Rap1p in bacteria**

Both pMT-1 and pMr-1 were transformed into BL21(DE3) bacterial cells. The bacterial cells were grown at 37°C in 500 ml LB broth with 50 mg/l carbenicillin. The cultures were induced at an OD600 of 0.3–0.4 by addition of 0.1 M IPTG to a final concentration of 0.3 mM and grown for 6–12 h at 22°C. Cells were harvested and cell pellets dissolved in 10 ml column buffer consisting of 100 mM Tris–Cl, pH 8, 200 mM NaCl, 0.005 mM DTT and stored at –80°C until further use. The bacterial cell pellet was thawed and sonicated for 6 × 10 s with 10 s intervals on ice for 2 min. The sonicated slurry was spun at 14,000 g for 10 min and the supernatant chromatographed on a 15 ml amylose resin column. The fusion proteins were purified at 4°C according to the manufacturer’s specifications (New England Biolabs). Protein quantitation was performed using the BioRad Bradford assay using BSA to construct the standard curve. The purified proteins were analyzed by SDS–PAGE and found to be 30–50% pure, with the major contaminant being a band that co-migrated with pure MBP (New England Biolabs). Western blotting used polyclonal anti-MBP Ab (New England Biolabs) and the BioRad Chemiluminescent kit.

**Preparation of DNA substrates**

Desalted purified oligomers of different lengths of telomeric and non-telomeric single-stranded (ss) DNA sequences were purchased from Ransom Hill Biosciences Inc. (Ramona, CA). The G-rich strand oligonucleotide was end-labeled using T4 DNA poly-nucleotide kinase (39). Duplexes were formed by mixing the end-labeled oligonucleotides with a 5-fold molar excess of the complementary strand for all oligonucleotides except R3-ST, where a 6-fold molar excess was used (40). Thus, in addition to the competitor DNA present in the binding reaction, each reaction also contains a 5 (or 6)-fold molar excess of competitor ssDNA from the C-rich strand. The DNAs were denatured at 95°C for 5 min and re-annealed by allowing cooling slowly to room temperature over a period of 6–12 h. The duplex DNAs were ethanol precipitated and stored at –80°C until further use. Non-radioactive competitor DNAs were also prepared with a 5-fold molar excess of the C-rich strand in the same manner.

**Gel retardation assays**

The 32P-labeled probe, 8 ng dsDNA (0.023 μM final concentration for the 26 bp duplex probes) and 32 ng unlabeled C-rich oligonucleotide were incubated with either bacterial cell lysate or partially purified protein in 20 μl binding buffer consisting of 25 mM HEPES, pH 7.5, 150 mM NaCl, 50 mM KCl, 1 mM DTT.
Table 1. Oligonucleotides used in the gel shift assay

<table>
<thead>
<tr>
<th>NAME</th>
<th>SEQUENCE</th>
<th>R1p Sites</th>
<th>Tel2p binding activity</th>
</tr>
</thead>
</table>
| R3-ST | GATCTGATCGGACCTGCGCGCGGCGGCGG | 5 | +++
| TELQ2 | GATCTGATCGGACCTGCGCGCGGCGGCGG | 2 | +
| TEF | GATCTGATCGGACCTGCGCGCGGCGGCGG | 1 | -
| TELQ1 | GATCTGATCGGACCTGCGCGCGGCGGCGG | 0 | +
| R2-ST | GATCTGATCGGACCTGCGCGCGGCGGCGG | 2 | ++
| R1-ST | GATCTGATCGGACCTGCGCGCGGCGGCGG | 1 | +
| YTC-A-IX | GATCTGATCGGACCTGCGCGCGGCGGCGG | 4 | ++++
| M13 | GATCTGATCGGACCTGCGCGCGGCGGCGG | 0 | -
| GAL4 | GATCTGATCGGACCTGCGCGCGGCGGCGG | 0 | -
| Y TEL JUNCTION | GATCTGATCGGACCTGCGCGCGGCGGCGG | 0 | -
| Human | GATCTGATCGGACCTGCGCGCGGCGGCGG | 0 | -
| Telomerase | GATCTGATCGGACCTGCGCGCGGCGGCGG | 0 | -
| OligoX | GATCTGATCGGACCTGCGCGCGGCGGCGG | 0 | -

R1p consensus sites are either underlined or in bold. Gel shifts were performed with partially purified MBP–Tel2p as described in Materials and Methods. The relative amount of shifted probe is indicated by the number of + signs, where each + indicates a visual increase in the amount of probe shifted relative to other probes; – indicates that no probe was shifted.

and 10% glycerol. To this reaction buffer were added 0.5 µg sonicated salmon sperm ssDNA (described in 41) and 0.1 µg GAL1–707 oligonucleotide (18 nt, 0.842 µM final concentration; sequence in 42) as non-specific competitors. End-labeled duplex oligonucleotide (50 000–80 000 c.p.m.) and 0.1 µg cell lysates were then added. After incubation for 20 min at room temperature, the mixture was run on a 5% native acrylamide gel in 1× TBE buffer at 10 V/cm for 2 h at room temperature. For the supershift reactions (Fig. 2C) 1.6 µg anti-MBP Ab (New England Biolabs) was added to the completed binding reaction and incubated on ice for 30 min prior to electrophoresis. Following electrophoresis, gels were dried under vacuum at 80°C. Protein–DNA complexes were visualized either by autoradiography (Table 1) or in a PhosphorImager (Molecular Dynamics) (Table 2). Quantitation of the amount of probe shifted was performed using the PhosphorImager by quantitating the amount of free and shifted probe region in reactions containing and lacking MBP–Tel2p or MBP–Rap1p, subtracting the c.p.m. for the region of the shifted probe in reactions lacking protein from the c.p.m. of shifted probe in reactions containing protein and dividing it by the total number of c.p.m. in the free probe in the reaction containing protein. The R1-ST value in each gel was then used as a standard for the mutant oligonucleotides (Table 2) on the same gel. Gel shift analysis was performed using partially purified MBP–Tel2p and analyzed for amount of binding relative to R1-ST.

Site truncation mutations were made by swapping the bold portions of the M13 sequence with the R1-ST sequence. The number of the bases in each strand of the Rap1p site is shown (after 46). Owing to some sequence similarities, some of the substitutions (bold bases) do not change all of the Rap1p consensus nucleotides (underlined bases). The lower portion of the table are mutations which eliminate G residues identified by methylation interference or that are in the minimal Tel2p recognition site. The mut3 oligonucleotide appears in both parts of the table. Gel shift analysis was performed using partially purified MBP–Tel2p and analyzed for amount of binding relative to R1-ST.

Methylation interference

Dimethyl sulfate (DMS) methylation of 32P-labeled dsDNA (100 ng, 108 c.p.m.) was carried out in a 200 µl reaction containing 50 mM sodium cacodylate, pH 8.0, 1 mM EDTA, 1 µg salmon sperm DNA and 1 µl 1 M DMS. The reactions were incubated for 5 min at room temperature and quenched by addition of 50 µl stop buffer consisting of 1 M 2-mercaptoethanol, 1.5 M NaOAc and 1 µg glycogen as a carrier (43). The DNAs were ethanol precipitated twice and dissolved in sterilized milliQ-purified water and stored at –80°C. Five MBP–Tel2p gel retardation assay reactions (described above) were loaded and, following electrophoresis, the shifted bands were cut out and cast in a 0.7% agarose gel sliced. The labeled DNAs were run into the DE81 paper and the paper removed from the gel. The DNAs were eluted in 1.0 M NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, ethanol precipitated as above and subjected to 0.1 M piperidine hydrolysis. Other methods of elution included significant amounts of

Table 2. Mutant oligonucleotides to define the Tel2p binding site in R1-ST

<table>
<thead>
<tr>
<th>NAME</th>
<th>SEQUENCE</th>
<th>Match to Rap1p site</th>
<th>MBP-Tel2p binding activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>0</td>
<td>0.0 X</td>
</tr>
<tr>
<td>R1-ST</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>13</td>
<td>1.0 X</td>
</tr>
<tr>
<td>Site Truncations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mut1</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>9</td>
<td>0.0 X</td>
</tr>
<tr>
<td>mut2</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>11</td>
<td>0.0 X</td>
</tr>
<tr>
<td>mut3</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>12</td>
<td>0.5 X</td>
</tr>
<tr>
<td>mut4</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>8</td>
<td>0.0 X</td>
</tr>
<tr>
<td>mut5</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>10</td>
<td>0.3 X</td>
</tr>
<tr>
<td>mut6</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>12</td>
<td>0.3 X</td>
</tr>
<tr>
<td>mut7</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>9</td>
<td>0.0 X</td>
</tr>
<tr>
<td>Point mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mut8</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>12</td>
<td>0.2 X</td>
</tr>
<tr>
<td>mut9</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>12</td>
<td>0.2 X</td>
</tr>
<tr>
<td>mut10</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>12</td>
<td>0.5 X</td>
</tr>
<tr>
<td>mut11</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>12</td>
<td>0.0 X</td>
</tr>
<tr>
<td>mut12</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>12</td>
<td>0.4 X</td>
</tr>
<tr>
<td>Minimal site mut13</td>
<td>GATCTGATCGGACCTGCGCGCGGCGGCGG</td>
<td>8</td>
<td>0.3X</td>
</tr>
</tbody>
</table>
acrylamide with the DNA that interfered with electrophoresis of the hydrolyzed DNA. The hydrolyzed DNAs were lyophilized under vacuum to remove trace amounts of piperdine. The purified DNAs were then dissolved in 80% formamide and resolved on a 7 M urea–18% acrylamide gel (39).

Other methods
Total RNA was isolated from exponentially growing KR95-2A cells (20) as described in McKinney et al. (44) and quantitated by OD260.

RESULTS
Expression of fusion proteins
Recombinant MBP–Rap1p and MBP–Tel2p were expressed in BL21(DE3) cells at various temperatures. Fusion proteins expressed at 37°C fractionated with the insoluble pellet. However, active soluble proteins were obtained by inducing expression at 22°C for 6–12 h. The predicted length of the Tel2p ORF is 688 amino acids. Fusion protein constructs containing full-length Tel2p were toxic to bacterial growth, even when cells were grown under repressing conditions. A 446 amino acid portion of the central region of Tel2p (amino acids 181–626) could be expressed in BL21 cells. The purified MBP–Rap1p and MBP–Tel2p proteins were 30–50% pure as judged by SDS–PAGE, with the major contaminating band being free MBP (as determined by Western blotting using anti-MBP as primary antibody; data not shown). Both the MBP–Rap1p and MBP–Tel2p purified fusion proteins migrated at the expected molecular weights of ∼140 and ∼100 kDa respectively (data not shown). The yields of final purified product were 0.3 mg for MBP–Tel2p and 0.5 mg for MBP–Rap1p per 500 ml culture.

Tel2p binds yeast telomeric duplex DNA in a sequence-specific manner
The binding specificity of Tel2p was initially determined in vitro using bacterial cell lysates and various duplex sequences (binding to single-stranded substrates will be considered elsewhere). These gel retardation assays all contained 0.5 µg sonicated salmon sperm DNA and 0.1 µg single-stranded oligonucleotide (∼40-fold molar excess over radiolabeled probes) as competitor DNAs. Using telomeric dsDNA probe R3-ST (Fig. 1 and Table 1) we observed a DNA–protein complex in the MBP–Tel2p lysate as well as the MBP–Rap1p lysate, but not in the MBP lysate. The MBP–Tel2p complex in lysates was specifically competed by a 20-fold molar excess of unlabeled probe (Fig. 1). Thus MBP–Tel2p can bind to the same telomeric DNA repeats as the known yeast telomere binding protein Rap1p.

The specificity of Tel2p binding was further tested with various DNA probes using partially purified MBP–Tel2p. The major yeast telomere binding protein is Rap1p, which has a strongly conserved consensus binding site (45). Because MBP–Tel2p can bind to TG1–3 repeats containing three Rap1p sites, Tel2p binding to sequences containing one (R1-ST), two (R2-ST), three (R3-ST) or four (YTCA-1X) Rap1p sites was examined (Table 1). MBP–Tel2p bound all of the yeast telomeric dsDNA sequences to give a single shifted band (Fig. 2 and Table 1). No binding was observed with purified MBP alone (data not shown). Competition analysis showed that binding to the R2-ST probe by MBP–Tel2p was specifically competed by a 5-fold molar excess of R2-ST (Fig. 2B, lanes 3–5), but not by a 50-fold molar excess of duplex M13 oligonucleotide. Similar results were obtained with a 100-fold mass excess of pUC19 DNA sonicated to an average size of 0.7 kb (data not shown). The amount of R1-ST probe (Table 1) shifted in the presence of a 100- or 1000-fold mass excess of total S. cerevisiae RNA was also monitored (not shown). When normalized to the amount of R1-ST shifted in the absence of RNA, 0.5× R1-ST probe was shifted in the 100-fold RNA excess reaction and 0.3× was shifted in the 1000-fold RNA excess reaction. We note that the reactions containing a 50-fold molar excess of the non-specific duplex competitor M13 (Fig. 2B, lanes 6–8) also contained a 200-fold molar excess of one M13 single strand (Materials and Methods). This 200-fold molar excess of a single-stranded non-telomeric oligonucleotide did not inhibit MBP–Tel2p binding to R2-ST. As there was little or no competition by RNA and non-telomeric DNA, Tel2p is not a general DNA or RNA binding protein. Addition of anti-MBP Ab to the completed MBP–Tel2p R1-ST binding reaction caused the appearance of a supershifted band (Fig. 2C), consistent with purified MBP–Tel2p causing the R1-ST band shift. R1-ST, R2-ST, R3-ST and YTCA-1X were bound by Rap1p, as expected, and multiple Rap1p–DNA retarded complexes were observed for DNAs with multiple Rap1p sites (data not shown), in agreement with previous studies (46). MBP–Tel2p and MBP–Rap1p did not bind non-telomeric duplex sequences (M13, GAL4 and Y Telo) and no binding was observed with heterologous human, Tetrahymena or Oxytricha telomeric DNA sequence in our in vitro analyses (Table 1). These data show that Tel2p is a double-stranded TG1–3 binding protein in vitro.
Figure 2. Partially purified MBP–Tel2p binds duplex DNAs that contain telomeric sequences. (A) The probe is listed above the pair of lanes. The lane containing the binding reaction with MBP–Tel2p is indicated and the MBP–Tel2p shifted band is indicated by an arrow. All lanes are from the same gel. The sequences of the probes are given in Table 1. Lane 1, R1-ST probe alone; lane 2, R1-ST + MBP–Tel2p; lane 3, R2-ST; lane 4, R2-ST + MBP–Tel2p; lane 5, R3-ST; lane 6, R3-ST + MBP–Tel2p; lane 7, M13; lane 8, M13 + MBP–Tel2p. (B) Partially purified MBP–Tel2p specifically binds telomeric DNA. Partially purified MBP–Tel2p specifically binds telomeric DNA. Partially purified MBP–Tel2p was used in the same experiments as in Figure 1A, performed using R2-ST as probe. The fold molar excesses of specific competitor, R2-ST, and non-specific competitor, M13, are given in Table 1. Lane 1, R2-ST probe alone; lane 2, R2-ST + MBP–Tel2p; lanes 3–5, as lane 2 with 5× (lane 3), 25× (lane 4) and 50× (lane 5) molar excess of unlabeled R2-ST added to the binding reaction; lanes 6–8, as lane 2 with 5× (lane 6), 25× (lane 7) and 50× (lane 8) molar excess of unlabeled M13 duplex added to the binding reaction. (C) Partially purified MBP–Tel2p was used to shift the R1-ST probe in the absence or presence of 1.6 μg polyclonal anti-MBP Ab. Arrows indicate the shifted band and supershifted band.

Figure 3. MBP–Tel2p requires 8 bp of the 13 bp telomeric Rap1p site in R1-ST to bind DNA. The probe used in each binding reaction is indicated above each pair of lanes and the presence of MBP–Tel2p in the reaction is indicated by +. Mut10 shows that the specific G·C bp that shows the strongest methylation interference is required for binding. The MBP–Tel2p band is indicated by a line. (A) A scanned autoradiograph of representative binding reactions quantitated in Table 2. (B) Comparison of MBP–Tel2p and MBP–Rap1p binding to R1-ST and mut13 probes. The amount of probe shifted relative to MBP–Tel2p/R1-ST shift (as in Table 2) for MBP–Rap1p is 32× for mut13, 14× for mut 6, 0.0× for mut 13 and 18× for R1-ST. Two shifted bands caused by a single Rap1p binding event have been observed previously (46,48).

The Tel2p–DNA complex migrated similarly to the fastest migrating Rap1p–DNA complex (see for example Figs 1 and 3B) and the intensity of the MBP–Tel2p shifted complex increased with increasing number of Rap1p binding sites and GT base pairs present in the DNA substrate (Fig. 2A, compare lane 2 with lanes 4 and 6). However, only a single MBP–Tel2p shifted band that increased in intensity with increasing protein levels irrespective of the number of Rap1p sites present on the DNA substrate (data not shown) was observed.

While Tel2p bound to the telomeric duplex sequences containing Rap1p sites, Tel2p did not bind the non-telomeric TEF DNA probe (Table 1). The TEF probe contains a non-telomeric Rap1p site from the TEF2 UAS, which is the second highest affinity Rap1p site known (35). In addition, Tel2p did not bind the M13, GAL4 or Y′ Telo junction or the heterologous telomeric DNA sequences from humans, Oxytricha and Tetrahymena (Table 1). Thus Tel2p has the in vitro properties of a novel yeast telomere-specific binding protein. The results with the TEF probe indicate that Tel2p DNA binding is distinct from Rap1p binding.
A minimum duplex telomeric sequence of 8 bp is required for Tel2p binding

To delineate the minimum Tel2p binding site within the telomeric Rap1p site, a series of mutations were introduced into the single Rap1p site in the R1-ST probe and tested for Tel2p binding. The mutations were constructed by taking two 26 bp duplexes, R1-ST and M13 (Table 1), and replacing portions of the R1-ST sequence with M13 sequences. The M13 duplex was not bound by Tel2p, so as residues critical for binding are altered, the gel shift should be greatly reduced. To comply with the previous nomenclature for the Rap1p site (46) and to relate these data to the methylation interference analysis (below), the bases in the Rap1p site in the CA-rich strand are numbered 1–13 in the 5’→3’ direction and in the TG-rich strand are numbered 13’→1’ in the 5’→3’ direction (Table 2). The fraction of probe shifted by a constant amount of MBP–Tel2p was measured in a phosphorimager and then normalized to R1-ST for a quantitative comparison of the different probes (Table 2).

Gel shift analysis of duplex oligomers with bases mutated at various positions revealed that bases 1’, 3’ and 4’ of the Rap1p binding site were not necessary for MBP–Tel2p binding, because binding was observed in mut5 (Fig. 3 and Table 2). Methylation interference data below and TEF probe binding (Table 1) suggested that the G at 2’ is also not required for MBP–Tel2p binding (confirmed below). Individual base pairs in positions 4–6 (CCC), which are essential for Rap1p–DNA interaction (37,47), were dispensable for Tel2p binding because the mut8 and mut9 probes were shifted (Fig. 3 and Table 2). Similar mutations abolished binding by Rap1p (47). Mutant duplexes with single base substitutions (mut8–mut12 and mut3) revealed that Tel2p binding was undetectable only when a G→C base substitution at position 12 was made (mut10). Other single base substitutions at G13 (mut3), G10 (mut11), G8 (mut12), G6 (mut8) or G5 (mut9) did not eliminate Tel2p–DNA interaction. However, Tel2p binding was abolished with multiple base substitutions in the combinations G13’ and G10’ (mut2), G12’ and G10’–G8’ (mut7) or G13’ and G10’–G8’ (mut1). The shortest telomeric sequence that could be bound by Tel2p in this sequence context was mut5 (Fig. 3A and Table 2), which contains a 9 bp contiguous tract identical to the Rap1p site and G2’. Because G2’ was not identified as an important base by methylation interference (below) and was present in the TEF duplex that was not bound by Tel2p (Table 1) and G13’ was not required for binding, this analysis suggested that the 8 bp GT tract from bp 5’ to 12’ of the Rap1p site was required for Tel2p binding.

To directly test the importance of G2’ in Tel2p binding, the mut13 probe bearing the 8 bp GT tract was made and tested. Tel2p shifted the mut13 probe as well as mut8, mut9 and several other probes (Fig. 3B and Table 2). Thus, G2’ was not required for Tel2p binding. The relative binding of equal amounts of partially purified MBP–Tel2p and MBP–Rap1p was compared using R1-ST, mut3, mut6 and mut13 substrates. Rap1p bound R1-ST, mut6 and mut3 efficiently as expected (47), but no binding of the mut13 probe was detected (Fig. 3B). An equal amount of MBP–Rap1p shifted 10- to 18-fold more R1-ST probe than MBP–Tel2p in independent experiments. These data further distinguish Rap1p and Tel2p binding of yeast telomeric DNA.

Tel2p contacts duplex telomeric DNA in the major groove

To further examine the specificity of MBP–Tel2p binding on the R1-ST probe, the DNA–protein contacts were studied by DMS interference analysis. DMS methylates the N7 position of guanines in the major groove of the DNA (39). Partially methylated DNA was used to form DNA–protein complexes with both MBP–Tel2p and MBP–Rap1p. The DNA from both complexes and the free probe were isolated and purified (Materials and Methods). As the free probe was in large excess over the amount of bound material, the free probe is equivalent to the input material. After piperidine cleavage the products were resolved on an 18% urea–acrylamide gel (Fig. 4). In agreement with previous reports (36,47,48), strong interference was observed at bases G5’, G6’, G12’ and G13’ in the Rap1p bound complex. The enhancement of bands near the 3’-end of the probe in the Rap1p bound material over the free probe has been observed previously by others (48) and did not alter the identification of bases critical for Rap1p binding. MBP–Tel2p showed a different pattern of DMS interference. Strong interference was observed at G12’ (Fig. 4). A comparison of protein–DNA contacts between the two proteins is shown in Figure 5. The strong interference at G12’ is consistent with the binding site mutations, as changing the G·C bp at position 12 to C·G (mut10, Table 2) eliminated Tel2p binding (Fig. 3). Thus the methylation interference data combined with the mutant oligo-
with Tel2p in DNA binding studies. Production and analysis of these proteins in combination molecular genetics should allow identification of Tel2p interacting clearly the potential Tel2p–DNA interactions.

Sequence and yeast cells would help answer these questions. However, since the increases its affinity for specific sites. Purification of Tel2p from alone or in conjunction with another protein that alters or regulation and cell viability.

constructing testable models of Tel2p function in telomere length kinase similar to DNA-PKcs (19,49). In contrast, Tel2p is a novel yeast genes that behave as genetic regulators of telomere length through Myb motifs (50). These data provide the first step for constructing testable models of Tel2p function in telomere length regulation and cell viability.

Presently, it is unknown if Tel2p binds DNA in vivo and, if so, alone or in conjunction with another protein that alters or increases its affinity for specific sites. Purification of Tel2p from yeast cells would help answer these questions. However, since the TEL2 sequence and GAL1–TEL2 and TEL2–lacZ fusions suggest that Tel2p is a very low abundance protein (20), this biochemical approach will be challenging. The information that Tel2p can bind yeast telomeric dsDNA in vitro combined with yeast molecular genetics should allow identification of Tel2p interacting proteins. Production and analysis of these proteins in combination with Tel2p in DNA binding studies in vitro will define more clearly the potential Tel2p–DNA interactions in vivo.

The MBF–Tel2p fusion protein could gel shift duplex telomeric oligonucleotides in the presence of a large excess of salmon sperm DNA and a single-stranded oligonucleotide present in each binding reaction (Materials and Methods) and a non-specific competitor (Figs 1 and 2 and Table 1). Competition for Tel2p binding of telomeric DNA was specific (Figs 1 and 2). In addition, not all duplex probes tested were bound by MBP–Tel2p (Table 1). These data and the methylation interference results indicate that MBP–Tel2p shows sequence specificity in its dsDNA binding. At least 8 bp of the 13 bp Rap1p telomeric site were required for Tel2p binding and the G-C bp at position 12 (Fig. 5) was essential (Fig. 3 and Table 2). In contrast, Rap1p did not bind to this 8 bp sequence (mut13, Fig. 3B). Because Tel2p could bind duplexes with point mutations at positions 6, 8 and 10 (mut8, mut12 and mut11, Table 2), Tel2p may make other contacts outside the major groove that compensate for these single base changes.

Tel2p has not been previously identified as part of a telomeric DNA binding activity in yeast extracts, most likely for two reasons. First, Rap1p is an abundant yeast telomere binding activity in yeast extracts (35), making detection of additional yeast telomere binding proteins difficult. Partial fractionation of yeast nuclear extracts has revealed the presence of a second non-Rap1p telomere binding activity called TBFβ (34), but this activity has not been further characterized. It is not yet known if Tel2p is TBFβ or part of TBFβ. Second, expression of Tel2p is toxic to bacteria. Indeed, even placing the TEL2 ORF behind strong yeast promoters, which are active in bacteria makes plasmids unstable in Escherichia coli such that transformants grow poorly and rearranged plasmids are frequently isolated (K.W. Runge, unpublished data). Only by constructing a truncated bacterial fusion that contains 65% of the TEL2 ORF were we able to obtain unrearranged plasmids in bacteria and produce protein. Thus, previous searches for yeast telomeric binding proteins with oligonucleotide probes (51) might not have found Tel2p because high level expression would inhibit bacterial colony formation.

The known homology of Tel1p with DNA–PKcs, the fact that TEL1 and TEL2 act in the same genetic pathway and these data showing that Tel2p can bind to telomeric DNA suggest a model for Tel1p and Tel2p function in telomere length regulation. DNA–PK is a DNA-dependent protein kinase in which the catalytic subunit, DNA–PKcs, is activated by binding to two DNA-bound subunits, Ku-70 and Ku-80, and can phosphorylate a number of proteins critical to cell cycle progression and DNA repair, including p53 (52). By analogy, a speculative model for Tel2p function is that Tel2p binds to telomeric DNA and, in conjunction with another protein bound to the chromosome terminus, activates Tel1p to phosphorylate telomere-associated proteins such as Rap1p, Rif1p (30), Rif2p (31), Sir2p, 3p and 4p (53), Est1p (32), Cdc13p/Est4p (33) or Ste11p (54) in the course of telomere length regulation. It is interesting to note that the point mutation in tel2-1 that causes cells to maintain short telomeres occurs in a part of the protein that is not present in the MBP–Tel2p fusion. In our model the tel2-1 protein would bind DNA but fail to bind to or activate the Tel1p kinase.

Deletion of TEL2 is lethal to cells, while deletion of TEL1 results in viable cells with short telomeres (19,20). Thus, Tel2p must have an essential function besides its activities in the TEL1 pathway. The physical similarity between telomeres and double-strand breaks, the evolutionary conservation of the telomere-related protein Tel1p and the double-strand break-activated kinase DNA–PKcs and the data presented here showing that Tel2p can bind telomeric DNA suggest that the essential function of Tel2p involves metabolism of DNA ends. Another Tel1p/DNA–PKcs homolog, Mec1p, controls cell cycle progression in response to DNA damage (55) and is essential for viability in some yeast strains (21). The present data on tel2 mutations and DNA binding are consistent with an essential role for Tel2p in this process. If so, TEL2 should show genetic interactions with components of the DNA damage signalling pathway. Thus, these data on Tel2p DNA binding suggest specific testable models for the function of this novel protein with no known homologs.

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**Figure 5.** Bases identified by methylation interference of MBP–Rap1p and MBP–Tel2p binding. The ovals indicate bases with strong interference. Underlined bases make up the Rap1p binding site. The base numbering on each strand is shown after the convention of Gilson et al. (46).
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