Functional Differentiation of tbf1 Orthologues in Fission and Budding Yeasts

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In Saccharomyces cerevisiae, TBF1, an essential gene, influences telomere function but also has other roles in the global regulation of transcription. We have identified a new member of the tbf1 gene family in the mammalian pathogen Pneumocystis carinii. We demonstrate by transspecies complementation that its ectopic expression can provide the essential functions of Schizosaccharomyces pombe tbf1 but that there is no rescue between fission and budding yeast orthologues. Our findings indicate that an essential function of this family of proteins has diverged in the budding and fission yeasts and suggest that effects on telomere length or structure are not the primary cause of inviability in S. pombe tbf1 null strains.

The phylogenetic pedigree of Pneumocystis species implies they are likely to employ conserved fungus-specific proteins in pathways that are essential for viability. Identification of such modules in the genome of the rat-specific pathogen P. carinii may help to identify novel targets for directed pharmaceutical intervention to treat opportunistic infection of immunocompromised human hosts by P. jirovecii. To identify conserved functional modules, we are assessing the ability of genes from P. carinii to function in the budding yeast Saccharomyces cerevisiae as well as in the fission yeast Schizosaccharomyces pombe, the closest evolutionary relationship for which molecular genetic analysis is well developed (25, 36).

In the course of this study, we identified a potential homologue of the S. pombe tbf1 (SpTbf1) and S. cerevisiae TBF1 (ScTBF1) genes. Sequence comparisons indicate that both SpTbf1p and ScTbf1p are members of a conserved fungus-specific family (50, 51). All the Tbf1 family members contain a C-terminal “telobox” DNA binding domain (8, 9) but bear additional significant homology throughout their coding sequences. The telobox, a particular variant of the Myb family (50, 51). All the Tbf1 family members contain a C-terminal “telobox” DNA binding domain (8, 9) but bear additional significant homology throughout their coding sequences. The telobox, a particular variant of the Myb family sequence are not the primary cause of inviability in S. pombe tbf1 null strains.

The loci in S. pombe were PCR amplified from whole-cell extracts as one of several activities that exhibited differential affinity in vitro for tandem copies of the human and S. pombe telomere repeat sequences (55, 62). The SpTbf1 gene is essential, although its full range of cellular functions is unknown. Overexpression of SpTbf1 has been shown to slightly increase the mean length of telomeres in vivo (51).

We have examined whether the P. carinii tbf1 homologue (PcTbf1) can rescue deletions of ScTBF1 and SpTbf1. Our data lead us to infer that SpTbf1p and PcTbf1p are also likely to be global regulators of chromatin structure in their respective organisms.

MATERIALS AND METHODS

Identification and cloning of the full-length P. carinii tbf1 gene. A partial sequence corresponding to the Pcb1 open reading frame (ORF) was identified in the unigene set of 1,042 expressed sequence tags (ESTs) isolated from a P. carinii cDNA library (the Pneumocystis Genome Project, http://pgp.cchmc.org) (14) by its homology to the SpTbf1 and ScTBF1 genes. Longer fragments of the Pcb1 gene were obtained from the randomly amplified cDNA library by PCR (GenomiPhl DNA amplification kit; GE Healthcare, Otelfingen, Switzerland) with primers corresponding to adjacent regions of the genomic sequence in conjunction with the T3 primer located upstream of the multiple-cloning site in the Uni-ZAP XR vector. The longest Pcb1 fragment that could be amplified from the cDNA library was obtained using the T3 and the PcONTIG609_5ter primers and was considered to contain the full-length ORF since the sequences between all potential further upstream start sites were punctuated by stop codons. The locus was also identified within the genomic databases of the Pneumocystis Genome Project. The Pcb1 gene contains six introns with the canonical donor and acceptor sites found in P. carinii.

All multiple-sequence alignments were constructed using MAFFT (G-INS-i mode) (28). HHalign was used with standard parameters (http://toolkit.tuebingen.mpg.de). The sequences used as input for HHalign are as follows: for the TRF domain, the full alignment of TRF domain provided by Pfarm (http://pfarm.sanger.ac.uk) or a multiple alignment of full-length vertebrate sequences containing both TRF and Myb DNA-binding domains (Q4ORH9_DANRE, Q4FZZ9_DANRE, Q6SGO4_DANRE, Q1WMD2_XENLA, Q71E7K7_XENLA, QL2K75_XENLA, TERF2_HUMAN, TERF1_HU MAN, Q5NHT6_HUMAN, Q5RSX2_PONPY, Q8CH10_MUSSP, Q5EB90_RAT, TERF1_CRIGR, Q3MYH0_BOVIN, Q8CH10_MUSSP, Q5EB90_RAT, TERF1_CRIGR, Q3MYH0_BOVIN).
Q59Y9_MUNMU, Q59Y6_MUNMU, Q53920_MUNMU, Q59Y5_MUNRE, Q59Y5_MUNRE, Q59Y7_MUNRE, Q71M47_CHICK, Q7T1R9_CHICK, TER2F2_CHICK, Q5SF3M6_CHICK, and Q8O022_CHICK); for fungal tbf sequences, the full alignment of 11 fungal tbf sequences is shown as Fig. S1 in the supplemental material (pc-tbf1/1-566, sp Q6E434 TRF1_SCHPO/1-485, sp Q02457 TBF1 YEAST/1-562, sp Q6FJX7 Q6FJX7_CANGA/1-525, sp Q5AHX1 Q5AHX1 CANAL/1-549, sp Q6FJX7 Q6FJX7_CANAD/1-815, sp Q6FJX7 Q6FJX7_CANAD/1-850, 1-505, and sp Q6E434 Q6E434_YPL128c/1-710 [all from Uniprot], gi 58921528 ref XP_955963.1-1146 [from NCBI], and C6d3 18830:1-817 [from GenDB version 2.1]).

The full-length cDNA Pbf1 ORF was amplified from the CDNA library using primers MC01 and MC02 for subcloning into the S. pombe expression vector pREP41 (5). Primers P6996StartEcoRI and P6996EndSall were also used to amplify the full-length Pbf1 ORF (1,701 bp) from the CDNA library, and the product was cloned into an S. cerevisiae centromeric expression vector, p1416scTBF1pro. The vector was derived from p1416GPD (47) by replacing GPD sequences with a 1,432-bp fragment immediately upstream of the ScTBF1 coding region. All cloned PCR fragments were verified by sequencing in the vectors.

Cloning of SpTbf1, ScTbf1 and CpTbf1. The SpTbf1 ORF (1,458 bp) was amplified from S. pombe 972 h genomic DNA, using primers MC04 and MC05, and cloned into pREP41 (5). The ScTBF1 ORF (1,689 bp) was amplified from S. cerevisiae BY4741 genomic DNA, using primers pQ02457StartBamHI and pQ02457EndSall, and cloned into p416tcTbf1pro. The Candida glabrata TBF1 (CgTBF1) ORF (1,578 bp) was amplified from Candida glabrata DS739 genomic DNA, using primers cgTBF1StartBamHI and cgTBF1EndSalI, and cloned into the vector p416tcTbf1pro. The ScTBF1 ORF was reamplified from the budding yeast expression vectors using primers MC40 and MC41 and subcloned into the vector pINT41-LEU2cassette (208 COCKELL ET AL. EUKARYOT. CELL).

Strains and media. S. pombe was grown on either complete (YE) medium or synthetic minimal (EMM2) medium supplemented as required (44). A diploid strain was grown on either complete (YE) medium or synthetic medium supplemented with leucine (29°C medium supplemented with leucine 29°C were examined for the presence of asyciotic tetrads. Tetrads were dissected using a Singer MSM micromanipulator, and spores were germinated on complete (YE) medium. The genotypes of spores from the budding yeast expression vectors using primers MC40 and MC41, and subcloned into the vector pINT41-LEU2cassette (208 COCKELL ET AL. EUKARYOT. CELL).

Southern blotting and telomere length comparisons. Genomic DNAs were prepared according to standard protocols (45). Twenty micrograms of each DNA was digested overnight with Apal. Telomere length was assessed by Southern blotting (12) using a synthetic telomere fragment (42) probe labeled by random priming.

Nucleotide sequence accession numbers. The genomic and cDNA sequences for Pbf1 have been deposited in GenBank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under accession numbers EU617328 and EU617329.

RESULTS

Identification of P. carinii tbf1. Searches of the S. cerevisiae, S. pombe, and vertebrate proteomes for homologues of the 1,042 unique P. carinii ESTs (http://pbg.cchmc.org/) (14) were performed under low-stringency conditions (E value of 1E−3) using the local alignment tool BLASTx. Several of the ESTs had homology with the C-terminal regions of ScTBF1 (YPL128C) and SpTbf1 (SPBC19G7.13), with maximum homology values of 1E−21 and 8E−23, respectively. Contiguous alignment of these overlapping P. carinii ESTs aligned with the C-terminal portion (110 amino acids) of a putative ORF within the genome sequence of P. carinii. PCR amplification was used to clone full-length genomic and CDNA clones of Pbf1 (see Materials and Methods) (Table 1). Automated sequence alignment of the SpTbf1 and ScTBF1 coding regions with putative homologues from several other fungal species indicated that in addition to containing a C-terminal Myb-like domain, all the fungal family members are characterized by the presence of a 230-amino-acid-domain (Pfam-B:010333, Pfam v23.0) in the N-terminal half which shows weaker but significant evidence of conservation (18). Pairwise sequence comparisons of the 79-amino-acid domains that encompass the Myb-like domain or telobox in the ScTbf1p (562 amino acids), SpTbf1p (485 amino acids), and PcTbf1p (566 amino acids) each show comparable degrees of identity and similarity (Fig. 1). The regions of S. pombe and P. carinii Tbf1p which correspond to the Pbf1-B P010333 domain align to each other with sequence identity of 44%, while their best alignments to the Pbf1-B P010333 domains of S. cerevisiae and C. glabrata Tbf1p each give sequence identities of approximately 22% (Fig. 1A and C).

The use of multiple alignment comparison algorithms to analyze Tbf1-like proteins from P. carinii and a wide variety of other fungal species has allowed us to reevaluate a recent
prediction of structural and functional conservation between the N termini of budding and fission yeast Tbf1 proteins and those of human telomere binding proteins TRF1 and TRF2. The predicted alignment of ScTbf1p and SpTbf1p based only on comparison with the human TRF proteins (51) is clearly different from and less accurate than the one arising from our multiple alignment of fungal Tbf1 protein sequences between multiple alignments of fungal Tbf1 protein sequences and the full alignment of the human TRF domain (extracted from Pfam v22.0) produce significant scores only in the C-terminal Myb domain, while the N-terminal region produces an alignment with a very low significance. Pairwise profile alignments are a very powerful tool for detecting distant homologies. The lack of a clearly significant score indicates that there has been little evolutionary constraint between the N-terminal Myb domain, while the N-terminal region produces a very high score only in the C-terminal Myb domain.

Expression of P. carinii tbf1 rescues the S. pombe tbf1 null mutant but not the S. cerevisiae tbf1 null mutant does not. To determine whether the P. carinii gene could rescue the tbf1 deletion mutant, the heterozygous tbf1/tbf1::KanMX4 diploid was transformed with the empty vector or a plasmid expressing Spbf1, Pcbf1, or ScTBF1 under the control of the thiamine-regulated nmt1-41 promoter and then starved to induce meiosis. Spores prepared from cells transformed with each of the plasmids were plated on media selective for the plasmid. Replica plating established that approximately 50% of the viable colonies that were recovered after transformation with the empty vector or ScBF1 with BMH1 linker. As expected, the other half of the colonies carried the G418-sensitive, genomic copy of the TBF1 allele. As expected, the other half of the colonies that arose after transformation with either empty vector or ScBF1 with BMH1 linker. To confirm the absence of the wild-type TBF1 allele, genomic DNA was prepared from the wild-type and rescued haploid strains, as well as from the tbf1/tbf1::KanMX4 diploid. PCR using primers flanking the tbf1::KanMX4 deletion amplified a single fragment corresponding to the expected size of the KANMX4 allele. The same primers amplified fragments containing both wild-type and null alleles in the heterozygous diploid (Fig. 2A).

TABLE 1. Oligonucleotide primers used for PCR amplifications and DNA sequencing analysis

| Primer | Nucleotide sequence (5'→3') | Description |
|--------|-----------------------------|-------------|
| MC01   | GCGCGCCGCTGACAAAAATGGAGGATAGTAGAC | First 20 nt of Pcbf1 with SalI linker |
| MC02   | CGCCCCTTGGATATCGCCTTTTTAATCTC | Last 20 nt of Pcbf1 with Smal linker |
| MC04   | GCGCGCGCCGCTGACAAAAATGGAGGATAGTAGAC | First 25 nt of Spbf1 with SalI linker |
| MC05   | CGCGATCTTTAATTCGCGAGGATGTCG | Last 20 nt of Spbf1 with SacI linker |
| MC17   | GCGCGATCTTTAATTCGCGAGGATGTCG | First 25 nt of Spbf1 with NheI linker |
| MC18   | GCGCGAGCTTTAATTCGCGAGGATGTCG | Last 20 nt of Spbf1 with SacI linker |
| MC20   | AGCTGAAAATCGCATTGGG | First 20 nt of Spbf1 with SalI linker |
| MC21   | GGCCTCGAGTTAGACCA | First 21 nt of Spbf1 Sall linker |
| MC41   | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | Last 21 nt of ScBF1 with BamH1 linker |
| pQ02457StartBamHI | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | Last 18 nt of ScBF1 with BamH1 linker |
| pQ02457EndSalI | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | Last 21 nt of ScBF1 with SalI linker |
| ScTBF1promStartSac | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | nt 1432–1414 upstream of the ScTBF1 ORF |
| ScTBF1promEndSphl | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | nt 20–1 upstream of the ScTBF1 ORF (1432) |
| CgTBF1StartSphl | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | First 22 nt of CgTBF1 with BamH1 linker |
| CgTBF1EndSphl | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | First 20 nt of CgTBF1 (1,578 bp) with SalI linker |
| pCwCONT1Gene5er | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | To extend EST 609 (Pcbf1) |
| pCCONT1Gene5er | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | to extend EST 609 (Pcbf1) |
| p609StartEcoRI | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | First 20 nt of Pcbf1 with EcoRI linker |
| p609EndSalI | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | Last 19 nt of Pcbf1 (1,701 bp) with SalI linker |
| T7      | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | In p416GPD, pRS405, pGEM-T Easy vector |
| T3      | GCGCGCGCTGACAAAAATGGAGGATAGTAGAC | Upstream of multicloning site in UniZAP XR vector, pRS405, p416GPD |

a Restriction enzyme sites are underlined and flanked by 5' to 8 random nucleotides to allow effective digestion. The fragments of each oligonucleotide that will anneal to the relevant genomic template and act as PCR amplification primers in bold.

b nt, nucleotides.
FIG. 1. Multiple alignment of P. carinii Tbf1p with other fungal homologues indicates a higher degree of similarity to S. pombe Tbf1p than to S. cerevisiae Tbf1p in the N-terminal domain. (A) A stick diagram illustrating the principal domains of conservation between P. carinii, S. pombe, S. cerevisiae, and C. glabrata Tbf1p. Black boxes indicate the telobox domains: Gray boxes indicate regions corresponding to the less strongly conserved Pfam-B PB010333 domain. (B) Amino acid alignment showing degree of conservation between the telobox domains of P. carinii, S. pombe, and S. cerevisiae Tbf1 proteins. Asterisks indicate positions (65% overall) conserved between all three species. Double dots represent positions containing similar residues. Single dots represent positions containing similar residues in at least two of the sequences. (C) Amino acid alignment showing the conserved domains of Pefb1, and the S. pombe, S. cerevisiae, and C. glabrata Tbf1 proteins from the Uniprot database. The hatched box indicates the Pfam-B PB010333 domain. The filled box represents the Pfam-A minimal Myb DNA-binding domain. A multiple alignment of Tbf1p sequences from 11 fungal species is shown in Fig. S1 in the supplemental material.
rate (data not shown). We conclude that even the repressed levels of expression of Spbfl and PcTbf1 are sufficient to rescue viability of the Spbfl null strain, while higher levels of their expression do not significantly impair the growth of the cells.

We also examined whether PcTbf1 could rescue the lethality of the TBF1 null mutant in S. cerevisiae. Constructs that carry either ScTBF1, the homologous ORF from C. glabrata (CAGL0M02761g; CgTBF1), or PcTbf1 were cloned into S. cerevisiae expression vectors and expressed in the heterozygous TBF1::tbf1::KanMX4 S. cerevisiae diploid strain. Upon sporulation and germination, heterologous expression of either ScTBF1 or CgTBF1 under the control of the S. cerevisiae TBF1 promoter, rescued viability of the S. cerevisiae TBF1::KanMX4 haploids; however, expression of PcTbf1 in the same vector did not (Fig. 2B).

Heterologous expression of PcTbf1 under the control of the strong constitutive S. cerevisiae GPD promoter was also unable to rescue viability (data not shown). Together, the results of transspecies complementation analyses in S. pombe and in S. cerevisiae demonstrate that some essential function of this family of related proteins has diverged over the course of evolutionary differentiation between the *Taphrina* and Saccharomyces branches of the fungal kingdom.

Ectopically expressed GFP-SpTbf1p or GFP-PcTbf1p rescues the *tbf1* null phenotype and shows nuclear localization patterns similar to that of GFP-ScTbf1p expressed in wild-type *S. pombe*. To localize the different orthologues of Tbf1p in *S. pombe* cells, we fused GFP tags to the C-termini of the Spbfl, PcTbf1, and ScTBF1 ORFs, which were then integrated into the *leu1* gene. When expression was induced from the medium-strength *nmt1-41* promoter (see Materials and Methods) in cells carrying the endogenous copy of *tbf1*, GFP-SpTbf1p, GFP-PcTbf1p, and GFP-ScTbf1p were each located in the nucleus, with enrichment throughout the euchromatic hemisphere, at all stages of the cell cycle (Fig. 3A and data not shown). In some cells (both mitotic and interphase), a few intense foci were also observed (Fig. 3A, panels 5 and 6, and E). When the GFP-SpTbf1p and GFP-PcTbf1p fusions were introduced into the *tbf1::KMX4* null strain (see Materials and Methods), a similar pattern was observed (Fig. 3C and D).

### Table 2. The essential function of *S. pombe* tbf1 can be provided by expression of *P. carinii* tbf1 but not *S. cerevisiae* TBF1

| Plasmid transformed | No. of viable colonies plated on minimal medium + Leu | No. of G418-resistant haploid colonies on replica plating | % of viable colonies that carry *tbf1::KanMX4* |
|---------------------|-----------------------------------------------|---------------------------------------------------|-----------------------------------------------|
| Vector              | 580                                           | 0                                                 | 0                                             |
| Spbfl               | 545                                           | 211                                               | 39                                            |
| PcTbf1              | 129                                           | 64                                                | 50                                            |
| ScTBF1              | 196                                           | 0                                                 | 0                                             |

* Spores from the *tbf1::KanMX4* diploid transformed with the indicated plasmids were plated at approximately equivalent densities and allowed to germinate on medium selective for the presence of plasmid. The number of haploid colonies (adenine prototrophs), able to grow on medium supplemented with G418, was established by replica plating and calculated as a percentage of the total number of viable colonies.

FIG. 2. The essential function of *S. pombe* *tbf1* can be provided by expression of *P. carinii* *tbf1* but not *S. cerevisiae* *TBF1*. (A) The presence of the *tbf1::KanMX4* allele and the absence of the wild-type (WT) *Spbfl* allele in the haploid colonies expressing ectopic *Spbfl* and *PcTbf1* was confirmed by PCR using locus-specific primers on genomic DNA from the following strains: 1, wild-type 972 h*"* haploid strain; 2, heterozygous *tbf1::KanMX4* diploid; 3, *tbf1::KanMX4* haploid expressing pREP41-*Spbfl*; 4, *tbf1::KanMX4* haploid expressing pREP41-*PcTbf1*. (B) The lethal phenotype of the *S. cerevisiae* *TBF1::KanMX4* strain is rescued by heterologous expression of *S. cerevisiae* *TBF1* or *C. glabrata* *TBF1* but not by that of *P. carinii* *tbf1*. *S. cerevisiae* diploid cells heterozygous for the *TBF1::KanMX4* null allele were transformed with plasmids p416ScTBF1pro-*PcTbf1* (rows a and b), p416ScTBF1pro-*ScTBF1* (rows c and d), or p416ScTBF1pro-*CgTBF1* (rows e and f), and allowed to sporulate. Tetraads from each transformant were separated (columns 1 to 4), grown on rich medium, and then replica plated to verify that viable colonies were dependent on the plasmid.
indicating that the GFP-tagged protein does not simply enter the nucleus by dimerizing with untagged Tbf1p. As observed with the native versions of SpTbf1 and PcTbf1, the GFP fusions of both proteins were able to rescue the tbf1::KMX4 null strain whether the nmt1-41 promoter was repressed or induced, while the GFP fusion of ScTbf1 did not rescue even when strongly expressed. The GFP-ScTbf1 fusion could be expressed in wild-type cells for >100 generations without noticeable effects on viability, indicating that it does not act as a dominant negative allele. Taken together, our observations suggest that lack of complementation by the S. cerevisiae homologue is unlikely to be due to either instability, mislocalization, or lack of DNA binding of the GFP-ScTbf1 fusion protein.

When the cultures are grown in thiamine, the GFP signals from all three fusion proteins are undetectable by fluorescence microscopy (Fig. 3B and data not shown). However crude estimates from previous work suggest that endogenous SpTbf1p is a relatively abundant protein (62) and is likely to be more closely approximated by the induced levels of the GFP fusions than by repressed levels.

Immunolocalization studies with S. cerevisiae have shown that ScTbf1p localizes to foci distributed throughout the non-nucleolar chromatin of interphase nuclei (33). Thus, although the two proteins are unable to functionally complement, both SpTbf1p and ScTbf1p appear to have broad distributions throughout chromatin.

Analysis of the phenotype of S. pombe cells lacking Tbf1p. In the course of the complementation experiments described above, we examined the phenotype of the null mutant of SpTbf1 in detail. In agreement with another study (51), we found that the gene is essential in more detail (Fig. 4A); however, in contrast to that report, we observed that the two spores carrying the tbf1::KMX4 allele routinely gave rise to microcolonies of up to 50 cells, which were elongated compared to wild-type cells. (Fig. 4B). This was not influenced by temperature, in the range 19°C to 32°C. Examination at intervals of germinating spores on plates at 25°C revealed that the null and wild-type colonies divided at similar rates for the first three divisions, with the null mutant undergoing several additional, significantly retarded divisions thereafter (not shown). These data are consistent with the hypothesis that the SpTbf1p inherited from the spore is diluted among the progeny in the course of the early division cycles and that the phenotype does not become apparent until its level decreases below a critical threshold.

S. pombe tbf1 null haploids arrest in interphase, prior to NETO (new-end takeoff), and have an aberrant chromatin structure. To examine the phenotype of the tbf1 null mutant cells in more detail, spores were first allowed to germinate and then grown for the equivalent of five generation times in medium containing G418 to prevent propagation of all germinated spores that carry the wild-type SpTbf1 allele (see Materials and Methods). The liquid cultures were then fixed and stained to analyze the state of the DNA, microtubules, and F-actin in the tbf1 null mutant cells.

The null mutant cells were swollen and elongated, consistent with a delay in cell cycle progression. DAPI staining showed that most contained a single irregular mass of chromatin, very different in appearance from the chromatin in wild-type cells at any stage of the cell cycle (Fig. 5A).

To determine whether or not the irregularly shaped DAPI-stained structures might reflect a problem of chromosome segregation or a delay in mitosis, we examined the distribution of tubulin and F-actin. We observed that the tbf1 null cells with aberrant DNA had microtubule structures characteristic of interphase, with long microtubules running the length of the cell; no mitotic spindles were observed (Fig. 5B; see Fig. S2a in the supplemental material) (21, 22).

Staining of cells with rhodamine-conjugated phalloidin, DAPI, and Calcofluor revealed that the tbf1::KMX4 null allele was allowed to undergo meiosis, and spores were dissected onto YE medium at 29°C. Colonies were allowed to form and were then replicated to the indicated media. (A) nine tetrads (a to i) are shown; the four spores are at positions 1 to 4. Note that all the surviving colonies are sensitive to G418 and are adenine auxotrophs, indicating that they are haploid, and that cells carrying the tbf1::KanMX4 allele cannot form a visible colony (B). The center and right panels show microcolonies derived from the germinating of tbf1::KanMX4 spores. The null mutant stopped dividing after forming a microcolony of 50 cells or fewer. The cells become elongated compared with cells in a tbf1+ colony, indicative of a cell cycle arrest or delay. For comparison, the left panel shows the edge of a colony of G418-sensitive cells from the same tetrad.

Fig. 4. Characterization of the phenotype of SpBF1 null haploids. An S. pombe diploid heterozygous for the tbf1::KanMX4 null allele was allowed to undergo meiosis, and spores were dissected onto YE medium at 29°C. Colonies were allowed to form and were then replicated to the indicated media. (A) nine tetrads (a to i) are shown; the four spores are at positions 1 to 4. Note that all the surviving colonies are sensitive to G418 and are adenine auxotrophs, indicating that they are haploid, and that cells carrying the tbf1::KanMX4 allele cannot form a visible colony (B). The center and right panels show microcolonies derived from the germinating of tbf1::KanMX4 spores. The null mutant stopped dividing after forming a microcolony of 50 cells or fewer. The cells become elongated compared with cells in a tbf1+ colony, indicative of a cell cycle arrest or delay. For comparison, the left panel shows the edge of a colony of G418-sensitive cells from the same tetrad.
FIG. 5. The Spbtf1 null haploids arrest in interphase. Spores prepared from a diploid heterozygous for the tbf1::KanMX4 null allele were grown in complete medium overnight at 19°C, when G418 was added to block further growth and division of tbf1null cells. At a time corresponding to four generations (see text for details), cells were fixed and the cytoskeleton was analyzed by indirect immunofluorescence. (A) The fixed cells were stained with DAPI. Note the presence of condensed chromosomes (cells marked 1), aberrant nuclear structure (cells marked 2), and problems in chromosome segregation (cells marked 3). Note also the presence of division septa that bisect the cell to generate an anucleate compartment (cells marked 4), indicating that cytokinesis has occurred without nuclear division. (B). The fixed cells were permeabilized and stained with DAPI and rhodamine-conjugated phalloidin. The right panels show tbf1 null cells. Note that F-actin patches are located only at one end of the cell, consistent with a delay early in the cell cycle. (C) The fixed cells were digested, and the TAT-1 antibody was used to analyze the microtubules, as described in Materials and Methods. DAPI is shown in red and microtubules in green. The right panels show the phenotype of elongated tbf1 null cells. Note that all of them have an interphase microtubule array. The scale bars for each panel represent 10 μm.

FIG. 6. Telomere length in both tbf1 null cells and cells rescued by GFP-SpTbf1 or GFP-PcTbf1 is slightly longer than wild type. Genomic DNA was extracted from the indicated strains and digested overnight with Apal. The DNA fragments were separated by electrophoresis overnight at 20 V (1 V/cm) through a 1.5% (wt/vol) agarose gel. The filter was probed with a synthetic telomere fragment as described in Materials and Methods. A portion of the filter is shown. The arrowheads indicate the positions of DNA markers visualized by ethidium bromide staining prior to transfer. The lane numbers are referred to in the text. Lanes 1 to 3, DNA from germinating tbf1 null spores at 24, 48, and 72 h after germination. Note that wild-type (WT) cells do not contribute significantly to the culture, as they are unable to divide in the presence of G418, while null spores undergo multiple divisions. Lane 4, wild-type cells. Lanes 5 and 6, DNA from tbf1::KanMX4 leu1::nmt1-41-GFP-SpTbf1(ura4+) cells grown in the absence (on) or presence (off) of thiamine. Lanes 7 and 8, DNA from tbf1::KanMX4 leu1::nmt1-41-GFP-PcTbf1(ura4+) cells grown in the absence (on) or presence (off) of thiamine. Lane 9, DNA from leu1::nmt1-41-GFP-PcTbf1(ura4+) cells grown in the absence (on) of thiamine. Lane 10, DNA from leu1::nmt1-41-GFP-SpTbf1(ura4+) cells grown in the absence (on) of thiamine. Note that lane 2 contains approximately three times as much DNA as the others.

activation of the DNA structure checkpoint permits time to repair defects in chromosome structure, then in the rad3 tbf1 double mutant one would expect to see fewer cells in the microcolony, since a division delay could no longer be imposed. However, the microcolonies with rad3+ or rad3Δ backgrounds were indistinguishable by either cell number or phenotype. These data suggest that the cell cycle delay of tbf1 null cells is not imposed by a rad3-dependent signaling pathway.

S. pombe telomeres are mildly elongated both in the tbf1 null and the rescued strains. S. pombe Tbf1p overexpression is reported to slightly increase mean telomere length, and it has been speculated that it could have an essential role at telomeres (51). Therefore, we examined whether telomeres had undergone any changes in tbf1 null cells that had ceased to divide. We found that the mean telomere repeat length was some 50 to 80 bp greater in tbf1 null cells than in the isogenic haploid wild-type strain (Fig. 6, lanes 1 to 3 compared with lane 4). There was no sign of end-to-end fusion events having occurred, and telomeres appeared similar in cultures maintained for up to 72 h under G418 selection. Therefore, we conclude that tbf1 null cells do not stop dividing because of rapid telomere shortening or chromosome end fusion.

We also compared the lengths of telomeres in cells rescued
by the *Pneumocystis* and *S. pombe* GFP-Tbf1 fusions (Fig. 6, lanes 5 to 8). Telomere length in the *thbf1* null cells rescued by GFP-SpTbf1 was also found to be slightly greater than wild type (approximately 50 to 80 bp) whether the *nmI1-4I* promoter was induced or not (Fig. 6, compare lanes 4, 5, and 6). This is consistent with the previous studies by Pitt et al., who found that prolonged expression of Tbf1 from the highly attenuated *nmI1-4I* promoter caused a discrete increase in telomere length (51). Interestingly, the increase in telomere length in the *thbf1* null cells rescued by GFP-PcTbf1 (approximately 100 to 150 bp) was somewhat larger than that in *thbf1* null cells rescued by GFP-SpTbf1 but also did not change upon induction of the promoter (Fig. 6, compare lanes 4, 7, and 8). Prolonged overexpression of either GFP-PcTbf1 or non-complementing GFP-ScTbf1 in the wild-type strain had no dominant effects on telomere length (Fig. 6, lanes 9 and 10).

The telobox motif is also found in the *S. pombe* telomere binding protein Taz1p (13). Loss of Taz1p function leads to a telomere erosion or entanglement (51). Interestingly, the increase in telomere length (50 to 80 bp) was somewhat larger than that in *thbf1* null cells rescued by GFP-SpTbf1 and also did not change upon induction of the promoter (Fig. 6, compare lanes 4, 7, and 8).

**DISCUSSION**

**Sequence divergence at interfaces mediating essential functions of Tbf1p in *S. cerevisiae* and *S. pombe/P. carinii*.** Our transspecies complementation assays indicate that the fission and budding yeast Tbf1 proteins have diverged significantly. We propose that this functional divergence is most likely to be correlated with the divergence in sequence and secondary structure observed at their N termini. Though not required for sequence-specific DNA binding, part of the equivalent region of ScTbf1p still confers telomere length regulation properties in *S. cerevisiae* when tethered there by means of a heterologous DNA binding motif (6). Thus, it could be important to gain a better understanding of the interactions mediated by this domain in the *S. pombe* and *P. carinii* orthologues. Proteins that participate in the essential function of Tbf1p via interactions with the N-terminal domain might also be conserved between the saprobe *S. pombe* and the mammalian pathogens *P. carinii* and *P. jiroveci*. However, the absence of a system to cultivate *S. pombe* null cells is unlikely to die because of a catastrophic loss of telomere capping function and exhibit no symptoms of either telomere erosion or entanglement.

**The Tbf1p family as global coordinators of gene expression.** It has recently been proposed that Tbf1p was an important contributor to the coordinated regulation of ribosomal protein genes in ancestral eukaryotes. This regulatory function was apparently taken over by Rap1p in the *S. cerevisiae* lineage but maintained in other Hemiascomycetes, including *S. pombe* (27). The essential role of ScTbf1p remains enigmatic. However, comparative genomics analyses of Tbf1p consensus binding sites in several closely related budding yeast species has revealed that they are highly conserved within many different regulatory regions (32). Tbf1p binding sites in these budding yeasts are often found adjacent to one of several stress response motifs (32), suggesting that many genes are regulated by combinatorial interactions between Tbf1p and the proteins that bind these stress response elements. In this context it is also noteworthy that ScTBF1 is one of only 17 genes in the *S. cerevisiae* genome with a profile of strong upregulation (>5-fold) in response to starvation for either phosphate, glucose, ammonium, or ethanol (64). This implies the existence of feedback loops linking ScTBF1 transcription to specific types of stress. It has been speculated that ScTbf1p and ScRap1p might, respectively, coordinate stress responses and global changes in protein synthesis levels with genome-wide epigenetic changes (32, 46).

The terminal phenotype observed in the *spbf1* null mutant is consistent with the view that the *S. pombe* protein is also likely to be a global regulator of transcription, i.e., more similar in function to the pleiotropic Tbf1 and Rap1 proteins of *S. cerevisiae* (10, 33, 35, 46, 54) than to the telobox protein Taz1p of *S. pombe* (13, 40, 41). In their respective species, SpTbf1p and ScTbf1p localize widely throughout the euchromatic nuclear hemisphere, supporting the assumption that both do more than regulate telomeres. Consistent with this, we have been unable to obtain any survivors with circular chromosomes from plating out large numbers of *thbf1* null haploids (data not shown), whereas this phenotype is characteristic of *S. pombe* mutants in which cell inviability is due principally to telomere erosion (48, 49). The null phenotype in *S. pombe* is also quite different from the phenotype produced by inhibiting protein synthesis with cycloheximide (52), suggesting that *spbf1* probably controls other regulons in addition to those of the ribosome and the subtelomeres. The reason for the cell cycle-specific arrest of *spbf1* null cells prior to NETO is still unclear. Perhaps genes required for this transition are among its regulatory targets.

**A conserved role for Tbf1p in the regulation of gene expression at telomere-proximal domains in fungi?** Tbf1 proteins from both the Taphrinidae and the Saccharomycetaceal fungal orders appear to mildly influence telomere length homeostasis (references 2 and 51 and this study). However our data clearly establish that the primary cause of death in the *spbf1* null mutants is not rapid telomere shortening or chromosomal end fusions. In addition, entanglement at chromosome ends, such as has been noted for *tas1* null mutants (40), does not seem to cause the *thbf1* null phenotype. Taken together, our data provide strong evidence that *thbf1* has an essential function in *S. pombe*, unrelated to telomere maintenance. If the role of *thbf1* at *S. pombe* telomeres is also essential but is masked by the pleiotropic effects of the null mutant, then the *Pneumocystis carinii* orthologue must be able to perform it too. If the role at
S. pombe telomeres is nonessential, we cannot assume that it is carried out by Pcbfl1, since S. pombe telomeres are slightly elongated both in tbf1 null cells and in the rescued strains.

Even if its telomeric role is not vital in S. pombe, a similar role for the Pcbfl1 orthologue at Pneumocystis carinii telomeres might have more critical consequences. In contrast to the degeneracy of telomeric repeats found in S. pombe (26), P. carinii telomeres consist of several different repeat types of identical repeats of TTAGGG. The subtelomeric regions harbor tandemly repeated copies of several gene families (1, 30, 31, 53), including the abundantly expressed major surface glycoproteins (MSGs), which play an important role in the host-parasite interaction and the escape from host responses during lung infection (20, 58). Only a single copy of approximately 100 or so MSG genes is expressed at any one time; telomeric recombination appears to mediate the switching of different antigenic variants into the unique subtelomeric locus from which expression takes place (29, 56, 57, 60). Whether the integrity of P. carinii telomere ends is maintained through telomerase-based addition of repeats or entirely by recombination is still unclear. There are no obvious homologues to the catalytic or RNA subunits of P. carinii telomerase is nonessential, we cannot assume that it is even if its telomeric role is not vital in S. pombe telomere elongation in budding yeast. EMBO J. 28:46–456.

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