Identification and Characterization of an Essential Telomeric Repeat Binding Factor in Fission Yeast*

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Telomeres are the dynamic DNA-protein complexes that make up the natural ends of eukaryotic chromosomes. Early cytological and genetic studies revealed that telomeres are necessary for chromosome stability and genome maintenance (1, 2). It was demonstrated that broken chromosomes fuse end-to-end and become dicentric, ring-formed, or adopt other unstable forms that cause genomic instability. The instability caused by broken chromosome ends contrasted with the stability of natural chromosome ends, and suggested that telomeres are essential structures that make the natural ends of eukaryotic chromosomes unique. Without telomeres eukaryotic chromosomes are unstable and suffer chromosomal rearrangements.

Telomeric DNA consists of extended arrays of tandem repeats with common endings. One strand is rich in guanines and thymines and forms a single-stranded 3’ extension (3). Consequently, the complementary strand is rich in cytosines and adenines, and has a recessed 5’-end. Moreover, telomeric repeat sequences appear well conserved through evolution. All vertebrates have an identical GGGTAC hexanucleotide telomeric repeat sequence (4) whereas the telomeric repeat sequence in Schizosaccharomyces pombe is somewhat degenerate. The general S. pombe consensus sequence is GGGTAC(A)(C)G0–6 with GGGTACA being the most frequently occurring repeat (5).

Conventional replication of linear chromosomes fails to make a complete copy of the lagging strand because of discontinuous DNA synthesis and the requirement for RNA priming (6), or because of loss of overhang at the end of leading strand synthesis (7), so in order to complete replication, unicellular eukaryotes like S. pombe depend on the telomerase enzyme (8). Telomerase is a dimeric reverse transcriptase that uses part of its intrinsic RNA moiety as a template to extend the guanine-rich strand of the telomeric DNA (9–12). In higher eukaryotes telomere extension occurs in all cells during early development, but is later restricted to a select subset of cells including the germline and stem cells. As a consequence, the length of the telomeric DNA gradually shortens in differentiated cells (3). It has been suggested that this provides a molecular clock that tells cellular age, and that telomere shortening serves as a tumor suppressor mechanism that prevents accumulation of mutations (13). A differentiated cell enters an irreversible state of arrested growth known as replicative senescence (14) when its telomere becomes critically short (15).

Telomere extension largely depends on the state of the telomere, whose conformation is modulated by proteins that bind telomeric DNA sequence specifically and fine tune the telomeric repeat sequence GGTGTA. Two structurally related proteins, telomeric repeat binding factors 1 (TRF1)4 (23) and 2 (TRF2) (24, 25), bind double-stranded telomeric DNA, whereas protection of telomeres 1 (POT1) bind telomeric single-stranded 3’ extensions (26–29).

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4 The abbreviations used are: TRF, telomeric repeat binding factors; POT, protection of telomeres; aa, amino acids; DTT, dithiothreitol; TEV, Tobacco Etch Virus.
A POT1 ortholog has been identified and characterized in *S. pombe* (26), and Taz1p was the first TRF1/TRF2 ortholog to be discovered in *S. pombe* (30). However, despite suggestions that also *S. pombe* contains two telomeric recognition factors (31, 32), a second TRF1/TRF2 ortholog remained to be identified. Here we report the identification and characterization of telomeric repeat binding factor 1 (Tbf1), a second TRF1/TRF2 ortholog in *S. pombe*.

**EXPERIMENTAL PROCEDURES**

Sequence Analysis, Cloning, and Protein Expression—A candidate *S. pombe* TRF1/TRF2 ortholog was identified from sequence homology. ClustalW sequence alignments were made using the BLOSUM62 substitution scoring matrix with open reading frame mutants (aa 1–411 and aa 405–456). The *taz1* gene was amplified from *S. pombe* chromosome I cosmid c16A10 (GenBank™ accession number Z97185). The PCR products were cloned into a modified Pet30a vector (Novagen) containing an N-terminal His6 tag/S-tag followed by a Tobacco Etch Virus (TEV) protease cleavage site, and the inserts were verified by sequencing (Geneservice). The constructed plasmids were transformed into *Escherichia coli* (E. coli) Rosetta cells (Novagen), and protein expression was induced by adding isopropyl-beta-D-thiogalactopyranoside to a final concentration of 0.5 mM when the cell cultures reached an optical density (λ = 600 nm) of about one. Cells were harvested by centrifugation (5000 × g for 15 min), and pellets were resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, 500 mM KCl, 1% Triton X-100, 2 mM DTT) containing protease inhibitors (Complete® EDTA-free protease inhibitor mixture tablet, Roche Applied Science). Cells were disrupted by sonication and following centrifugation (25,000 × g for 30 min), the supernatant was incubated with lysis buffer equilibrated Ni-NTA resin (Qiagen) for 60 min. After centrifugation (200 × g for 3 min), the Ni-NTA resin was washed with wash buffer (50 mM Tris/HCl, pH 8.0, 500 mM KCl, 10 mM imidazole, 2 mM DTT, 10% glycerol), and the protein was stepwise eluted with wash buffer containing 200 mM imidazole. Protein containing fractions were pooled and dialysed into digestion buffer (500 mM Tris/HCl, pH 8.0, 500 mM KCl, 2 mM DTT, 10% glycerol). TEV proteolytic digestion was performed overnight, with TEV protease at approximately a ratio of 1:100 to eluted protein, to remove the N-terminal His6 tag/S-tag. The TEV protease was itself His6-tagged, which allowed the cleaved protein to be purified from TEV protease and cleaved N-terminal His6 tag/S-tag by binding to Ni-NTA resin equilibrated with digestion buffer. The proteins were further purified by gel filtration on a Superdex 200 column also equilibrated with digestion buffer, and dialysed into binding buffer (50 mM Tris/HCl, 125 mM KCl, 5 mM DTT, 10% glycerol).

Proteolysis, Western Blotting, and N-terminal Sequencing—Purified full-length protein was digested at room temperature using a protease mixture containing papain, trypsin, and chymotrypsin, each at final concentration of 25 μg/ml, and aliquots were removed into 2× SDS loading buffer at 15-min intervals. Proteolytic fragments and prestained markers were separated using standard SDS-PAGE, and the protein content of the gel was transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore) for N-terminal sequence analysis by automated Edman degradation in a Procise 494 Protein Sequencer.

**Electrophoretic Mobility Shift Assays—**Synthetic oligonucleotides (Sigma) containing common *S. pombe* telomeric repeat sequences were purified by polyacrylamide gel electrophoresis and hybridized in equimolar amounts to make up double-stranded telomeric DNA. The pNSU70 plasmid containing cloned *S. pombe* telomeric DNA (originally constructed by Neal Sugawara, PhD thesis, Harvard University, 1989) was digested by Apal and SacI (New England Biolabs) to produce a 295-bp fragment consisting of 263-bp telomeric DNA, equivalent to 30 naturally occurring *S. pombe* telomeric DNA repeats, and 32-bp telomere-associated sequence. The restriction fragment was purified by standard agarose gel electrophoresis. DNA was radiolabeled using 5’-[γ-32P]triphosphate (Amer sham Biosciences) and T4 polynucleotide kinase (New England Biolabs). Unincorporated probe was removed on Bio-Spin P30 columns (Bio-Rad). Radiolabeled DNA (1 nM) was incubated with protein in binding buffer (50 mM Tris/HCl, pH 8.0, 125 mM KCl, 5 mM DTT, 10% glycerol) containing 100 μg/ml bovine serum albumin (New England Biolabs), 10 μg/ml sheared *E. coli* DNA for 30 min at +4 °C, and reaction mixtures were analyzed with native agarose gel electrophoresis (0.5–3% agarose, 0.25× TB) at 75 V/cm for up to 80 min at +4 °C. Gels were dried onto DE81 anion exchange chromatography paper (Whatman) and scanned using a Typhoon 8600 imaging system (Amer sham Biosciences).

**S. pombe Strain Construction and Culture Conditions**—All strains used in this study (Table 1) were cultured in YE or EMM medium as described (33). The diploid strain JCF1101 was made by mating JCF22 and JCF24. A linear DNA fragment carrying the kanMX module (34) was generated by PCR using primers corresponding to sequences flanking the open reading frame of the identified gene (fwd: AGCAATCGAT TAATCA-GACT GCTTCTCACC TATAGTTTTG ATTTTCTCACC AT- CAATTGAA AAACCTGAT TTCCAGAAA CGGATC- CCCG GATTTTAAA, rev: AATGCTCTA GCTTTAAG TTTAGTATTT AAAAAAAT CGGAAAGG ATCAAGAGAT AAATGAACTC TCTAGCTAAGA GATTT- CGAGTCGTTTTAAA) (Table 1). This was used to transform the diploid to G418 resistance, and deletion of one allele was confirmed by Southern hybridization (Table 1).

**Protein Overexpression and Telomere Length Measurement**—The following plasmids, carrying the identified gene under control of the NMT81 promoter, were constructed using the pNMT TOPO Expression kit (Invitrogen): pNMT81-tbf1,

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**Table 1**

| Strain | Genotype |
|--------|----------|
| JCF22  | h+ ade6-M216 leu1-32 ura4-D18 |
| JCF24  | h- ade6-M210 leu1-32 ura4-D18 |
| JCF1101| h+ ade6-M216 leu1-32 ura4-D18/h- ade6-M210 leu1-32 ura4-D18 |
| JCF109 | h+ ade6-M216 his3-D1 leu1-32 ura4-D18 |
FIGURE 1. Pairwise sequence alignments of SpTbf1p with human TRF1 (A), human TRF2 (B), and budding yeast TBF1 (C) indicate a high degree of structural and functional conservation. Identical amino acids are indicated by black dots, similar residues are indicated by circles. Residues belonging to α helices are highlighted with red. Positions of secondary structure elements in the human TRF1 and TRF2 were taken from their structures (38, 55) and secondary structure elements of the S. pombe Tbf1p and the S. cerevisiae TBF1 were predicted using the PROF algorithm (56), which accurately predicts the positions of the α helices in both TRF1 and TRF2 (not shown). Amino acids corresponding to the TRF1 and TRF2 dimerization domains are underlined, and amino acids corresponding to the C-terminal DNA-binding MYB/homeodomains are shown in italics.
pNMT81-tbf1-V5, pNMT81-tbf1-ΔMYB-V5 (V5 = V5 epitope tag). These were used to transform JCF109 to leucine prototrophy in the presence of thiamine. Single colonies were re-isolated while maintaining plasmid selection and thiamine repression, and then grown to late log phase in EMM-leucine, with or without thiamine (5 μg/ml). Samples were isolated for Western blot and Southern blot analyses. Mouse ANTI V5-TAG (Serotec MCA1360) was used as primary antibody in the detection of the V5 epitope, with anti-mouse IgG-HRP (GE Healthcare NA931V) as secondary antibody. Loading control was Cdc2 p34 PSTAIRE, detected with rabbit polyclonal primary antibody (Santa Cruz Biotechnology SC-53), with anti-rabbit IgG-HRP (Amersham Biosciences NA934) as secondary antibody.

Telomere length was assessed as described (30), except that Southern blots were probed with a random prime-labeled 450-bp synthetic telomeric fragment (35).

RESULTS AND DISCUSSION

Identification of a Candidate TRF1/TRF2 Ortholog in S. pombe—A candidate TRF1/TRF2 ortholog from S. pombe was identified from sequence homology. Sequence comparisons between human telomeric repeat binding proteins, and their S. pombe orthologs reveal that the amino acid sequences of human POT1 and S. pombe Pot1p share around 19% identity and 32% homology. The Taz1p amino acid sequence shares 16% identity and 30% homology with TRF1 as well as TRF2, while the identified S. pombe Tbf1p shares in the region of 17% identity and 30% homology with both TRF1 and TRF2 (Fig. 1).

Despite moderate overall sequence homology, secondary structure prediction using a combination of the Profile-based neural network prediction of protein structure (PredictProtein) (36) and Predictor of natural disordered regions (PONDR) (37) algorithms reveal striking similarities between the human TRF1 and TRF2 proteins and the spTbf1p (Fig. 1). The human TRF1 and TRF2 proteins both contain unstructured N termini. Similarly, the N terminus (aa 1–64) of the spTbf1p is predicted to lack fixed secondary structure. The human TRF1 and TRF2 proteins both have large homodimerization domains that consist of 9 α-helices, which despite low sequence homology are structurally conserved (38). The spTbf1p has a corresponding structural domain (aa 71–287), which consists of 9 predicted α-helices with very high probability scores. The homodimerization domains of the human TRF1 and TRF2 proteins are connected to C-terminal DNA-binding MYB/homeodomain via flexible linker peptides, which contains putative nuclear localization domains. The spTbf1p has a predicted C-terminal DNA-binding MYB/homeodomain (aa 405–456), with a link-
Figure 3. The spTbf1p binds double-stranded telomeric DNA. A, titrations of three common S. pombe telomeric DNA repeat sequences with spTbf1p reveal that it can bind different telomeric repeat sequences, and that it may have a slight preference for telomeric repeats containing three or more consecutive guanines. B, titrations of the same three S. pombe telomeric repeat sequences with Taz1p. C, titrating a DNA fragment containing 30 naturally occurring S. pombe telomeric DNA repeats shows that spTbf1p can polymerize along S. pombe telomeres despite a degenerate repeat sequence. D, spTbf1p does not bind single-stranded telomeric DNA (left panel), nor double-stranded non-telomeric G-rich DNA (right panel). Faint slower migrating telomeric ssDNA bands, which form independently of protein concentration, likely corresponds to higher order G-quadruplex DNA structures (57). E, spTbf1p MYB/homeodomain on its own binds S. pombe telomeric DNA with one MYB/homeodomain per telomeric repeat (top panel), whereas the spTbf1p homodimerization domain exhibits no detectable affinity for telomeric DNA (bottom panel).
A Telomeric Repeat Binding Factor in S. pombe

**A**

| Organism       | Amino Acid Sequence |
|----------------|---------------------|
| Human (P54274) | RQAMV1WEDKNKRSERVRKYGEGNWKLLHYKPN--NTSGMV1RGGTNK |
| Cattle (B0P46373) | RQAMVWEDKNKRSERVRKYGEGNWKLLHYEFP--NTSGMV1RGGTNK |
| Hamster (O55036) | RQAMWDEGTNKKRSERVRKYGEGNWKLLHYKPN--NTSGMV1RGGTNK |
| Mouse (P70371) | RQWAVDEGTNKKRSERVRKYGEGNWKLLHYKPN--NTSGMV1RGGTNK |
| Rat (XP_236387) | RQWAVDEGTNKKRSERVRKYGEGNWKLLHYKPN--NTSGMV1RGGTNK |
| Chicken (AAQ47666) | RQFWTVEGRKQKGGWREWFGVGNWKKTLGHPDN--NTSGMV1RGGTNK |
| Frog (AAQ98349) | RQFWTVEGRKQKGGWREWFGVGNWKKTLGHPDN--NTSGMV1RGGTNK |
| Zebrafish (AAM26210) | RRWAVVEQKLAIQGQHGKLGKIVGGKGGKEPPLTTNL |
| S. cerevisiae (Q62457) | qpptkdek derselelgkkgvlecglvlgpsksktenellkdqemo |
| N. crassa (CAD21129) | RPFPFDWEXAIAMMLDMVKGPHWOLTELPGFQSGVSDDLKQTOALM |
| S. pombe (AAD51726) | RRSQKTKEKAAELDELDLVMKPFSQQLELYGPPGKESVLEKYNQNG |

**B**

![FIGURE 4. The TRF1/TRF2 family of proteins binds double-stranded telomeric DNA via conserved MYB/homeodomains.](image)

**A**

- **Human TRF2** (Q15554) KQXKTVVEESLYKQAVKYGEEKGNWAAIASKYFVY---/NRTAVMIKDOMRTMK
- **S. pombe Ta21p** (F79005) RKKWTVVEEHLKEMISQWG-CCWSKHLWQKLENGPP-LKTFPTQIKMKALIK

**B**

The spTbf1p Has the Same Architecture as Mammalian TRF1 and TRF2—The results from the sequence analyses and secondary structure predictions indicate that the spTbf1p, just like the spTa21p, is a TRF1/TRF2 ortholog. The spTbf1p is predicted to contain twelve α-helical secondary structure elements, organized in two domains which are connected by an unstructured linker (Fig. 1). This domain organization is conserved in the human TRF1 and TRF2 proteins (38, 39).

We next compared the information obtained from the proteolysis study with the predicted structural organization of the identified TRF1/TRF2 ortholog. The spTbf1p is predicted to contain twelve α-helical secondary structure elements, organized in two domains which are connected by an unstructured linker (Fig. 1). This domain organization is conserved in the human TRF1 and TRF2 proteins (38, 39).

The amino acid sequence MNQGMDYAA, representing the N terminus of the ~25-kDa proteolysis fragment, falls within the first predicted α-helix of the domain that contains the nine predicted α-helices (aa 76–287). The calculated molecular mass of the predicted domain is ~24 kDa, which corresponds very well with the experimentally determined molecular mass of the ~25-kDa proteolysis fragment. This probably corresponds to a highly structured domain similar to those found in the human TRF1 and TRF2 proteins, in which nine α-helices pack tightly to form a compact homodimerization domain (38). The amino acid sequence SWTKEEE, representing the N terminus of the ~10 kDa proteolysis fragment, is found within the first predicted α-helix of the domain that contains the remaining three predicted α-helices (aa 407–485). The calculated molecular mass of this domain is 9 kDa, which corresponds well with the experimentally determined molecular mass of the ~10-kDa proteolysis fragment. From sequence homology and available structural information (39), this domain would be expected to fold into a three α-helix bundle that makes up the C-terminal DNA-binding MYB/homeodomain.

We conclude from the proteolysis study that the spTbf1p, just like the human TRF1 and TRF2 proteins (38, 39) and the spTa21p (32), contains two distinct structural domains. The larger N-terminal domain is likely responsible for the formation of homodimers, whereas the smaller C-terminal domain is a conserved MYB/homeodomain that could mediate sequence-specific recognition of telomeric DNA.
The spTbf1p Binds S. pombe Telomeric DNA in Vitro—Because the spTbf1p appeared to have the same overall architecture as the human TRF1 and TRF2 proteins, we proceeded to investigate whether it bound S. pombe double-stranded telomeric DNA sequence specifically. Binding was investigated using cloned S. pombe telomeres and synthetic oligonucleotides, each containing two repeats of the most frequently occurring S. pombe telomeric repeat sequences (ACACAGGTTACAGGTTACG, ACACAGGTTACAGGTTACG, and ACACAGGTTACAGGTTACG) (5) flanked by AC dinucleotides and a 3′-G to prevent oligonucleotides from forming multimers.

Electrophoretic mobility shift assays show that full-length spTbf1p (Fig. 3A), just like Taz1p (Fig. 3B), binds double-stranded S. pombe telomeric DNA with high affinity and specificity in vitro. The spTbf1p polymerizes along a DNA fragment containing 30 naturally occurring S. pombe telomeric DNA repeats without significant cooperativity (Fig. 3C). The spTbf1p thus coats S. pombe telomeric DNA in the same way that TRF1 coats human telomeric DNA in vitro (40, 41). The spTbf1p does not bind single-stranded telomeric DNA, nor does it bind non-telomeric G-rich DNA (Fig. 3D). Moreover, while the MYB/homedomain of spTbf1p binds S. pombe telomeric DNA on its own, the Tbf1p dimerization domain exhibits no detectable affinity for telomeric DNA (Fig. 3E). Electrophoretic mobility shift assays using oligonucleotides containing two telomeric repeats and full-length spTbf1p produce a single slow migrating band (Fig. 3A). In contrast, the same experiment using only the MYB/homedomain of spTbf1p yields two bands (Fig. 3E), further supporting the notion that full-length spTbf1p preferentially forms homodimers.

The high degree of sequence homology between the DNA-binding MYB/homedomains within the telomeric recognition factor family of proteins is particularly noteworthy (Fig. 4). All family members, including the S. cerevisiae TBF1 protein, use a conserved MYB/homedomain motif for sequence specific recognition of double-stranded DNA (42).

Taken together, the DNA binding studies demonstrate that the spTbf1p binds S. pombe double-stranded telomeric DNA sequence specifically, and thus may function at telomeres in vivo.

The tbf1 Gene Is Essential for Survival—To explore in vivo functions of the spTbf1p, the entire open reading frame of one copy of the tbf1+ gene was replaced with the KanR marker in a diploid S. pombe strain (JCF1101). While ascis dissected from the parental JCF1101 diploid each yielded four colonies (all of which were Kan−), each ascus dissected from tbf1−/− diploids yielded only two colonies and these were both Kan− (Fig. 5), indicating that the gene is essential. Microscopic examination of tbf1Δ spores that failed to produce colonies revealed that they produced only one or two cells before dying. Albeit, unlike fission yeast whose telomeres erode due to loss of telomerase (trt1) or pot1 (26, 43), survivors harboring circular chromosomes do not arise in tbf1Δ strains. Similar observations have been reported for the mammalian trf1 gene. Targeted deletion of exon 1 of the trf1 gene causes early embryonic lethality in mice (44). The trf1 gene family thus appears to share a common essential function, and we proceeded to investigate a role of the spTbf1p in telomere length regulation.

The tbf1p Affects Telomere Length in Vivo—To address the role of spTbf1p in telomere length regulation, we overexpressed versions of the tbf1+ gene on plasmids under control of the NMT81 promoter. Expression from this promoter can be repressed by growing in medium containing thiamine. For clarity, here we say that the culture is induced if it lacks thiamine and repressed if it contains thiamine.

Transformants were isolated under repressing conditions, then induced or repressed in liquid medium for 6 days. A transformant carrying epitope-tagged tbf1+ (pNMT81-tbf1-V5) displayed telomere elongation by ~100–150 bp when induced (Fig. 6A, lane 2, relative to the same strain carrying an empty vector (Fig. 6, lane 11). Similar elongation was seen when untagged tbf1+ (pNMT81-tbf1) was induced (Fig. 6, lane 8). The elongation seen for full-length tbf1+ was not observed in a C-terminal deletion epitope-tagged tbf1 (pNMT81-tbf1-ΔMYB-V5), which lacks the last 82 MYB-containing amino acids (Fig. 6, lane 5). We saw intermediate telomere elongation at day 0 (repressed) for transformants carrying full-length tbf1+ constructs (Fig. 6, lanes 1 and 7). This is probably due to incomplete repression of the NMT81 promoter by thiamine. Indeed, after a further 6 days growth in repressing medium, we see a further slight increase in telomere length (Fig. 6, lanes 3 and 9), consistent with incomplete repression. We also found that the stronger promoter, pNMT41, led to equivalent telomere elon-
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FIGURE 6. Overexpression of tbf1 leads to MYB-dependent telomere elongation. Fission yeast transformed with plasmids containing thiamine-repressible (NMT81 promoter) tbf1, tbf1ΔMYB, or empty vector grown in medium containing thiamine (+ thi; expression repressed) or lacking thiamine (− thi; expression induced). V5, C-terminal fusion with V5 epitope tag. A, Southern blot analysis for telomere length: Lane 1, tbf1-V5 0 days + thi; lane 2, tbf1-V5 6 days + thi; lane 3, tbf1-V5 6 days + thi; lane 4, tbf1ΔMYB-V5 0 days + thi; lane 5, tbf1ΔMYB-V5 6 days − thi; lane 6, tbf1ΔMYB-V5 6 days + thi; lane 7, tbf1 0 days + thi; lane 8, tbf1 6 days − thi; lane 9, tbf1 6 days + thi; lane 10, empty vector 0 days + thi; lane 11, empty vector 6 days − thi; lane 12, empty vector 6 days + thi. B, Western blot analysis of samples in A (same loading order). Calculated MW of Tbf1p-V5 is 57.6 kDa, Tbf1pΔMYB-V5 is 48.2 kDa. Loading control, Cdc2, MW is 34.4 kDa.

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