The 3’ Overhangs at *Tetrahymena thermophila* Telomeres Are Packaged by Four Proteins, Pot1a, Tpt1, Pat1, and Pat2

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Although studies with the ciliate *Tetrahymena thermophila* have played a central role in advancing our understanding of telomere biology and telomerase mechanisms and composition, the full complement of *Tetrahymena* telomere proteins has not yet been identified. Previously, we demonstrated that in *Tetrahymena*, the telomeric 3’ overhang is protected by a three-protein complex composed of Pot1a, Tpt1, and Pat1. Here we show that Tpt1 and Pat1 associate with a fourth protein, Pat2 (Pot1 associated *Tetrahymena* 2). Mass spectrometry of proteins copurifying with Pat1 or Tpt1 identified peptides from Pat2, Pot1a, Tpt1, and Pat1. The lack of other proteins copurifying with Pat1 or Tpt1 implies that the overhang is protected by a four-protein Pot1a-Tpt1-Pat1-Pat2 complex. We verified that Pat2 localizes to telomeres, but we were unable to detect direct binding to telomeric DNA. Cells depleted of Pat2 continue to divide, but the telomeres exhibit gradual shortening. The lack of growth arrest indicates that, in contrast to Pot1a and Tpt1, Pat2 is not required for the sequestration of the telomere from the DNA repair machinery. Instead, Pat2 is needed to regulate telomere length, most likely by acting in conjunction with Pat1 to allow telomerase access to the telomere.

Telomere proteins are essential for genome stability, because they sequester the DNA terminus from unwanted DNA repair reactions that lead to end-to-end fusion of chromosomes (1, 2). They also function in telomere replication by aiding the passage of the replication fork through the telomeric duplex DNA and by regulating the access of enzymes such as telomerase, which are needed to replicate the extreme DNA terminus (3). Telomeric DNA generally consists of tandem repeats of a simple GC-rich sequence that extends to form an overhang on the 3’ G-rich strand. In mammals and fission yeast, the telomeric DNA is protected by a multisubunit protein complex (shelterin) that contains both telomere duplex and 3’ overhang binding proteins in addition to various linker subunits (4, 5). In other organisms, such as *Saccharomyces cerevisiae*, the telomere duplex and 3’ overhang are protected by separate but slowly cooperating complexes (6, 7).

The telomere proteins that bind the 3’ overhang (e.g., POT1-TTP1 in vertebrates and Cdc13-Stn1-Ten1 in budding yeast) have several important roles (3, 4). First, they exclude replication protein A (RPA) from the overhang, thus preventing the recruitment of ATR and the activation of a DNA damage response (8–10). Second, they engage with telomerase and DNA polymerase α (pol α) to regulate the replication of the DNA terminus (11–13). During telomere replication, telomerase compensates for the inability of DNA polymerase to fully replicate the chromosome 5’ end by adding additional DNA to the 3’ overhang. The extended overhang is then partially filled in through the action of pol α (3, 14, 15). In mammalian cells, the overhang binding protein POT1 and its partner subunit TTP1 have opposing effects on telomerase: POT1 excludes telomerase from the overhang, while TTP1 recruits telomerase and enhances telomerase processivity (16–18).

In budding yeast, Cdc13 anchors and activates telomerase at the overhang, while Cdc13 and Stn1 appear to mediate subsequent fill-in synthesis of the C strand by pol α (12, 13, 19–21).

Ciliated protozoa have played an important role in our understanding of telomere biology, because these organisms have an unusual genomic organization that results in each cell containing thousands of telomeres and an abundance of telomerase (22). The ciliate *Tetrahymena thermophila* has been a particularly valuable player because it is amenable to both genetic manipulation and biochemical analysis (23, 24). As a result, the composition and function of *Tetrahymena* telomerase subunits are well characterized, and studies with the *Tetrahymena* enzyme continue to establish paradigms for the enzymatic mechanism (25–29).

*Tetrahymena* cells contain two types of nuclei: the germ line micronucleus and the transcriptionally active macronucleus (30). The macronucleus is diploid and contains five chromosomes with telomeres of >2.5 kb (31). The macronucleus is polypliod and is formed from a copy of the micronucleus during sexual reproduction. As part of this process, the micronuclear chromosomes are subdivided into smaller pieces, telomeres are added to the new DNA termini, and the resulting macronuclear chromosomes are subject to endoreduplication (22). The outcome is ~20,000 macronuclear chromosomes, of which ~9,000 make up a ribosomal DNA (rDNA) minichromosome of 21 kb (32). The telomeres on macronuclear chromosomes consist of 250–350-bp T₆G₆C₄A₂ repeats that terminate in a 3’ G-strand overhang of ~14 or 20 nucleotides (nt) (33). The telomere is packaged into a nonnucleosomal DNA protein complex; however, the protein components of the complex are only partially characterized (34).

We previously identified the 3’-overhang binding protein...
Pat1 on the basis of sequence identity to *Oxytricha nova* TEBPα and human POT1 (35). We then identified two proteins, Tpt1 and Pat1, that associate with Pot1a (36). Neither Tpt1 nor Pat1 binds DNA directly. Tpt1 interacts with Pot1a and appears to be the *Tetrahymena* ortholog of human TTP1, Schizosaccharomyces pombe Tpz1, or *Oxytricha* TEBPβ. The Pot1a-Tpt1 dimer is important for telomere protection and negative regulation of telomerase action, as evidenced by the fact that even partial depletion of either Pot1a or Tpt1 results in cell cycle arrest and rapid telomere elongation. Pat1 interacts with Tpt1 to form a Pot1a-Tpt1-Pat1 complex. Pat1 is a unique protein that appears to be required for telomerase to gain access to the DNA terminus, since depletion of Pat1 causes gradual telomere shortening without affecting telomerase levels (36). Here we report the identification of a fourth component of the G-overhang binding complex, Pat2 (Pot1-associated *Tetrahymena* 2), which also appears to facilitate telomerase action at the chromosome terminus.

**MATERIALS AND METHODS**

*Tetrahymena* growth and transformation. *Tetrahymena thermophila* cells were grown in 1.5× PPSY medium at 30°C as described previously (33). TAP-Tpt1 and TAP-Pat1 cells have been described previously (36). In each cell line, the endogenous TPT1 or PAT1 promoter and 5′ coding sequence are replaced by the calcium-inducible *MTT1* promoter and a sequence encoding a 6-His motif followed by 2 protein A motifs, a tobacco etch virus (TEV) cleavage site, and the start of the Tpt1 or Pat1 coding sequence. Cells expressing Pat2-FLAG-His and TAP-tagged Pat2 were generated by using biotic transformation to introduce a gene replacement construct into the native PAT2 gene locus (see Fig. 1). The FLAG-His tag encodes a FLAG peptide followed by 6 histidines. The TAP tag encodes 6 histidines followed by 2 protein A motifs and a TEV cleavage site. To allow selection for clones with gene replacement, the gene replacement construct contained the Neo3 cassette, which encodes the NEO1 gene driven by the *MTT1* promoter (37). Cells were selected with paromomycin in the presence of 2 μg/ml CdCl2, in order to obtain full gene replacement. Clones were checked at regular intervals to ensure that they retained the full gene replacement and had not reverted to wild type (WT) (see Fig. S2A in the supplemental material). In each case, the residual WT band remained at <5% of the level seen in control cells, indicating that it corresponded to signal from the micronuclear gene. For growth curves, the culture was adjusted regularly to keep the cells in log phase (2 × 10^8 cells/ml).

**Mass spectrometry.** Nuclear extracts were prepared from TAP-Pat1, TAP-Tpt1, and WT cells as described previously (36). Briefly, nuclei (chromatin) were isolated from cells expressing the TAP-tagged protein and were extracted with 20 mM Tris (pH 7.5), 200 mM NaCl, and 1.5 mM MgCl2 plus protease inhibitors for 1 h at 4°C. The clarified supernatant was collected and protein complexes were released from the beads by digestion with TEV protease. Samples were precipitated with trichloroacetic acid (TCA), separated by SDS-PAGE, and visualized by colloidal Coomassie staining. The entire lane was excised, divided into 16 pieces, and prepared for mass spectrometry (MS) by in-gel reduction, alkylation, and trypsin digestion. The eluted samples were analyzed by reverse-phase nanoelectrospray tandem MS as described previously (38). Spectra from the gel slices were searched against tryptic peptides predicted from the *Tetrahymena* genome as described previously (36).

**Immunoprecipitation and ChIP.** Common immunoprecipitation studies were performed as described previously (36). Nuclear extracts from Pat2-FLAG-His cells were prepared as described above except that salt was added to 300 mM NaCl. The clarified extracts were incubated with Ni-Sepharose beads (GE Healthcare) and washed with 20 mM Tris (pH 7.5), 300 mM NaCl, and 1.5 mM MgCl2 plus 8 or 40 mM imidazole. Proteins were released by boiling in SDS-PAGE sample buffer. Micrococcal nuclease (MNase) digestion was performed on clarified extracts prior to the addition of the Ni-Sepharose beads. Samples were incubated at 30°C for 30 min with 180 U MNase per ml extract. Copurifying Tpt1 and Pat1 were identified by Western blotting using previously generated antibodies (36). For chromatin immunoprecipitation (ChIP) analysis, cells were fixed with formaldehyde, the DNA was sheared, and the soluble chromatin fraction was prepared as described previously (35). Precipitation was performed for 1 h at 4°C with IgG Sepharose for TAP-Pat2, with M2 anti-FLAG resin for Pat2-FLAG-His, or with an antibody and protein A Sepharose for endogenous proteins. Precipitates were washed sequentially with radioimmunoprecipitation assay (RIPA) buffer, a high-salt solution, and a LiCl solution (35). DNA was isolated from the precipitate using Chelex resin (Chelex 100; Bio-Rad) (39). Fifty microliters of a 10% Chelex slurry in double-distilled water (ddH2O) was added to the washed beads and was boiled for 10 min; the suspension was allowed to cool; proteinase K (100 μg/ml) was added; and the sample was incubated for 30 min at 55°C with shaking. Samples were boiled for another 10 min, centrifuged, and the supernatant collected. The residual Chelex-IgG Sepharose/protein A bead fraction was then reextracted with 50 μl water and was centrifuged, and the first and second supernatants were combined. The supernatant was used directly as a template in real-time quantitative PCR (qPCR). Real-time PCR was performed using SYBR Advantage qPCR Premix from Clontech. Conditions for PCR were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 30 s. Primers were against the 26S rRNA subtelomeric sequence (GAACCT CAACCTTTTGACTAGC and AATTTCTTGGACATTGAAATGAAGTTA TTATT) or the internal, nonsubtelomeric sequence (TGAAATTGCAA GTTAGGTTTC and CATAGTACTCCGCGCCGT).

**Telomere length analysis.** Telomere length was determined by Southern hybridization to HindIII-digested genomic DNA using a subtelomeric probe to the rDNA telomere (40).

**RESULTS AND DISCUSSION**

Identification of Pat2. In the original experiments designed to identify Pot1a interaction partners, we used mass spectrometry to identify proteins that copurified with TAP-tagged Pot1a. Based on peptide abundance and the percentage of gene coverage, Tpt1 and Pat1 were the most obvious candidates. To determine whether the Pot1a-Tpt1-Pat1 complex contains additional proteins, we repeated the analysis with tagged Tpt1 and Pat1. Nuclear extracts were prepared from wild-type cells and from cells expressing TAP-Tpt1 or TAP-Pat1, and the tagged Tpt1 or Pat1 was purified on IgG Sepharose. Bound proteins were released by digestion with TEV protease and were analyzed by MS (36). Peptides were compared to the predicted *Tetrahymena thermophila* proteome. MS was performed on two independent samples from TAP-Tpt1, TAP-Pat1, and WT cells. Proteins identified in the WT control sample were subtracted from the TAP-Tpt1 and TAP-Pat1 samples.

MS analysis of each TAP-Tpt1 or TAP-Pat1 sample consistently identified peptides from Tpt1, Pat1, Pot1a, and a new protein since named Pat2 (Pot1a-associated *Tetrahymena* 2) (see Tables S1 and S2 in the supplemental material). Multiple unique peptides were identified for each protein; 9 to 28 Pat2 peptides were identified in the four samples analyzed. Reverse transcriptase PCR (RT-PCR) was then used to identify the full cDNA sequence of Pat2 (see Fig. S1 in the supplemental material). The predicted protein has a molecular mass of 69 kDa. It was striking that no other *Tetrahymena* proteins consistently copurified with both TAP-Tpt1 and TAP-Pat1 and that the peptide coverage of the remaining proteins identified by MS was substantially lower than that observed for Pat2 (see Table S2 in the supplemental material). This finding suggests that the proteins present at the 3′ overhangs.
of Tetrahymena telomeres exist in a four-protein complex consisting of Pot1a, Tpt1, Pat1, and Pat2.

**Verification that Pat2 is a telomere protein.** To confirm that Pat2 is a telomere protein, we sought to verify the interaction with Tpt1 and Pat1 and to demonstrate localization to telomeres. To achieve this, we generated cell lines that expressed FLAG-His- or TAP-tagged Pat2 (Fig. 1). Cells expressing Pat2-FLAG-His were generated by gene targeting using a construct that placed a sequence encoding the tag in frame with the 3′ coding sequence of Pat2. These cells retained the endogenous promoter. Cells expressing TAP-Pat2 had the endogenous promoter replaced by the cadmium-inducible MTT1 promoter, and a 6-His, 2-protein-A tag was added to the 5′ coding sequence (Fig. 1AII). Following selection in paromomycin, cells with full replacement of the endogenous gene were obtained for both the 5′- and 3′-tagged alleles (Fig. 1B), indicating that the tags did not interfere with protein function. Moreover, the gene replacements were stable during growth in the absence of drug; the residual WT band remained at <5% of the level seen in control cells, indicating that it corresponded to signal from the micronuclear gene (see Fig. S2A in the supplemental material; also data not shown).

We verified that Pat2 is present in complexes with Tpt1 and Pat1 through pulldown experiments using nuclear extracts from Pat2-FLAG-His-expressing cells. Pat2 was bound to Ni-Sepharose beads, and copurifying proteins were identified by Western blotting (Fig. 2A). Tpt1 and Pat1 both copurified with Pat2. To ensure that the interaction with Tpt1 and Pat1 was not mediated through DNA or RNA, control samples were treated with micrococcal nuclease before application to Ni-Sepharose. This treatment did not disrupt the interaction, indicating that Tpt1 and Pat1 are present in a complex with Pat2.

To test for telomere localization, we performed ChIP with Pat2-FLAG-His and TAP-Pat2 cells. The cells were cross-linked with formaldehyde and sonicated, and Pat2-associated chromatin was isolated with an agarose-linked anti-FLAG antibody or with IgG Sepharose. Telomere association was monitored by real-time PCR using primers corresponding either to a region immediately adjacent to the rDNA telomere or to an internal region of the rDNA (Fig. 2B). Preferential enrichment of telomeric over non-telomeric DNA was observed with both the anti-FLAG resin and IgG Sepharose (Fig. 2BII and III). The enrichment was particularly apparent for TAP-Pat2 cells grown with cadmium, and it was lost if the cells were grown without cadmium to repress TAP-Pat2 expression (see below). We therefore conclude that Pat2 is a telomere protein.

While sequence analysis of Pat2 failed to reveal orthologous proteins in other organisms, analysis using secondary-structure prediction and structure-threading programs suggested a high alpha-helical content with a possible Myb motif in the N terminus. Since Myb repeats are characteristic of proteins that bind the telomere duplex, we expressed Pat2 in Escherichia coli and tested whether the purified protein bound telomeric DNA. However, we were unable to detect significant binding to either single-stranded or double-stranded DNA (data not shown). This finding indicates that Pat2, like Pat1 and Tpt1, associates with the telomeric 3′ overhang via Pot1a. It further suggests that the 3′ overhang-binding complex may be physically separate from any complex that binds to the telomere duplex DNA.

**Depletion of Pat2 leads to gradual telomere shortening.** To assess the effects of Pat2 removal from the telomere, we initially attempted to generate cells with a disruption of the PAT2 gene. We were able to obtain only 80 to 90% gene replacement, suggesting that Pat2 is essential (see Fig. S2B in the supplemental material). Nonetheless, analysis of telomere length in the knockdown cells revealed slight telomere shortening (see Fig. S2C in the supplemental material). We next examined the effect of Pat2 depletion by using the conditional TAP-Pat2 cell line. Cells were kept in continuous culture with or without cadmium for as long as 15 days and were monitored for changes in growth rate or telomere length. Pat2 depletion did not cause a noticeable change in the growth rate (Fig. 3A). However, Southern blot analysis of telomeric restriction fragments revealed gradual telomere shortening during the first ~10 days in culture (Fig. 3B). The length shortened from 350 to 400 bp to about 150 bp and then stabilized. The rate and extent of telomere shortening were similar to those observed previously after conditional depletion of either Pat1 or telomerase reverse transcriptase (TERT) (36). When Pat1 and TERT conditional cells are grown without cadmium, the length of telomeres gradually declines and then stabilizes due to partial re-activation of the MTT1 promoter in cells with short telomeres and the resulting reexpression of Pat1 or TERT. When we examined PAT2 mRNA levels by RT-PCR, we did not see significant reactivation of the MTT1 promoter in TAP-Pat2 cells grown without Pat2 expression.
However, repression of TAP-Pat2 expression was incomplete, and a low level of \( \text{PAT2} \) mRNA remained throughout the experiment (see Fig. S2D in the supplemental material). This residual Pat2 expression likely explains why TAP-Pat2 cells grown without cadmium fail to show a growth defect despite the essential nature of the \( \text{PAT2} \) gene.

The lack of rapid growth arrest and the gradual nature of the telomere shortening seen after Pat2 depletion indicate that Pat2 is probably not required to protect the telomere from degradation or DNA repair activities (35, 36). Thus, this function seems to be reserved for Pot1a-Tpt1. The similarities in the rate and extent of telomere shortening seen after Pat2 and TERT (36) depletion instead suggest that Pat2 is needed for telomerase to add repeats to the chromosome terminus. Since loss of Pat1 also causes gradual telomere shortening, it remained possible that Pat2 depletion had an indirect effect on telomere length by causing Pat1 to dissociate from the telomere. To test for this scenario, we performed ChIP with TAP-Pat2 cells grown with or without cadmium by using antibodies to Pat1 and Tpt1. As shown in Fig. 2C, the association of Pat1 and Tpt1 with the telomere is unaffected by Pat2 removal. Chromatin from TAP-Pat2 cells grown for 3 days with or without cadmium was precipitated with an antibody to Pat1 (I) or Tpt1 (II). Three experiments were performed; error bars indicate standard errors of the means.

Overall, our results indicate that the 3’ overhangs of \( \text{Tetrahymena} \) telomeres are packaged by a four-protein complex containing cadmium. However, repression of TAP-Pat2 expression was incomplete, and a low level of \( \text{PAT2} \) mRNA remained throughout the experiment (see Fig. S2D in the supplemental material). This residual Pat2 expression likely explains why TAP-Pat2 cells grown without cadmium fail to show a growth defect despite the essential nature of the \( \text{PAT2} \) gene.

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FIG 2 Pat2 interacts with Tpt1 and Pat1 and localizes to telomeres. (A) (I) Western blots showing copurification of Tpt1 and Pat1 with Pat2 on Ni-Sepharose beads. Nuclear extracts were made from WT or Pat2-FLAG-His cells. Lanes 2 and 3 and lanes 5 and 6 are from duplicate immunoprecipitation reactions. PPT, precipitate. (II) Nuclear extracts from Pat2-FLAG-His cells were treated with MNase prior to the addition of the beads. (B) ChIP analysis showing the presence of Pat2 at telomeres. (I) Schematic showing positions of real-time PCR primers on the rDNA chromosome. CDS, coding sequence; Int, internal, nontelomeric DNA; Tel, telomeric DNA. (II and III) Results of real-time PCR analysis with ChIP samples from Pat2-FLAG-His or TAP-Pat2 cells. (II) Chromatin from Pat2-FLAG-His cells precipitated with anti-FLAG agarose. Two experiments were performed; error bars indicate minimum and maximum. (III) Chromatin from TAP-Pat2 cells grown with or without cadmium for 3 days was precipitated with IgG Sepharose. Three experiments were performed; error bars indicate standard errors of the means. (C) ChIP analysis showing that the association of Pat1 and Tpt1 with the telomere is unaffected by Pat2 removal. Chromatin from TAP-Pat2 cells grown for 3 days with or without cadmium was precipitated with an antibody to Pat1 (I) or Tpt1 (II). Three experiments were performed; error bars indicate standard errors of the means.

FIG 3 Effects of Pat2 depletion on growth rate and telomere length. (A) Growth curves for TAP-Pat2 cells grown with or without cadmium. Results are averages for two experiments. (B) Southern blots showing the lengths of rDNA telomeres from TAP-Pat2 cells grown with or without cadmium for 1 to 15 days.
taining Pot1α, Tpt1, Pat1, and Pat2. Previous studies have indi-
cated that the complex is anchored to the overhang via Pot1α and
that the Pot1α-Tpt1 dimer serves to prevent the overhang from
activating a DNA damage response and limits extension of the
overhang by telomerase. In contrast, Pat1 and Pat2 are needed for
telomerase to maintain the length of telomeres. Currently it is
unclear how Pat1 and Pat2 facilitate the action of telomerase.
However, one possibility is that they coordinate the removal of
Pot1α with telomerase binding to ensure that the overhang is al-
ways protected by one complex or the other. The Tetrahymena
telomerase holoenzyme binds to the overhang through the oligo-
nucleotide/oligosaccharide binding (OB) fold-containing subunit
Teb1, which also serves as a processivity factor (25, 41). Since most
Tetrahymena 3′ overhangs are 14 or 20 nt long, and since both Pot1α and Teb1 require at least two T2G4 repeats for high-affinity
binding (42; B. R. Linger and C. Price, unpublished results), it is
unlikely that Pot1α and Teb1/telomerase can bind the overhang
concurrently.

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