DNA-Binding Properties of the Replication Telomere Protein†

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ABSTRACT: The replication Telomere Protein, rTP, is a nuclear protein from the ciliate *Euplotes crassus* that appears to be a novel telomere replication factor. rTP shares extensive amino acid sequence identity with the two proteins that bind and protect the macronuclear telomeres from the ciliates *Oxytricha* and *Euplotes.* Since the most extended regions of conservation fall within the DNA-binding domains of the telomere-binding proteins, when rTP was first identified it was predicted to be another structural telomere-binding protein. However, subsequent research demonstrated that rTP transcripts accumulate only during DNA replication and the rTP protein localizes to the sites of DNA replication within *Euplotes* macronuclei. We have now expressed rTP in a heterologous expression system and have examined the DNA-binding properties of the recombinant protein. We show that rTP binds specifically to the G-strand of *Euplotes* telomeric DNA and hence has some of the same DNA-binding characteristics as the *Euplotes* and *Oxytricha* telomere-binding proteins. However, other aspects of rTP binding are unique. In particular, the protein exhibits a very high off-rate and can bind double-stranded DNA as well as internal tracts of telomeric sequence. We conclude that rTP and the telomere-binding proteins are members of a class of proteins that have a conserved DNA-binding motif tailored to bind the G-strand of telomeric DNA. However, the unique DNA-binding characteristics of rTP indicate that the protein has evolved to fulfil a specialized role during telomere replication.

The telomeres of eukaryotic chromosomes are nucleoprotein complexes that contain repeated sequence DNA and specialized telomere-binding proteins (*1*). The telomeric DNA usually consists of tandem repeats of a simple G-rich sequence, and in some (maybe all) organisms, the extreme 3′ terminus of the DNA is single-stranded (*2*, *3*). The ciliates *Oxytricha* and *Euplotes* have very short telomeres that consist of 20 or 28 bp of G_4T_4•C_4A_4 sequence with the G-strand extending an additional 16 or 14 nucleotides beyond the C-strand to form a 3′ overhang (*4*). Much of telomere function is mediated by proteins that bind specifically to either the double- or the single-stranded regions of the telomeric DNA. These proteins form protective complexes that prevent degradation or fusion of chromosome ends (*5*), regulate telomerase access to the DNA terminus (*6*, *7*, *8*) and organize complex telomeric chromatin structures that regulate both telomere length and expression of telomere adjacent genes (*9*, *10*).

Double-stranded telomere-binding proteins such as Rap1, TRF1, and Taz1 bind along the duplex region of the telomere and participate in many different aspects of telomere function. For example, Rap1 from *S. cerevisiae* not only plays a key role in organizing telomeric chromatin structure but also regulates telomere length, chromosome stability, and telomere position effects (*11*). Although Taz1 from *S. pombe* and the human telomeric repeat factor TRF1 share little sequence identity with Rap1, these proteins are probably functional homologs as they also influence telomeric chromatin structure and play a role in telomere length regulation (*10*, *12*, *13*, *14*, *15*). One common feature of Rap1, Taz1, and TRF1 is that they all contain Myb-type repeats in the DNA-binding domain (*13*, *15*, *16*). Thus, while this class of telomere-binding proteins appears to have evolved quite rapidly, some critical features such as the DNA-binding domain have been conserved.

Single-strand telomere-binding proteins are a diverse group of proteins that are involved in various aspects of telomere maintenance. For example, the yeast G-strand-binding proteins Cdc13 and Est1 both control telomere repeat addition by telomerase (*7*, *8*, *17*). Cdc13 also plays an important role in protecting telomere integrity by preventing C-strand degradation (*18*). The telomere-binding proteins from *Euplotes* and *Oxytricha* are two other proteins that are known to play a protective role at the telomere. Both these proteins bind the G-strand overhang tightly but noncovalently, and form a protective cap over the end of the telomeric DNA (*5*, *19*). The two proteins recognize not only the sequence and structure of the G-strand but also the DNA terminus. The resulting nucleoprotein complex is extremely stable and protects the DNA from degradation. The *Euplotes* protein is a 51 kDa monomer (*20*) while the *Oxytricha* protein exists as a heterodimer with a 56 kDa α subunit and a 41 kDa β subunit (*21*). The α subunit shares extensive sequence identity with the *Euplotes* protein (*22*) and is largely responsible for the DNA-binding specificity (*23*).

While studying the *Euplotes* telomere-binding protein, we identified a 53 kDa *Euplotes* protein that has extensive sequence similarity to both the *Euplotes* telomere-binding protein and the α subunit of the *Oxytricha* protein (*22*). For reasons described below, this protein has since been named rTP or the replication Telomere Protein. rTP shares 35−36% amino acid identity and 54−56% similarity to the two telomere-binding proteins. The sequence identity is particularly striking because all of the extended regions of conservation fall within the N-terminal section that comprises the DNA-binding domain of the two telomere-binding

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proteins (*5*, *20*, *22*). Furthermore, two of the three amino acids that can be UV cross-linked to telomeric DNA in the *Oxytricha* protein are conserved in rTP (*24*). Given the extensive conservation between rTP and the DNA-binding domain of the two telomere-binding proteins, it is highly likely that rTP also binds telomeric DNA.

Despite their obvious sequence similarity, rTP and the *Euplotes* telomere-binding protein differ in a number of ways (*25*). First, rTP is much less abundant than the telomere-binding protein and, unlike the bulk of the telomere-binding protein, can be extracted from *Euplotes* macronuclei using only 150 mM NaCl. Second, the pattern of rTP and telomere-binding protein transcript accumulation is different. This is particularly apparent during macronuclear development, a process that takes place as part of the sexual stage of the *Euplotes* life cycle. Telomere-binding protein transcripts accumulate at multiple stages during development including at meiosis, at the time of new telomere addition, and throughout the subsequent period of macronuclear DNA replication (*26*). In contrast, rTP transcripts accumulate only during the period of macronuclear DNA replication (*25*). Finally, the two proteins differ in their cellular location. As might be expected for a protein that binds to all macronuclear telomeres, the telomere-binding protein is located throughout the macronucleus. However, this is not the case for rTP which is only present in the replication bands of developing and mature macronuclei (*25*). Replication bands are the visible "replication machines" that are responsible for DNA replication in hypotrichous ciliates (*27*). Thus, the localization of rTP to this organelle, together with the timing of rTP expression, strongly suggests that rTP is somehow involved in DNA replication.

We have now expressed rTP in a heterologous expression system and have examined the DNA-binding properties of the recombinant protein. We show here not only that rTP binds specifically to telomeric DNA but also that a number of its DNA-binding properties are similar to those of the *Euplotes* and *Oxytricha* telomere-binding proteins. However, we also show that rTP has some unique DNA-binding characteristics that are not shared by either telomere-binding protein. In particular, rTP has an extremely high off-rate, shows no preference for the DNA terminus, and binds to double-stranded DNA. Our findings indicate that although the telomeric DNA-binding motif has been conserved between rTP and the two telomere-binding proteins, rTP has evolved to fulfill a very different function at the telomere.

EXPERIMENTAL PROCEDURES

Expression of rTP in S. cerevisiae. The rTP expression vector was created using a novel expression system developed by Dr. Judith Berman. The system relies on recombination between the bacterial vector pRSET (Invitrogen) and pSE402. Yeast GALset, pSE402 is a 6.473 kb *Saccharomyces cerevisiae* expression vector that contains the selectable LEU2 gene and a CEN marker. Expression of the foreign gene is driven by the inducible GAL10 promotor.

The entire 1.6 kb gene encoding rTP (*22*) was cloned in-frame into pRSETA as a *Bam*HI−*Pst*I restriction fragment. This places the first codon of rTP immediately downstream of a his_6-tag. Both the resulting clone, pDLC11, and pSE402 were linearized and cotransformed (*28*) into *S. cerevisiae* strain INVSc-1 (Invitrogen). The cells were then plated on SD minimal medium lacking leucine. Individual transformants were isolated; the plasmid was rescued using the boiling STET method (*29*) and used to transform *E. coli* DH5α. DNA from a correct clone, as analyzed by restriction analysis, was then retransformed into INVSc-1. To induce rTP expression, the resulting strain, DLY-4, was grown in modified SC medium (6.7 g of yeast nitrogen base, 50 mL of glycerol, and 1 g of glucose per liter) to a density of approximately 0.2 OD_{260}. Galactose was added to a final concentration of 2%, and the cells were grown for an additional 3 h. Uninduced controls were grown under identical conditions in the absence of galactose.

Crude extracts were prepared essentially as described by Dunn and Wobbe (*30*). Cells were suspended in 0.3 M NH_3SO_4, 20 mM Tris, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, and 1× protease inhibitors, and vortexed with 0.45 mm glass beads. The 100× protease inhibitor solution consisted of 10 μg/mL chymostatin, 200 μg/mL aprotinin, 100 μg/mL pepstatin A, 110 μg/mL phosphoramidon, 720 μg/mL E-64, 50 μg/mL leupeptin, 250 μg/mL antipain, 10 mM benzamidine, and 10 mM sodium metabisulfite. The cells were vortexed for 1 min and then placed on ice for 1 min; this cycle was repeated 3 or 4 times. Cell breakage was monitored by microscopy as most rTP activity was obtained when 60−70% of the cells were broken. The broken cells were incubated at 4 °C for 30 min to extract the rTP, the cell debris was removed by centrifugation at 10 000 rpm for 1 h, and rTP was then isolated from the crude extract.

Isolation of rTP and Telomere-Binding Protein. rTP-containing yeast extracts were dialyzed against prechilled wash buffer (0.01 M $NaHPO_4$, pH 7.2, 0.15 M NaCl, 10% glycerol, 1 mM PMSF, and 1× protease inhibitor solution) and then applied to a HiTrap affinity column (Pharmacia) that had been charged with 0.1 M $NiSO_4$ and equilibrated with 5 column volumes of wash buffer. rTP was eluted from the column with 3 column volumes of wash buffer containing 0.5 M imidazole. The elute fractions were dialyzed against prechilled wash buffer to remove the imidazole and then frozen at −80 °C. rTP-containing fractions were identified by Western blotting using affinity-purified anti-peptide antibody made against the central nonconserved region of rTP (*25*). *Euplotes* telomere-binding protein was isolated as previously described (*20*).

Preparation of Oligonucleotides. Oligonucleotides were synthesized, purified and 5′-end-labeled as previously described (*20*). Blunt-end duplexes were made by annealing a 2-fold excess of nontelomeric 10mer to a complementary nontelomeric sequence at the 3′ end of the relevant C-strand oligonucleotide. Klenow was then used to extend the 10mer to the end of the C-strand oligonucleotide. Nt_{10} was hybridized to ^{32}P-labeled CA_{52} or $rTet_{52}$ to make the GT_{52} and Tet_{52} duplexes; $NT2_{10}$ was hybridized to Luc to make the Luc duplex.

Mobility Shift Assays. Mobility shift assays were performed as previously described (*20*). The 20 μL binding reactions contained 0.1−0.01 pmol of end-labeled oligonucleotide, 5−10 μL of rTP-containing extract or telomere-binding protein, 1 μg of autoclaved calf-thymus DNA, and TE or TE + 15 mM EGTA. The radiolabeled oligonucleotide was boiled for 2 min prior to adding the calf thymus DNA and rTP or telomere-binding protein in order to remove any secondary structure.The rTP-binding reactions were

incubated for 30 min at 37 °C, while the telomere-binding protein reactions were incubated for 1 h at 37 °C. After binding was complete, 0.5 volume of 30% glycerol, 0.25% bromphenol blue, and xylene cyanol was added, and the reactions were electrophoresed on 10% nondenaturing polyacrylamide gels at 15 mA in 0.5× TBE. To perform the competition experiments, cold competitor DNA or RNA was mixed with the 5′-end-labeled GT_{52} prior to rTP addition. The amount of DNA−protein complex formed in the presence and absence of competitor was determined using a PhosphorImager (Molecular Dynamics).

Derivation of the Equilibrium Binding Constant. A constant amount of partially purified rTP (10 μg total protein as determined by Bradford assay) was incubated with increasing amounts of GT_{52}. Each binding reaction was done in triplicate. The resulting rTP·GT_{52} complexes were then separated from free DNA using mobility shift gels, and the amount of bound versus free DNA was measured using a PhosphorImager. The amount of complex formed was plotted relative to the amount of free DNA, and the plot was used to determine the free DNA concentration at half-saturation. Since $K_D = ([\text{protein}]_{free} \cdot [\text{DNA}]_{free})/[\text{complex}]$ for a complex with a 1:1 molar ratio of protein to DNA, at half-saturation the amount of free protein and the amount of complex are equal so the K_D is equal to the concentration of free DNA.

To accurately convert the pixel volume measurements obtained with the PhosphorImager to molar concentrations, a standard curve was generated for each experiment by loading known quantities of ^{32}P-labeled GT_{52} on a gel and then exposing the gel to the same PhosphorImager cassette as the gel containing the titration curve. The pixel volume was then plotted versus DNA concentration, and the slope of the standard curve was determined by linear regression. The pixel volume measurements obtained for the DNA−protein complexes were then divided by the slope of the standard curve in order to determine the molar concentration of each complex. The K_D was calculated using several different protein preparations containing varying amounts of rTP.

Derivation of the Relative Binding Constants by Competition. The relative K_D for rTP binding to the GA_{32}, Tet_{52}, and Hum_{52} oligonucleotides was determined by competition analysis; 0.05 pmol of ^{32}P-labeled GT_{52} was incubated with a constant amount of partially purified rTP (approximately 10 μg of total protein), in the presence of an increasing amount of competitor oligonucleotide (15 concentrations spanning 3 log units). The final volume of all binding reactions was 20 μL. The amount of complex remaining was determined using a PhosphorImager (Molecular Dynamics). The relative dissociation constant was calculated using the equation:

$$\frac{[\text{protein}\cdot\text{DNA}]}{[\text{protein}]_{total}} = \frac{(1 - f_{easy} - f_{poor})[\text{DNA}]}{[\text{DNA}] + K_D(1 + [\text{competitor}]/K_c)} + f_{poor}$$

where K_C represents the relative K_D of the competitor in question, f_{easy} represents a normalization factor that accounts for the fraction of the protein that was readily competed by the competitor DNA, and f_{poor} represents a normalization factor for the fraction of rTP−GT_{52} complexes that were competed poorly. The normalization factor was necessary as the competitor oligonucleotides were only able to efficiently compete ∼70% of rTP binding. Given that a number of different rTP−GT_{52} complexes are observed in mobility shift gels, it is not surprising that there was variation in the efficiency with which different populations of complex were competed.

Data Equation Fitting. To calculate the K_D and relative K_D values for each oligonucleotide, the data were fit to their respective equations by the nonlinear least-squares approach using Excel 5.0 (Microsoft Corp.) and the FIT.XLM program (WindowChem Software, Fairfield, CA).

Off-Rate Measurements. Off-rates were determined essentially as described by Cardenas et al. (1993) (*31*). A 400 μL reaction containing rTP or telomere-binding protein, 2 pmol ^{32}P-labeled GT_{52}, and 20 μg of calf thymus DNA was incubated at room temperature for 10 min. Two 20 μL samples were then removed from the reaction, and one was loaded immediately onto a running gel. The other sample was loaded on the same gel 10 or 30 min later when the challenge experiment was completed. The challenge experiment was initiated by adding a 100-fold excess (200 pmol) of unlabeled GT_{52} or Luc to the main reaction. Twenty microliter samples were then removed at successive time points and loaded onto the running gel. The amount of radiolabeled DNA−protein complex was determined using a PhosphorImager.

RESULTS

Expression of rTP in Saccharomyces cerevisiae. When we tested the DNA-binding specificity of native rTP isolated from *Euplotes* replication bands, the protein did not bind telomeric DNA (*25*). However, we suspected that this lack of binding might be artifactual because telomere-binding protein that was coextracted from the replication band was also unable to bind G-strand oligonucleotides. To circumvent this potential isolation problem as well as the very low abundance of rTP in *Euplotes* cells, we chose to express rTP in a heterologous expression system. Attempts to produce soluble protein in *E. coli* and *Baculovirus* were unsuccessful, but we were able to obtain his_6-tagged rTP using a *Saccharomyces cerevisiae* expression system. The 1.6 kb gene encoding rTP was cloned into the yGalset vector, pSE402, and transformed into *S. cerevisiae*, and rTP expression was induced with galactose. Following induction, rTP was isolated by breaking the yeast cells and extracting them with 0.3 M ammonium sulfate. The crude extract was then applied to a HiTrap chelating column. As shown in Figure 1, the extract from galactose-induced cells contained a protein that was specifically recognized by antibody to rTP while the extract from uninduced cells did not. The protein recognized by the antibody was ∼54 kDa, the estimated size of the rTP−pSE402 fusion protein. The rTP antibody also detected a less intense band of ∼35 kDa which appeared to be a degradation product of the 54 kDa protein.

Although the HiTrap affinity matrix yielded protein preparations that were significantly enriched for rTP, the protein was only partially pure. The incomplete purification was not surprising because a number of yeast proteins are known to bind nonspecifically to the HiTrap matrix. This problem was exacerbated by the low level of rTP expression.

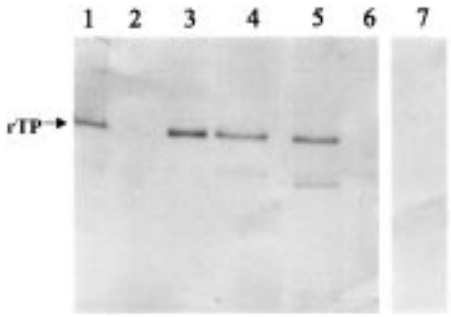

FIGURE 1: Western blot showing expression of rTP in *S. cerevisiae*. Lane 1, positive control showing *Baculovirus*-expressed rTP. Lanes 2−4, fractions from the HiTrap affinity purification. Lane 2, wash fraction. Lanes 3 and 4, the first two rTP-containing fractions eluted with 0.5 M imidazole. Lane 5, crude yeast extract from galactose-induced cells. Lanes 6 and 7, crude yeast extract from uninduced cells. The blot was probed with an affinity-purified anti-peptide antibody to rTP.

Table 1: Sequence of the Oligonucleotides Used To Examine the DNA-Binding Specificity of rTP

GT_{14}	$T_4G_4T_4G_2$
GT_{22}	$T_4G_4T_4G_4T_4G_2$
GT_{30}	$T_4G_4T_4G_4T_4G_4T_4G_2$
GT_{52}	$^{a}CG_2CT_2A_2GCG_4T_4G_4T_4G_4T_4G_4T_4G_4T_4G_2$
GT_{46}	$CG_2CT_2A_2GCG_4T_4G_4T_4G_4T_4G_4T_4G_4$
GA_{32}	$A_4G_4A_4G_4A_4G_4A_4G_4$
TG perm	$G_4T_4G_4T_4G_4T_4G_4T_4$
CA_{38}	$C_4A_4C_4A_4C_4A_4C_4GCT_2A_2GC_2G$
CA_{52}	$C_2A_4C_4A_4C_4A_4C_4A_4C_4A_4C_4GCT_2A_2GC_2G$
Hum_{52}	$CG_2CT_2A_2GCT_2AG_3T_2AG_3T_2AG_3T_2AG_3T_2AG_3T_2AG_3T_2AG_3$
Tet_{52}	$CG_2CT_2A_2GCT_2G_4T_2G_4T_2G_4T_2G_4T_2G_4T_2G_4T_2G_4$
$rTet_{52}$	$A_2C_4A_2C_4A_2C_4A_2C_4A_2C_4A_2C_4A_2C_4GCT_2A_2GC_2G$
$3'NtGT_{52}$	$G_4T_4G_4T_4G_4T_4G_4T_4G_4T_4G_2CGA_2T_2CG_2C$
$5'3'NtGT_{52}$	$CG_2CT_2A_2GCG_4T_4G_4T_4G_4T_4G_4T_4G_4T_4G_2CGA_2T_2CG_2C$
Luc	$TA_4G_2C_2A_2GA_2G_3CG_2A_3GTC_2A_3T_2GTA_2T_3CATAGAT_2ATAC$
Nt_{10}	$CG_2CT_2A_2GC$
$Nt2_{10}$	$AT_4C_2G_2GT$

[a] Nontelomeric sequences are in small capitals.

Fortunately, the column did remove almost all of the native yeast G-strand-binding proteins (see next section).

rTP Binds to the G-Rich Strand of Euplotes Telomeric DNA. To test whether rTP can bind to telomeric DNA, rTP-containing fractions from the HiTrap column were analyzed for DNA-binding activity using mobility shift assays. When rTP-containing extracts were incubated with a pair of complementary oligonucleotides annealed to mimic a natural *Euplotes* telomere (GT_{52}·CA_{38}, see Table 1), a group of strong band shifts was observed (Figure 2A, lane 1). DNA−protein complexes were also observed when the extract was incubated with a single-stranded oligonucleotide (GT_{52}) that

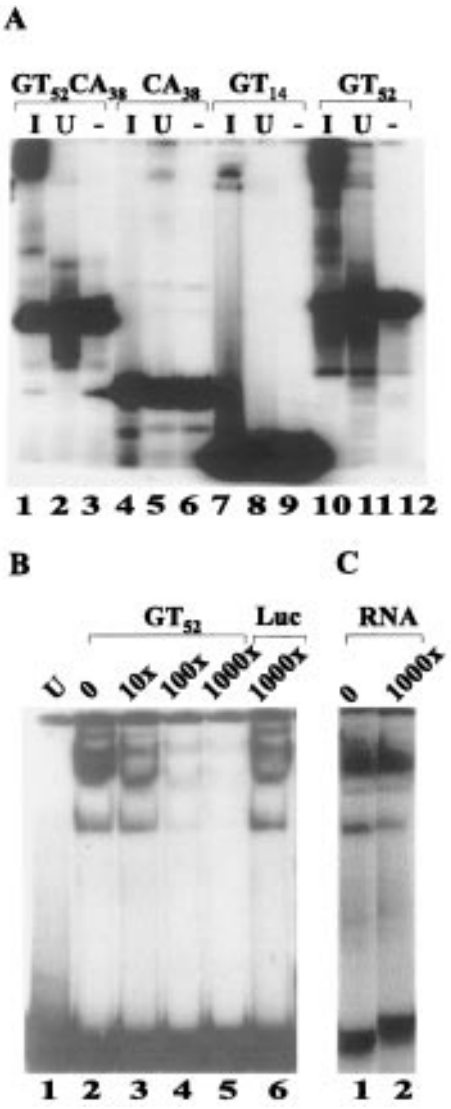

FIGURE 2: Binding of rTP to telomeric and nontelomeric DNA. (A) Mobility shift gel showing the migration of various telomeric oligonucleotides in the presence of affinity-purified yeast extract from induced (I) and uninduced (U) cells or in the absence of yeast extract (−). Lanes 1−3, the duplex GT_{52}·CA_{38} that mimics wild-type telomeres. Lanes 4−6, the C_4A_4 oligonucleotide CA_{38}. Lanes 7−9, the short G_4T_4 oligonucleotide GT_{14}. Lanes 10−12, the longer G_4T_4 oligonucleotide GT_{52}. The sequences of the oligonucleotides are shown in Table 1. (B) Mobility shift gel showing that binding of rTP to ^{32}P-labeled GT_{52} is competed by addition of excess cold GT_{52} (lanes 2−5) but not cold Luc oligo (lane 6).The molar excess of cold competitor added to each binding reaction is shown at the top of each lane. 100-fold excess represents 5 μM DNA. Lane 1 contains extract from uninduced cells. (C) Mobility shift gel showing that binding of rTP to ^{32}P-labeled GT_{52} is not competed by 1000-fold excess (50 μM) yeast RNA.

had the same length of G_4T_4 sequence as the G-strand of a *Euplotes* telomere (lane 10). In contrast, incubation of an equivalent amount of extract from uninduced cells with these same oligonucleotides resulted in little specific complex formation (lanes 2 and 11). If the rTP-containing extracts were treated with proteinase K prior to gel electrophoresis, all band shifts were abolished (data not shown).

The rTP-containing extracts demonstrated a strong preference both for the G-strand of the telomeric DNA and for an extended length of DNA. No band shifts were observed when the extract was incubated with an oligonucleotide (CA_{38}) that mimicked the C-strand of a *Euplotes* telomere

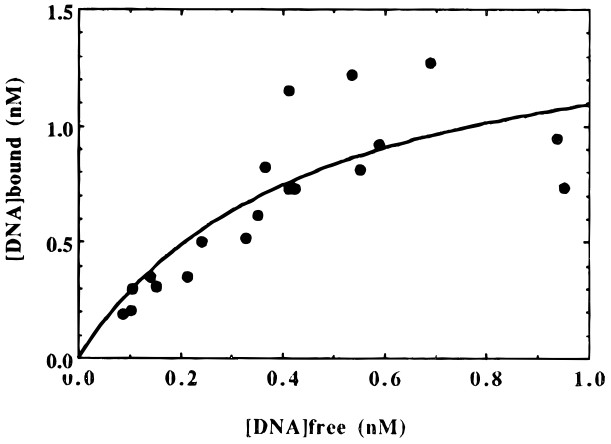

FIGURE 3: Binding curve for rTP binding to GT_{52}. The plot shows the quantity of bound versus free DNA that accumulated when various amounts of labeled GT_{52} were incubated with a set amount of rTP.

(lane 4), and only very weak complex formation was observed with a short oligonucleotide (GT_{14}) that mimicked the length and sequence of the natural 3′ overhang (lane 7). In competition experiments, binding to ^{32}P-labeled GT_{52} was not competed by the addition of a 1000-fold excess of a cold nontelomeric oligonucleotide (Luc) (Figure 2B, lane 6) or a 1000-fold excess of total yeast RNA (Figure 2C, lane 2), but efficient competition was observed by the addition of a 100-fold excess of cold GT_{52} (Figure 2B, lane 4). In all experiments where rTP-containing extracts gave rise to strong band shifts, incubation of the same oligonucleotides with extract from uninduced cells resulted in little or no complex formation. In cases where the uninduced cell extract gave rise to weak band shifts, these could easily be competed away with a nontelomeric oligonucleotide (data not shown). From these results, we conclude not only that rTP binds to *Euplotes* telomeric DNA, but also that the protein has a strong preference for the telomeric G-strand.

Measurement of the Equilibrium Binding Constant. The binding affinity of rTP for the oligonucleotide GT_{52} was determined by measuring the amount of specific rTP−DNA complex formed as a function of DNA concentration. This titration method, where the protein concentration is kept constant and the DNA concentration is varied, has the advantage that one does not need to know the concentration of the protein that is being studied (*32*). Assuming that there is a 1:1 ratio of rTP to DNA in the complex, the K_D is equal to the concentration of free DNA at half-saturation of the protein. To determine how much rTP−DNA complex was formed at different GT_{52} concentrations, the DNA−protein complexes were separated from the free DNA using mobility shift gels, and the amount of bound versus free DNA was determined using a PhosphorImager. The data were plotted using the nonlinear least-squares fit approach to calculate the curve that best fit the data. A representative binding curve is shown in Figure 3. The best theoretical curve was obtained when we used a protein to DNA ratio of 1:1. The resulting K_D was $(0.5 \pm 0.2) \times 10^{-9}$ M. This K_D is an average for all the rTP−DNA complexes observed in a mobility shift gel and does not distinguish between the contributions made by each species. It is similar to the K_D that was determined for the *Oxytricha* telomere-binding protein binding to macronuclear DNA ($\sim 0.1 \times 10^{-9}$ M) or a 36 nucleotide telomeric oligonucleotide (19×10^{-9} M) (*32*).

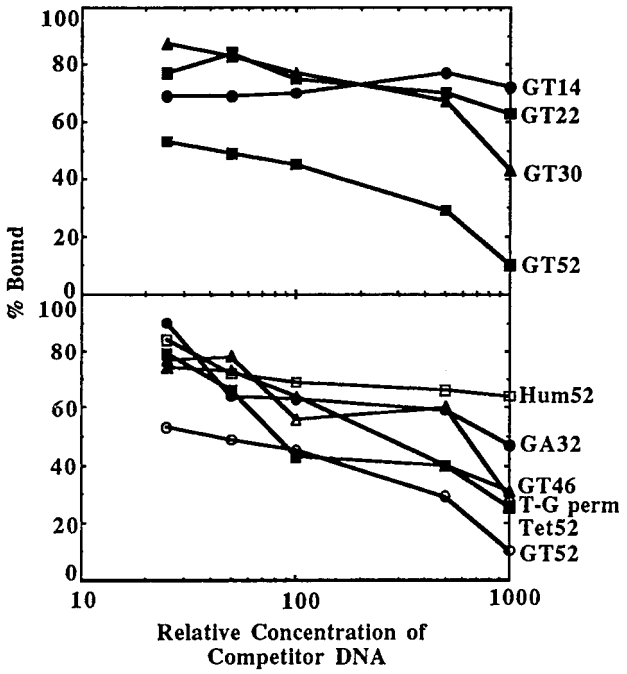

FIGURE 4: Competition for rTP binding by various telomeric oligonucleotides. The two plots show the relative amount of GT_{52} bound by rTP in the presence of 25−1000-fold excess (62.5 nM to 2.5 μM) of cold competitor DNA. 100% bound corresponds to the amount of labeled GT_{52} bound in the absence of competitor DNA.

DNA Length and Sequence Requirements for rTP Binding. To further investigate the DNA-binding properties of rTP, we performed a series of competition experiments to determine the relative preference of rTP for different lengths and sequence variations of telomeric G-strand DNA. In these experiments, rTP-containing fractions were incubated with the ^{32}P-labeled G-strand oligonucleotide GT_{52} in the presence of increasing quantities of cold competitor oligonucleotide. The samples were then separated on mobility shift gels and the amount of ^{32}P-labeled DNA−protein complex was determined using a PhosphorImager. The results of this experiment are shown in Figure 4.

The low affinity of rTP for the short GT_{14} oligonucleotide suggested that a minimum length of DNA was necessary for binding. To test this hypothesis, we performed a series of competition experiments using G-strand oligonucleotides of increasing length (GT_{14}, GT_{22}, GT_{30}, GT_{52}) as the competitor DNA. As shown in Figure 4, there was a clear correlation between increased length and an increase in ability to compete. This length requirement for rTP binding is similar to that observed for the *Euplotes* telomere-binding protein which needs ~30 nucleotides of G_4T_4 sequence to bind efficiently (*20*).

To learn more about the sequence requirements for rTP binding, we tested whether the protein would bind either human or *Tetrahymena* telomeric DNA or to a variation of the *Euplotes* telomeric sequence where the T's were replaced with A's. As shown in Figure 4, the protein bound the *Tetrahymena* telomeric sequence (Tet_{52}) quite well, but bound poorly to the human telomeric oligonucleotide (Hum_{52}). Binding to the G_4A_4 sequence (GA_{32}) appeared to be intermediate between binding to the human and *Tetrahymena* sequences. To determine the relative K_D values for binding

to Tet_{52}, Hum_{52}, and GA_{32}, we performed a more detailed competition analysis with 15 different concentrations of competitor DNA stretching over 3 log units (data not shown). When the resulting competition curves were fit using the same nonlinear least-squares approach as was used to calculate the K_D for rTP binding to GT_{52}, we obtained a relative K_D value of $(0.2 \pm 0.1) \times 10^{-9}$ M for Tet_{52}, $(97 \pm 57) \times 10^{-9}$ M for Hum_{52}, and $(15 \pm 9) \times 10^{-9}$ M for GA_{32}. Since these relative K_D values only take into account the population of rTP−GT_{52} complexes that could be competed most readily (~70% of the total, see Experimental Procedures), they may be somewhat low. Nonetheless, this result suggests that the presence of alternating blocks of G and T residues is important for binding, but the spacing between the blocks of G-residues is less critical.

rTP Binds to Duplex DNA. Initially we tried to determine whether rTP binds duplex DNA by annealing GT_{52} to CA_{52} and using the resulting duplex in competition experiments. However, these experiments gave variable results. Since we suspected that the variability was caused by misalignment of the G- and C-strand oligonucleotides, we devised a way to make perfect duplexes using primer extension. A 10 base nontelomeric oligonucleotide (Nt_{10}) was annealed to the nontelomeric DNA at the 3′ end of oligonucleotide CA_{52} and then extended with Klenow (see Table 1). Since extension of Nt_{10} did not go to completion, the reaction products consisted of a mixture of GT_{52}·CA_{52} duplexes and Nt_{10} hybridized to CA_{52}. However, no single-stranded G_4T_4 sequence was generated by this procedure. As rTP does not bind CA_{52} or Nt_{10} (data not shown), the presence of Nt_{10}·CA_{52} in the subsequent binding reaction did not matter.

As shown in Figure 5A, rTP bound efficiently to the Klenow generated GT_{52}·CA_{52} duplex (note, much less GT_{52} duplex was used in the binding reaction than single-stranded GT_{52}). Interestingly, the resulting complexes migrated differently from those observed with rTP and single-stranded GT_{52} (compare lanes 2 and 4). This indicates that the duplex DNA had not simply become denatured and the single-stranded GT_{52} bound by rTP. For comparison, we also tested the ability of the *Euplotes* telomere-binding protein to bind both GT_{52} and the GT_{52}·CA_{52} duplex. As expected, the telomere-binding protein recognized the single-stranded GT_{52} but did not bind the duplex DNA (Figure 5B). To investigate the sequence specificity of rTP binding to duplex DNA, we used the primer extension method to generate duplexes with the Tet_{52} and Luc oligonucleotides. As shown in lanes 7 and 10, rTP bound the *Tetrahymena* telomeric duplex quite efficiently but bound poorly to the nontelomeric (Luc) duplex. These results indicate that rTP can bind to double-stranded telomeric DNA with much the same sequence specificity as it binds single-stranded telomeric DNA. This is in direct contrast to the *Euplotes* and *Oxytricha* telomere-binding proteins which can bind only single-stranded DNA (*5*, *20*).

To assess how well rTP binds duplex versus single-stranded telomeric DNA, next we determined the binding constant for rTP binding to GT_{52}·CA_{52} duplexes. As described earlier, we measured the amount of specific rTP−DNA complex formed as a function of DNA concentration. Figure 6 shows the plot of bound versus free duplex DNA. The K_D that was determined from the best-fit plot is $(0.9 \pm 0.3) \times 10^{-9}$ M. Since this is very similar to the K_D obtained with single-stranded GT_{52} (0.5×10^{-9} M), we conclude that rTP binds single-stranded and double-stranded DNA with a similar high specificity.

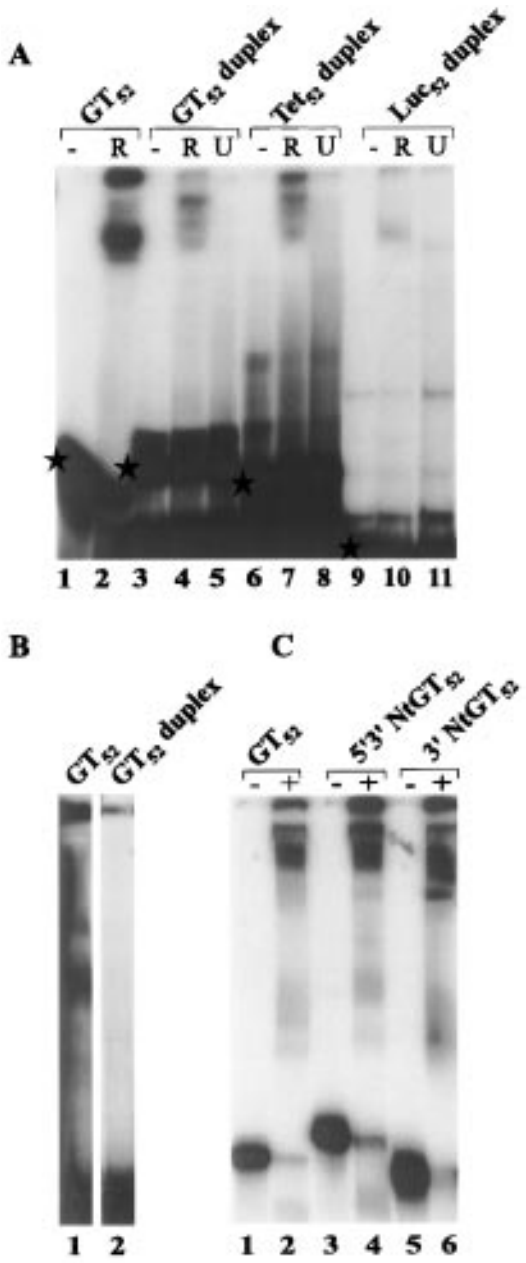

FIGURE 5: Binding of rTP to duplex DNA and internal telomeric tracts. (A) Mobility shift gel showing the migration of single-strand and duplex telomeric DNA in the absence of protein (−) or in the presence of affinity-purified rTP (R) or affinity-purified yeast extract from uninduced cells (U). Lanes 1 and 2, the single-stranded oligonucleotide GT_{52}; lanes 3−5, Nt_{10}·CA_{52} extended with Klenow to make the GT_{52} duplex; lanes 6−8, Nt_{10}·$rTet_{52}$ extended to make the Tet_{52} duplex; lanes 9−11, $Nt2_{10}$·Luc extended to make the Luc duplex. The stars mark the position of the unbound full-length duplexes. (B) Mobility shift gel showing binding of the *Euplotes* telomere-binding protein to single-stranded GT_{52} (lane 1) but not to the GT_{52}·CA_{52} duplex (lane 2). (C) Mobility shift gel showing rTP binding to single-strand oligonucleotides with nontelomeric DNA at the 5′ terminus (GT_{52}), at the 5′ and 3′ termini (5′3′ $NtGT_{52}$), or at the 3′ terminus (3′ $NtGT_{52}$). Lanes 1, 3, and 5, oligonucleotide alone (−). Lanes 2, 4, and 6, oligonucleotide plus rTP (+).

rTP Binds to Internal Tracts of G_4T_4 Sequence. The *Euplotes* telomere-binding protein displays great specificity for the DNA terminus of native *Euplotes* telomeres. In particular, the protein recognizes the G_2 dinucleotide present at the end of the G-strand (*20*). To determine whether rTP

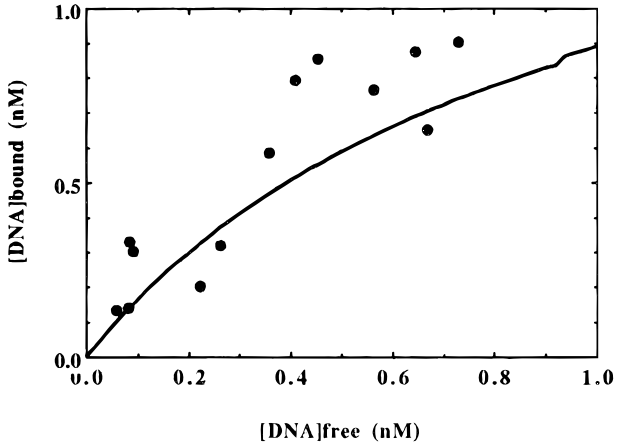

FIGURE 6: Binding curve for rTP binding to the GT_{52}·CA_{52} duplex. The plot shows the quantity of bound versus free DNA that accumulated when various amounts of labeled GT_{52}·CA_{52} were incubated with a set amount of rTP. The amount of full length duplex was quantified after it was generated using the primer extension method.

also has a preference for the natural DNA terminus, we performed competition experiments with G-strand oligonucleotides that ended at several different places in the G_4T_4 repeat sequence. As shown in Figure 4B, both the oligonucleotides GT_{46} and TG perm competed quite well for rTP binding. Since GT_{46} ends in a 3′ G_4 and TG perm ends in a 3′ T_4, this result demonstrated that the identity of the 3′-terminal nucleotides(s) is relatively unimportant for rTP binding. This finding led us to question whether rTP might be capable of binding internal tracts of G_4T_4 sequence.

To investigate this possibility, we determined whether rTP could bind G-strand oligonucleotides that had nontelomeric DNA at the 3′ end (3′$NtGT_{52}$, see Table 1) or at both the 3′ and 5′ ends (5′3′$NtGT_{52}$). As shown in Figure 5C, rTP bound equally well to 3′$NtGT_{52}$, 5′3′$NtGT_{52}$, and GT_{52}. In contrast, the *Euplotes* telomere-binding protein bound only GT_{52} (data not shown; see Price et al.) (*20*). From these results, we conclude that rTP is capable of binding internal tracts of G_4T_4 sequence. This conclusion is supported by data from methylation interference assays where methylation of telomeric DNA caused a general decrease in rTP binding, but no obvious footprint at the DNA terminus (R. Venkatesan, unpublished results). The ability of rTP to bind to internal tracts of G_4T_4 sequence may explain why in mobility shift assays we routinely see a group of shifted bands rather than a single band. Binding of the protein to different positions along the G-strand oligonucleotides could result in DNA−protein complexes of various mobilities.

rTP−DNA Complexes Exhibit a High Off-Rate. As might be expected of proteins that are designed to protect the telomere, both the *Euplotes* and *Oxytricha* telomere-binding proteins form extremely stable complexes with telomeric DNA (*21*, *32*, *33*, *34*). To investigate whether rTP−DNA complexes exhibit a similar stability, we used a series of challenge experiments to determine the off-rate of the protein. In these experiments, a 100-fold excess of cold competitor DNA was added to preformed ^{32}P-labeled GT_{52}−rTP complexes; samples were then removed at successive time points and loaded onto a running gel (*31*). The disappearance of the GT_{52}−rTP complex with time was then quantified.

When the challenge experiment was performed using GT_{52} as the cold competitor, it became apparent that the rTP−

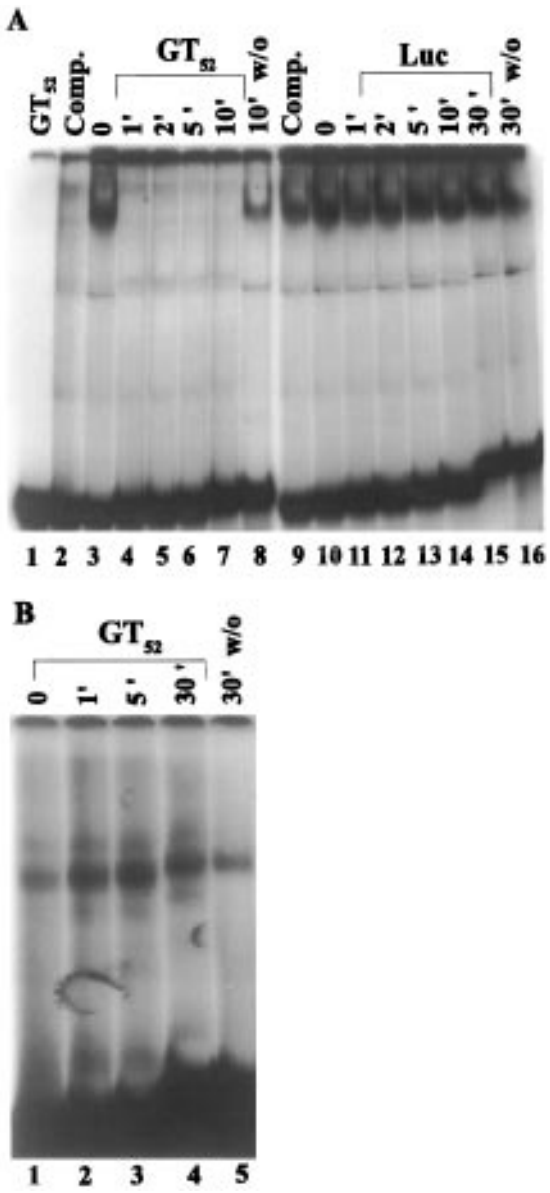

FIGURE 7: Kinetic stability of rTP−DNA complexes. (A) A 100-fold excess (5 nM) of cold GT_{52} or Luc was added to preformed rTP−[^{32}P]GT_{52} complexes. Samples were then removed and loaded on the running gel after a 1 min (lanes 4 and 11), 2 min (lanes 5 and 12), 5 min (lanes 6 and 13), 10 min (lanes 7 and 14), or 30 min challenge (lane 15). Lane 1, GT_{52} alone; lanes 2 and 9, controls showing a standard competition experiment where cold GT_{52} or Luc was mixed with the ^{32}P-labeled GT_{52} prior to rTP addition. Lanes 3 and 10, the amount of preformed complex present at the start of the challenge experiment. Lanes 8 and 16, the amount of preformed complex remaining after a further 10 or 30 min incubation in the absence of competitor. (B) The identical experiment to (A) except cold GT_{52} was added to preformed complexes of GT_{52} and *Euplotes* telomere-binding protein. Samples were removed and loaded on the running gel after a 1 min (lane 2), 5 min (lane 3), or 30 min challenge (lane 4). Lane 1 shows the amount of preformed complex present at the start of the challenge while lane 5 shows the amount remaining after a 30 min incubation in the absence of competitor DNA.

GT_{52} complex was not kinetically stable. As shown in Figure 7, lane 4, most of the labeled complex disappeared after only a 1 min challenge. This disappearance of the rTP−GT_{52} complex was not merely due to inactivation of the rTP during the 37 °C incubation because in parallel reactions where the competitor DNA was omitted, there was little decrease in the amount of complex even after a 10 min incubation (lane

8). Furthermore, when the experiment was repeated using a 100-fold excess of the nontelomeric Luc oligonucleotide, less than 2% of the preformed complex dissociated even after a 30 min challenge (Figure 7, lane 15).

For comparison, we also performed the challenge experiment with the *Euplotes* telomere-binding protein. As before, DNA−protein complexes were preformed with labeled GT_{52}, and a 100-fold excess of cold GT_{52} was used for the challenge. As anticipated, the telomere-binding protein−DNA complexes were extremely stable with 100% of the original complex remaining after a 30 min challenge (Figure 7B, lane 4). Very little dissociation was observed even after a 24 h incubation (data not shown). From these results, we conclude that rTP has a much higher off-rate than the telomere-binding protein. Note that this high-off rate is not inconsistent with the observed tight binding; it mearly places limits on the K_{on}.

DISCUSSION

Recent studies of yeast and vertebrate telomeres have revealed the presence of a surprising number of telomere proteins. While the precise function of many of these proteins is still unknown, it is clear that they act in concert to perform a variety of functions that are critical for telomere maintenance. For example, the yeast proteins Cdc13, Est1, and Rap1 all play an important role in regulating access of telomerase to the DNA terminus (*6*, *8*, *17*). Although telomeres from the ciliate *Euplotes* are much smaller and simpler than yeast or vertebrate telomeres, our studies indicate that they too incorporate multiple proteins. These proteins include the telomere-binding protein that binds and protects the 3′ single-strand overhang on mature macronuclear telomeres, and rTP, the replication telomere protein that binds both single- and double-stranded telomeric DNA. Yeast and vertebrate telomere proteins display a surprising lack of sequence conservation even between proteins that apparently share a common function. In contrast, rTP and the *Euplotes* and *Oxytricha* telomere-binding proteins are clearly members of a family of proteins that share a highly conserved DNA-binding motif. However, even this family of proteins has evolved significantly, as rTP exhibits some unique DNA-binding characteristics that are not shared by either telomere-binding protein. Presumably these novel properties are required for rTP to function during telomere replication.

Like the *Euplotes* and *Oxytricha* telomere-binding proteins, rTP exhibits a clear preference for the G-strand of *Euplotes* telomeric DNA. The protein binds tightly to oligonucleotides that contain G_4T_4 repeats but will not bind C_4A_4 repeats or to nontelomeric DNA. Interestingly, the DNA-binding specificity of rTP binding is slightly more relaxed than that of the *Euplotes* telomere-binding protein as rTP binds *Tetrahymena* telomeric DNA just as well as *Euplotes* telomeric DNA.

The overall similarity in the DNA-binding specificity of rTP and the two telomere binding proteins is not surprising given the high degree of sequence conservation between the N-terminal domains of rTP, the *Euplotes* telomere-binding protein, and the α subunit of the *Oxytricha* protein (*22*, *24*). X-ray crystallography of the *Oxytricha* telomere-binding protein has revealed that this N-terminal domain makes up one side of a deep DNA-binding cleft (Steve Schultz, personal communication). Further, a number of the amino acids that are conserved in both rTP and the *Euplotes* telomere-binding protein are a part of this deep cleft, and in the *Oxytricha* DNA−telomere−protein cocrystal, some of these amino acids make specific contacts with bases in the telomeric DNA. Thus, although the *Oxytricha* telomere-binding protein forms a heterodimer in the presence of DNA and the *Euplotes* telomere-binding protein binds as a monomer, it is highly likely that rTP and the *Euplotes* telomere-binding protein have a DNA-binding cleft that is structurally similar to that of the *Oxytricha* protein.

Despite the many similarities between rTP and the two telomere-binding proteins, rTP has some DNA-binding characteristics that are unique. These unique properties include a high off-rate, the ability to bind double-stranded DNA, and the lack of preference for a DNA terminus. The high off-rate is particularly striking because it is in such stark contrast to the extremely low off-rate exhibited by the two telomere-binding proteins. The speed with which rTP dissociates from telomeric DNA argues against this protein serving a structural or protective role at the telomere and suggests that the protein is involved in a more transient aspect of telomere biochemistry.

Our data concerning the ability of rTP to bind internal stretches of T_4G_4 sequence must be interpreted cautiously because, as was observed with the *Oxytricha* telomere-binding protein, even a slight misfolding of the recombinant rTP might reduce the end-binding specificity (*23*). Nonetheless, the ability to bind internal stretches of T_4G_4 sequence again suggests that rTP has a distinct function from that of the *Euplotes* and *Oxytricha* telomere-binding proteins. While their stringent end-binding specificity is almost certainly necessary for them to form a protective telomeric DNA−protein complex (*20*, *31*), this property would probably not be needed by a protein such as rTP that plays another role at the telomere.

The ability of rTP to bind double-stranded telomeric DNA is fascinating as both the native and recombinant telomere-binding proteins have an absolute requirement for single-stranded DNA (*20*, *26*). The crystal structure of the *Oxytricha* protein readily explains this requirement as there is clearly no room for a DNA duplex in the narrow DNA-binding cleft (S. Schultz, personal communication). Given this situation, one wonders whether the section of rTP that makes up the less conserved portion of the DNA-binding cleft is more flexible so under some conditions the cleft can accommodate a stretch of duplex DNA. Alternatively, a different region of the protein may be responsible for the duplex binding.

Just how the double-strand DNA-binding specificity is related to rTP function is intriguing. Given that our previous work suggested a role for rTP in DNA replication (*25*), one possibility is that rTP binds to the junction between the 3′ overhang and the double-stranded region of the telomere during a specific stage in telomere replication. A protein bound in this position could serve a number of purposes. For example, it might act like Cdc13 to prevent C-strand degradation (*18*), or it might determine the length of the G-strand overhang by protecting a set length of G_4T_4 sequence from nuclease digestion. Alternatively, it might form part of a telomeric replication initiation complex.

In conclusion, it is now clear that rTP and the previously identified *Oxytricha* and *Euplotes* telomeric end-binding

proteins share a similar DNA-binding motif. However, the unique DNA-binding characteristics of rTP strongly suggest that this protein has evolved to fulfil a cellular function that is quite different from that of the telomere-binding proteins. Given the other properties of rTP, this function most likely involves either the initiation or the completion of telomeric DNA replication.

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