Published in final edited form as:

Biochemistry. 2017 August 15; 56(32): 4210–4218. doi:10.1021/acs.biochem.7b00584.

Human CST Prefers G-Rich but Not Necessarily Telomeric Sequences

Robert A. Hom and Deborah S. Wuttke*

Department of Chemistry and Biochemistry, UCB 596, University of Colorado, Boulder, Colorado 80309, United States

Abstract

The human CST (CTC1–STN1–TEN1) heterotrimeric complex plays roles in both telomere maintenance and DNA replication through its ability to interact with single-stranded DNA (ssDNA) of a variety of sequences. The precise sequence specificity required to execute these functions is unknown. Telomere-binding proteins have been shown to specifically recognize key telomeric sequence motifs within ssDNA while accommodating nonspecifically recognized sequences through conformationally plastic interfaces. To better understand the role CST plays in these processes, we have produced a highly purified heterotrimer and elucidated the sequence requirements for CST recognition of ssDNA *in vitro*. CST discriminates against random sequence and binds a minimal ssDNA comprised of three repeats of telomeric sequence. Replacement of individual nucleotides with their complement reveals that guanines are specifically recognized in a largely additive fashion and that specificity is distributed uniformly throughout the ligand. Unexpectedly, adenosines are also well tolerated at these sites, but cytosines are disfavored. Furthermore, sequences unrelated to the telomere repeat, yet still G-rich, bind CST well. Thus, CST is not inherently telomere-specific, but rather is a G-rich sequence binder. This biochemical activity is reminiscent of the yeast t-RPA and *Tetrahymena thermophila* CST complexes and is consistent with roles at G-rich sites throughout the genome.

Graphical Abstract

The management of single-stranded DNA (ssDNA) is an essential element of several key cellular processes, including telomere maintenance, DNA replication, repair, and

*Corresponding Author: deborah.wuttke@colorado.edu. Phone: 303-492-4576. Fax: 303-492-8425.

Notes

The authors declare no competing financial interest.

recombination. Performing these functions requires both specific and nonspecific ssDNA binding activities.^{1,2} When ssDNA is transiently exposed during replication, repair, and recombination, it is managed primarily by RPA, a heterotrimeric protein that tightly yet nonspecifically recognizes ssDNA through multiple oligosaccharide/oligonucleotide/oligopeptide-binding (OB) fold motifs spanning its subunits. In this complex, RPA32 and RPA14 modulate the ssDNA binding activity of the main core binding protein RPA70.^{2,3}

In contrast, the long, conserved G-rich 3'-ssDNA overhang at telomeres is specifically recognized in most eukaryotes by the POT1-TPP1 complex, a component of the uniquely purposed shelterin complex.⁴⁻⁷ The recognition of ssDNA telomere sequences by human POT1 is achieved through a DNA-binding domain (DBD) comprised of two OB folds⁸ that bind single-stranded telomere sequence with low nanomolar affinity.⁸ TPP1 interacts with the C-terminal domain of POT1, providing a bridge to the double-stranded DNA telomere-binding factors while also increasing the avidity of POT1 for single-stranded telomeric sequences.^{4,7,9,10}

Curiously, single-stranded regions of telomeres in the budding yeast are not maintained by an analogous shelterin complex but are instead maintained through the action of a heterotrimer comprised of the proteins Cdc13, Stn1, and Ten1. Extensive structural and biochemical evidence revealed that the *Saccharomyces cerevisiae* Cdc13-Stn1-Ten1 complex contains a domain architecture that parallels that of RPA;^{11,12} however, the Cdc13 component possesses tight picomolar affinity and sequence specificity uniquely suited to the particular heterogeneity exhibited by yeast telomeres.^{11,13-15} This specificity, however, is not widely retained even within *Saccharomycotina*.¹⁶ On the basis of its structural homology with RPA but functions associated with telomeres, this complex has been renamed t-RPA for a telomere-specific RPA.^{17,18}

A heterotrimer similar to this yeast t-RPA has been identified in humans and other eukaryotes. Originally identified because of its role as an accessory factor for DNA polymerase α -primase in humans and named AAF for this activity,^{19,20} this heterotrimeric complex is comprised of three subunits, CTC1, STN1, and TEN1, and also appears to adopt an RPA-like domain organization²¹⁻²⁴ (Figure 1A). In particular, there are strong sequence and structural similarities between hSTN1-TEN1, yeast Stn1-Ten1, and RPA32-RPA14 proteins.²⁵⁻²⁹ CTC1 is more divergent, lacking readily identifiable sequence relationships with either RPA70 or Cdc13, but like those proteins is predicted to contain several OB fold motifs.^{12,30}

At telomeres, CST coordinates C-strand synthesis, which is stimulated after telomerase extension through interaction of CST with polymerase α -primase and reminiscent of a similar activity exhibited by yeast Cdc13.^{19,23,31-33} Inhibition of telomerase activity has also been proposed through CST preventing access of telomerase to free 3'-ssDNA ends or by directly interacting with the POT1-TPP1 complex.³⁴ Unlike POT1 or t-RPA, CST is not required for telomere end protection. Deletion of CTC1 in human cells leads to shortened telomere ends,^{21,22} and human cells depleted of STN1 or TEN1 exhibit defects in telomere maintenance.^{21,22} Deletion of CTC1 in mice results in early death due to complete bone

marrow failure, a consequence of rapid telomere shortening due to telomere replication defects.³⁵

CST also plays roles beyond telomere maintenance. Deletion of each CST component promotes replication fork stalling,^{21,36–38} while overexpression of the complex can allow cells to recover from DNA damage and replication stress.³⁹ At telomeres and other places in the genome, CST can rescue stalled replication forks.³⁶ The rates of DNA replication are affected by CST availability, as deletion of either STN1 or TEN1 results in decreased rates of origin firing for replication forks while overexpression of CST increases the rates of origin firing.^{21,36,38} Finally, mutations within CTC1 and STN1 have been associated with several rare human diseases such as dyskeratosis congenita and Coats plus syndrome, a pleiotropic disorder characterized by a range of abnormalities throughout the brain, bones, and gastrointestinal system as well as aging phenotypes.^{40–42} Several of these mutations do not appear to affect telomere length,⁴¹ suggesting these mutations exert their pathology through defects in the proposed global roles of CST.

These dual roles in telomere maintenance and throughout the genome are consistent with the observation that only 15–20% of CTC1 and STN1 foci localize to telomeres.²² When replication stress is induced, STN1 was found to localize to both telomeric and nontelomeric sequences, and through ChIP-Seq, STN1 was enriched at ~90% sequences containing repetitive sequences.⁴³ Many of these repetitive sequences contained G-rich repeats. These data suggest that CST is able to recognize a diverse range of ssDNA sequences.

Consistent with a role at telomeres, the CST heterotrimer has been shown to bind telomeric ssDNA tightly.^{22,34} Also analogous to RPA, CST appears to have two binding modes, a short, specific one and a longer, nonspecific mode.^{44,45} We focused on the specific binding mode, as the longer, nonspecific mode is likely achieved through plasticity at the protein–ssDNA interface due to the inherent flexibility of the ssDNA ligand. The precise minimal length for specific binding is not known. (TTAGGG)₃ has been reported to bind well, while (TTAGGG)₂ does not;²² the elements of sequence specificity have not been fully determined. To refine our understanding of what biochemical features CST inherently possesses, we have systematically investigated the ssDNA binding activity of the highly purified CST heterotrimer complex. We have found that CST is an effective ssDNA binder of both short telomeric sequence and nontelomeric oligonucleotides that are rich in G, but not necessarily rich in G-tract. These binding preferences support CST's proposed roles at G-rich sequences throughout the genome.

MATERIALS AND METHODS

Chemicals, Reagents, and Proteins.

All chemicals and reagents were purchased from Fisher Scientific unless otherwise indicated. All enzymes were purchased from New England Biolabs. All oligonucleotides were synthesized and obtained from Integrated DNA Technologies. The [γ -³²P]ATP was purchased through PerkinElmer. Prof. Carolyn Price (University of Cincinnati, Cincinnati, OH) generously provided the cDNA for full length human CTC1, STN1, and TEN1 (UniProt entries Q2NJK3, Q9H668, and Q86WV5, respectively).

Cloning of Human CST into a Baculovirus Expression System.

The N-terminal 2xFLAG-tagged version of CTC1 was subcloned into acceptor plasmid pACEBAC1 using the primers indicated below. The vectors were digested with SalI-HF and NotI-HF and transformed into DH5 α competent cells. N-Terminally His₆-tagged STN1 and TEN1 were subcloned into the donor plasmids of pIDK and pIDS. The vectors were digested with XhoI-HF and NheI-HF and transformed into pirHC competent cells: 2xFLAG CTC1 to pACEBAC1 forward primer, GGGTCGACAATGGACTACAAGGACGACGACG; 2xFLAG CTC1 to pACEBAC1 reverse primer, GGGCGGCCGCTTATTAACAGGAGGAAGCAAGGATC; His₆ Stn1 to pIDK forward primer, GGCTCGAGAATGCACCACCACCACCAC; His₆ Stn1 to pIDK reverse primer, GGGCTAGCTTATCAGAACGCTGTGTAGTAGTGCT; His₆ Ten1 to pIDS forward primer, GGCTCGAGAAATGTCTACTACCATCACCATCAC; His₆ Ten1 to pIDS reverse primer, GGGCTAGCTTACTACTGGCTGCCGCC.

The multigene plasmid containing 2xFLAG CTC1, 6x-His STN1, and 6x-His TEN1 was generated through Cre-Lox recombination of the parent plasmids. For a 20 μ L reaction, 0.5 μ g of CTC1 pACEBAC1, STN1 pIDK, and TEN1 pIDS were added to 1x Cre recombinase reaction buffer and 2 units of Cre recombinase. The reaction mixture was incubated at 37 °C for 1 h. The reaction mixture was then transformed into DH5 α competent cells and allowed to recover overnight at 37 °C. Cells were then plated on gentamycin, kanamycin, and spectinomycin resistant plates. Colonies were picked and grown overnight at 37 °C in 5 mL of Luria-Bertani broth (LB) with gentamycin, kanamycin, and spectinomycin antibiotics and mini prepped (Omega). All plasmids were fully sequenced to ensure the integrity of the clones.

This recombinantly generated CST plasmid was transposed into the MultiBac baculoviral DNA of EmBacY *Escherichia coli* cells. The transposition of the CST genes was implemented through a Tn7 transposition pathway. Cells were allowed to recover overnight and then plated on gentamycin, kanamycin, tetracyclin, isopropyl β -D-1-thiogalactopyranoside, and X-Gal plates. After 48–72 h, white colonies were picked and restreaked to ensure the desired CST construct was transposed into the bacmid. Colonies were again picked and grown overnight in 10 mL of LB with gentamycin, kanamycin, and tetracycline antibiotics, and CST MultiBac bacmid was then isolated for baculovirus generation.

Expression and Purification of the Human CST Protein.

Transfections of CST MultiBac bacmid were performed using Cellfectin II reagent (Invitrogen), according to the manufacturer's instructions. Amplification of baculovirus from P1 to P3 was performed by pipetting 2 mL of P1 virus to 15 mL of a suspension of 2×10^6 Sf9 cells/mL in Sf-900 III SFM medium (Gibco) into a 500 mL shaker flask at 27 °C for 72 h at 130 rpm. After 72 h, 45 mL of a suspension of 2×10^6 cells/mL mixture in Sf-900 III SFM medium was added and incubated at 27 °C for an additional 72 h at 130 rpm. Cells were spun down, and supernatant with virus was stored at 4 °C for up to 6 months. Using the generated CST MultiBac baculovirus stocks, 1 L of Sf9 cells at a density of 2×10^6 cells/mL in Sf-900 III SFM medium or ESF 921 medium (Expression Systems) was infected with baculovirus. The 1 L of cells was incubated at 27 °C for 48–96 h at 130 rpm. The cells were

spun down at 3000 rpm and gently resuspended in 1x PBS to remove materials that would decrease the level of binding of the hexahistidine tag to the nickel beads. The cells were spun back down at 1500 rpm, frozen in liquid nitrogen, and stored at -70°C until protein purification was performed.

Protein Purification of the CST Complex.

A pellet from a 1 L growth of CST was resuspended with 25 mL of lysis buffer [50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 15 mM β -mercaptoethanol (β ME), 10% glycerol, 10 mM imidazole, and a complete EDTA free protease inhibitor tablet (Roche)] at 4°C . Cells were lysed by Dounce homogenization and clarified by being spun at 18000 rpm for 40 min at 4°C . The CST complex was affinity-purified by passing the clarified CST cell extract over a gravity flow column packed with Ni-NTA resin at 4°C . The resin was washed with 25 column volumes of lysis buffer. The CST complex was eluted off the beads with 20 mL of elution buffer [50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 15 mM β -mercaptoethanol (β ME), 10% glycerol, and 150 mM imidazole]. The flow-throughs were concentrated to 2 mL in a Vivaspin 10000 molecular weight cutoff concentrator (Sartorius). The CST was further purified on a HiLoad 16/600 Sephadex 200 (S200) gel filtration column in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 15 mM β ME, and 5% glycerol, concentrated down, flash-frozen, and stored at -70°C . Typical yields of the recombinant CST heterotrimer complex were in the range of 15 nmol/L.

Double-Filter Binding Experiments.

Apparent binding constants ($K_{D,app}$) were determined using double-filter binding. Binding buffer consisted of 50 mM Tris (pH 7.4), 150 mM KCl, 150 mM LiCl, 1 mM dithiothreitol (DTT), 0.1 mg/mL bovine serum albumin, and 15% glycerol. These conditions were found to help maintain the stability of the CST heterotrimer complex, which is less stable under low-salt conditions. ssDNA oligonucleotides (Integrated DNA Technologies) were resuspended in nuclease free water and 5'-end-labeled using [γ - ^{32}P]ATP with T4 polynucleotide kinase. G25 spin columns (GE Healthcare) were used to remove free ATP, and labeled ssDNAs were diluted to a final concentration of 2x in binding buffer. Final ssDNA concentrations were held at a fixed concentration below the $K_{D,app}$ between 50 and 300 pM. Double-filter binding experiments were performed with a 96-well vacuum manifold dot blot apparatus (GE Healthcare), nitrocellulose, Hybond XL filters (charged nylon; GE Healthcare), and 3 mm chromatography filter paper (GE Healthcare). All filters were presoaked in 50 mM Tris (pH 7.5), and protein-ssDNA complexes were preincubated in a 96-well plate on a cold block in ice for incubation times varying from 2 to 18 h, with no impact observed of incubation times in this range on the measured $K_{D,app}$. The 96-well MultiScreens were placed under vacuum, and 20 μL of protein and ssDNA were loaded onto the filter paper in the wells with a multichannel pipet. The filter paper was washed three times with 100 μL of 50 mM Tris (pH 7.5), dried with heated air, and transferred to phosphorimaging screens (GE Healthcare). The phosphorimaging screens were scanned after 2–3 days on either a TyphoonXL or a FLA9500 phosphorimager. Data were quantitated by ImageQuantTL (GE Healthcare) and processed with Microsoft Excel, and Kaleidagraph (Synergy Software) was used to both plot and fit the data for a modified $K_{D,app}$ as described previously.¹³

RESULTS

The CST Complex Minimally Binds a Telomeric Sequence of 18 Nucleotides.

We have optimized the recombinant protein expression and purification of the human CST complex (hereafter termed CST) from insect cells (Sf9) through the use of the MultiBac multiexpression system.^{46,47} The MultiBac system is tailored for the expression of multiple proteins by multigene expression on the same baculovirus. This system has greatly increased our protein yield and simplified the process of recombinant expression of the CST complex when compared to co-infecting the Sf9 cells with three separate viruses. Additionally, we use a robust, two-step protocol to purify the CST complex through both affinity purification and size exclusion chromatography (Figure 1B). This protocol produces highly purified protein devoid of any higher-order aggregates and is suitable for quantitative binding analysis.

To investigate how CST interacts with telomeric and related ssDNAs, we used a high-throughput double-filter binding assay.⁴⁸ We found that the $K_{D,app}$ for CST bound to the 18mer (TTAGGG)₃ was 21.6 ± 1.0 nM (Figure 2). The $K_{D,app}$ value from our double-filter binding assay falls within the range of the previously reported $K_{D,app}$ values of 18.8 nM²² and 0.6 nM.³⁴ This discrepancy in previously reported $K_{D,app}$ values is likely due to the difference in the oligonucleotide sequences used in each experiment and specific differences in binding assay conditions.

Investigation of CST sequence specificity required first defining the minimal binding length, as single-stranded oligonucleotides containing additional nucleotides have the capacity to structurally rearrange to meet the binding requirements.⁴⁹ Previous work suggests that CST can bind to oligonucleotides containing multiple repeats of telomeric oligonucleotides with no change in the $K_{D,app}$ value until a sequence of two repeats is reached.^{22,34} To pinpoint the minimal binding length, we systematically removed nucleotides from the 5'-end and the 3'-end of the 18mer (TTAGGG)₃ sequence and determined the changes in binding affinity. Removing up to two nucleotides from the 5'-end showed modest changes in binding affinity, whereas removal of three nucleotides to create a 15mer led to an ~6-fold weaker binding affinity (Table 1). In contrast, removing one and two guanines from the 3'-end revealed an ~2–3-fold change in $K_{D,app}$ values and a more dramatic ~14-fold reduction when all three guanines are removed from the 3'-end of the 18mer (TTAGGG)₃ to make a 15mer. Thus, the minimal length for binding of CST to the telomeric sequence lies in the range of 16–18 nucleotides. Furthermore, the significant loss of binding affinity upon removal of the guanines at the 3'-end suggests that the 3'-end guanines are important for binding of CST to telomeric sequences, prompting us to investigate the specificity of binding.

The Specificity Profile for CST Reveals a Preference for G-Rich Sequences.

Characterization of the cellular activities of CST suggests roles in telomere maintenance in addition to a genomewide role in DNA damage and replication response.^{33,36,38} Understanding the intrinsic binding specificity exhibited by CST is needed to understand CST's role in these different activities. To characterize the binding preferences of CST, we first compared binding to short random sequences, learning that CST discriminates for

telomeric sequence by 3.6–6-fold (Table 2). Surprisingly, we found that the poly-T (TTTTTT)₃ sequence exhibited no detectable binding, indicating the limits to which CST can accommodate ssDNA and differentiating its action from that of RPA that binds poly-T oligonucleotides quite well in a length-dependent manner.⁵⁰

To test which contacts within the ssDNA are important for CST binding, we measured the specificity profile for the cognate (TTAGGG)₃ ligand. Each single-nucleotide position of the (TTAGGG)₃ was changed to its complement sequence, and the $K_{D,app}$ value for each altered ssDNA ligand was measured (Table 2 and Figure 3). The ratio of the $K_{D,app}$ value for the noncognate pool and the free energy of binding to the cognate (TTAGGG)₃ ligand reveal which bases are specifically recognized, and values ranged from no change to 17-fold. We found that guanines are the most discriminated for, with G5, G11, G12, and G16 making the largest contributions to the free energy of binding for CST. We set a threshold of significance for $G > 0.5$ kcal/mol, corresponding to a 3-fold difference in binding affinity. Using this threshold, we found that the complement changes at positions 2, 4, 5, 10–12, 16, and 17 result in significant changes in free energy. These data define a recognition profile for CST to (TTAGGG)₃ of **TTAGGGTTAGGGTTAGGG**, with specifically recognized nucleotides underlined and bolded. This profile reveals that seven of the nine guanines within the sequence make significant contributions to binding, with only the thymine at position 2 making the other significant contribution. The specificity-determining elements are distributed rather evenly across the oligonucleotide, in contrast to the 5'-preference observed with the telomere-binding proteins of *S. cerevisiae* Cdc13 and hPOT1.^{8,14,15}

The CST Complex Can Accommodate a Variety of Sequence Changes.

The specificity profile for CST reveals a preference for G-rich sequences, suggesting that CST could accommodate a variety of nontelomere sequences. We tested whether there was an additive or cooperative effect upon additional mutations within the telomeric oligonucleotide sequence. To achieve this, we altered each repeating position within the (TTAGGG)₃ sequence to give us the following oligonucleotides to test (site of alteration bolded): (ATAGGG)₃, (TAAGGG)₃, (TTTGGG)₃, (TTACGG)₃, (TTAGCG)₃, and (TTAGGC)₃ (Table 3). The triple-position alterations of the first three bases of the repeat, (ATAGGG)₃, (TAAGGG)₃, and (TTTGGG)₃, weakened the binding affinity by 3–5-fold. The more dramatic changes in binding affinity occurred with the triple-guanine alterations for sequences of (TTACGG)₃, (TTAGCG)₃, and (TTAGGC)₃. For (TTACGG)₃ and (TTAGGC)₃, 20- and 36-fold weaker binding affinity was observed. No detectable binding was observed to (TTAGCG)₃, defining the G5 position as the most important site recognized in the telomeric repeat. The results were roughly, but not precisely, additive, suggesting that the recognition is largely noncooperative, with some possible accommodation at positions 2 and 4.

To refine our understanding of the specificity observed for guanine nucleotides, we tested whether triple-purine nucleotide base alterations of guanine to adenine can rescue the dramatic decreases in binding affinity observed with the change in the purine nucleotide base of guanine to the pyrimidine nucleotide base of cytosine. Surprisingly, an only 3–7-fold change in binding affinity was observed for (TTAAGG)₃, (TTAGAG)₃, and (TTAGGA)₃. The ligand (TTAAGG)₃ bound ~3-fold tighter than (TTACGG)₃ and ~8-fold tighter for (TTAGGA)₃

than for (TTAGGC)₃, and (TTAGAG)₃ was able to largely rescue the lack of binding observed for (TTAGCG)₃ (Table 3). These data suggest that the purine base plays a vital role in CST recognition of oligonucleotides and conversely that pyrimidines are disfavored. This degree of accommodation raises the possibility of a plastic interface for CST and the oligonucleotides, as seen with other telomere end-binding proteins.^{1,49}

The specificity profile of CST does not strictly define the characteristic telomere repeat, suggesting that the CST complex could also productively bind diverse short G-rich yet non-telomeric sequences. We first tested a repeating 18mer (TTAGG)_{3,6} sequence (Table 3) to ascertain if deletion of the last guanine within the core repeat sequence would lead to a loss in binding affinity. Surprisingly, this repeating (TTAGG)_{3,6} sequence showed an only modest 1.4-fold change in affinity when compared to the precise telomere repeat-based sequence (Table 3). We next tested a nontelomeric sequence composed of repeating (TGTGTG)₃ that revealed an only 2.4-fold change in binding affinity. The 18mer TG sequence was randomized and showed an only 1.5-fold change compared to (TGTGTG)₃ and a 3.6-fold change when compared to (TTAGGG)₃ (Table 3). Given that adenosines were tolerated at G sites, we also queried whether (TATATA)₃ can bind. No binding to this oligonucleotide was observed up to 2.5 μM protein, consistent with CST having a preference for G rather than simply purines (Table 3). These results suggest that the specific mode of the ssDNA-binding interface of the CST complex can accommodate a more diverse range of G-rich sequences than other telomere end-binding proteins can.^{8,15,51}

DISCUSSION

CST has been implicated in several pathways of chromatin maintenance, including at telomeres and throughout the genome. Our characterization of the ssDNA binding activity for the CST complex reveals differences in ssDNA recognition relative to the known ssDNA telomere-binding proteins in yeast or humans. The minimal length of 16–18 oligonucleotides required for full CST binding activity (Table 1) is longer than that reported for other telomere-binding proteins, including human POT1 (10 nucleotides),⁸ *Schizosaccharomyces pombe* Pot1 (12–15 nucleotides),^{51,52} and *S. cerevisiae* Cdc13 (11 nucleotides)^{15,53,54} yet shorter than that of the *Tetrahymena thermophila* CST (24 nucleotides).⁵⁵ Because of a lack of sequence homology and structural characterization, it is unclear if the CTC1 DNA-binding domain consists of one OB fold, as found in Cdc13, two OB folds, as observed in POT1, or even more, as observed in RPA70.^{8,28,53,56–58} The low nanomolar binding affinity of CST is much weaker than those of the low-picomolar binders of *S. cerevisiae* Cdc13 and *Sc. Pombe* Pot1^{15,51,53} but is comparable to that of the low-nanomolar binder of human POT1⁸ and also that of RPA.³ These similar binding affinities suggest that CST should be considered in developing models of how these various ssDNA-binding proteins work together to regulate telomeres.⁵⁹

We show that G-rich sequences are the main requirement for interaction of CST with ssDNA (Figure 3), but that these guanines do not appear to be required in the context of a specific sequence motif and may be partially substituted with adenosines without severe impacts on affinity. Curiously, nontelomeric sequences that contain repetitive elements exhibit relatively small changes in binding affinity when compared to the (TTAGGG)₃ sequence. In contrast,

swapping guanines with cytosines within the (TTAGGG)₃ sequence dramatically changed the binding affinity, suggesting that CST strongly discriminates against C-rich sequences, including the type present on the opposite strand of the telomere (Tables 2 and 3). We postulate that by using the minimal length of ssDNA we are restraining the ability of CST to accommodate other bases within the ssDNA, and that the cytosine provides unfavorable interactions within the context of the minimal ssDNA length and CST. This leads to the prediction that the CST complex can accommodate more favorable interactions on longer ssDNA ligands with G-rich sequences, consistent with previous work.^{22,34} This accommodation could be achieved perhaps by utilizing plasticity at the interface as has been shown for other telomere end-binding proteins.^{49,60}

The finding that a core telomeric repeat is not required for high-affinity binding contrasts with the specificity exhibited by hPOT1, which is for the central TAGG element⁸ and is instead highly reminiscent of that of yeast t-RPA and the *T. thermophila* CST. In the yeast t-RPA, ssDNA binding activity is conferred by the Cdc13 component. Cdc13 also recognizes predominantly G bases, with a 5'-GxGT motif that is necessary for Cdc13 recognition of the minimal 11mer sequence.^{14,15} Nucleotide substitutions within the Cdc13 specificity-determining motif lead to several hundred-fold reductions in binding affinity. Accompanying preferences for G-rich sequences downstream of the core motif make more modest contributions, with substitutions at G7 and G9 of the minimal GTGTGGGTGTG ligand leading to 20–30-fold changes in binding affinity.^{14,15} The preference of human CST for guanines is even less pronounced than that observed in *S. cerevisiae* Cdc13, although Cdc13 proteins from other related families are also less sequence-specific,^{16,61} suggesting that a modest preference for guanines is a common feature of this family. Likewise, the recently discovered *Tetrahymena* CST,^{55,62} which preferentially binds a longer *Tetrahymena* telomeric sequence (TTGGGG)₄ with an affinity of 96 nM, exhibits marked sequence preference for the guanosines, with the largest impact on affinity observed upon substitution of G5 and G6 in all repeats, with the G6 position inferred to be the most important.⁵⁵ RPA, and other RPA-like complexes, are believed to utilize multiple binding modes involving the engagement of different numbers of OB folds in ssDNA binding.² The fact that specificity is distributed equitably across the substrate suggests that hCST may not access this range of binding modes in a similar way.

The specificity exhibited by CST further allows it to tolerate sequence alterations that occur at telomeres as a result of a lack of fidelity by telomerase and when an alternative, recombination-based telomere maintenance pathway (ALT) is active.^{63,64} Deep sequencing of human telomeres shows that variations due to telomerase error occur most frequently at positions 1 and 3 of the TTAGGG repeat.⁶⁴ Variant repeats are more common in ALT cells, but they also tend to maintain the GGG repeat and are characterized by differing sequences for the TTA segment. Thus, the tolerance shown by CST would allow it to continue to support replication at these altered sequences.

We have found that CST is neither a nonspecific nor a telomere-specific binder of ssDNA; rather, CST is a tight binder of ssDNA with a preference for G-rich sequences. This biochemical activity effectively explains its involvement with both telomere maintenance and genome replication. On the basis of its observed sequence specificity, we propose that

CST is associated with the replication of myriad G-rich sequences in the genome, including at telomeres. As the domain architecture of CST is similar to that of RPA, this further suggests that CST functions as a G-specific RPA. Thus, just as the replication machinery uses several polymerases to replicate DNA, the RPA component of the replication machinery may also be tailored to the sequences being replicated.

ACKNOWLEDGMENTS

We thank Carolyn Price for providing cDNA for the proteins in this study. We are grateful to Dylan Taatjes for providing reagents for this study. We thank Chen Davidovich for technical assistance and Neil Lloyd and Leslie Glustrom for experimental support and comments on the manuscript. We gratefully acknowledge helpful comments provided in the review of the manuscript.

Funding

National Institutes of Health (Grants GM059414 to D.S.W. and F32 GM100532 to R.A.H.).

REFERENCES

- (1). Dickey TH, Altschuler SE, and Wuttke DS (2013) Single-stranded DNA-binding proteins: multiple domains for multiple functions. *Structure* 21, 1074–1084. [PubMed: 23823326]
- (2). Fanning E, Klimovich V, and Nager AR (2006) A dynamic model for replication protein A (RPA) function in DNA processing pathways. *Nucleic Acids Res.* 34, 4126–4137. [PubMed: 16935876]
- (3). Wold MS (1997) Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu. Rev. Biochem* 66, 61–92. [PubMed: 9242902]
- (4). Xin H, Liu D, Wan M, Safari A, Kim H, Sun W, O'Connor MS, and Songyang Z (2007) TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. *Nature* 445, 559–562. [PubMed: 17237767]
- (5). Wang F, Podell ER, Zaug AJ, Yang Y, Baciú P, Cech TR, and Lei M (2007) The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature* 445, 506–510. [PubMed: 17237768]
- (6). Palm W, and De Lange T (2008) How shelterin protects mammalian telomeres. *Annu. Rev. Genet* 42, 301–334. [PubMed: 18680434]
- (7). Schmutz I, and De Lange T (2016) Shelterin. *Curr. Biol* 26, R397–R399. [PubMed: 27218840]
- (8). Lei M, Podell ER, and Cech TR (2004) Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. *Nat. Struct. Mol. Biol* 11, 1223–1229. [PubMed: 15558049]
- (9). Chen C, Gu P, Wu J, Chen X, Niu S, Sun H, Wu L, Li N, Peng J, Shi S, Fan C, Huang M, Wong CCL, Gong Q, Kumar-Sinha C, Zhang R, Pusztai L, Rai R, Chang S, and Lei M (2017) Structural insights into POT1-TPP1 interaction and POT1 C-terminal mutations in human cancer. *Nat. Commun* 8, 14929. [PubMed: 28393832]
- (10). Rice C, Shastrula PK, Kossenkova AV, Hills R, Baird DM, Showe LC, Doukov T, Janicki S, and Skordalakes E (2017) Structural and functional analysis of the human POT1-TPP1 telomeric complex. *Nat. Commun* 8, 14928. [PubMed: 28393830]
- (11). Lewis KA, and Wuttke DS (2012) Telomerase and telomere-associated proteins: structural insights into mechanism and evolution. *Structure* 20, 28–39. [PubMed: 22244753]
- (12). Rice C, and Skordalakes E (2016) Structure and function of the telomeric CST complex. *Comput. Struct. Biotechnol. J* 14, 161–167. [PubMed: 27239262]
- (13). Anderson EM, Halsey WA, and Wuttke DS (2003) Site-directed mutagenesis reveals the thermodynamic requirements for single-stranded DNA recognition by the telomere-binding protein Cdc13. *Biochemistry* 42, 3751–3758. [PubMed: 12667066]
- (14). Eldridge AM, Halsey WA, and Wuttke DS (2006) Identification of the determinants for the specific recognition of singlestrand telomeric DNA by Cdc13. *Biochemistry* 45, 871–879. [PubMed: 16411763]

- (15). Lewis KA, Pfaff DA, Earley JN, Altschuler SE, and Wuttke DS (2014) The tenacious recognition of yeast telomere sequence by Cdc13 is fully exerted by a single OB-fold domain. *Nucleic Acids Res.* 42, 475–484. [PubMed: 24057216]
- (16). Mandell EK, Gelinas AD, Wuttke DS, and Lundblad V (2011) Sequence-specific binding to telomeric DNA is not a conserved property of the Cdc13 DNA binding domain. *Biochemistry* 50, 6289–6291. [PubMed: 21668015]
- (17). Gao H, Cervantes RB, Mandell EK, Otero JH, and Lundblad V (2007) RPA-like proteins mediate yeast telomere function. *Nat. Struct. Mol. Biol* 14, 208–214. [PubMed: 17293872]
- (18). Paschini M, Mandell EK, and Lundblad V (2010) Structure prediction-driven genetics in *Saccharomyces cerevisiae* identifies an interface between the t-RPA proteins Stn1 and Ten1. *Genetics* 185, 11–21. [PubMed: 20157006]
- (19). Goulian M, and Heard CJ (1990) The mechanism of action of an accessory protein for DNA polymerase alpha/primase. *J. Biol. Chem* 265, 13231–13239. [PubMed: 2376593]
- (20). Goulian M, Heard CJ, and Grimm SL (1990) Purification and properties of an accessory protein for DNA polymerase alpha/primase. *J. Biol. Chem* 265, 13221–13230. [PubMed: 2165497]
- (21). Surovtseva YV, Churikov D, Boltz KA, Song X, Lamb JC, Warrington R, Leehy K, Heacock M, Price CM, and Shippen DE (2009) Conserved telomere maintenance component 1 interacts with STN1 and maintains chromosome ends in higher eukaryotes. *Mol. Cell* 36, 207–218. [PubMed: 19854131]
- (22). Miyake Y, Nakamura M, Nabetani A, Shimamura S, Tamura M, Yonehara S, Saito M, and Ishikawa F (2009) RPA-like mammalian Ctc1-Stn1-Ten1 complex binds to single-stranded DNA and protects telomeres independently of the Pot1 pathway. *Mol. Cell* 36, 193–206. [PubMed: 19854130]
- (23). Casteel DE, Zhuang S, Zeng Y, Perrino FW, Boss GR, Goulian M, and Pilz RB (2009) A DNA polymerase- α -primase cofactor with homology to replication protein A-32 regulates DNA replication in mammalian cells. *J. Biol. Chem* 284, 5807–5818. [PubMed: 19119139]
- (24). Price CM, Boltz KA, Chaiken MF, Stewart JA, Beilstein MA, and Shippen DE (2010) Evolution of CST function in telomere maintenance. *Cell Cycle* 9, 3177–3185.
- (25). Bryan C, Rice C, Harkisheimer M, Schultz DC, and Skordalakes E (2013) Structure of the human telomeric Stn1-Ten1 capping complex. *PLoS One* 8, e66756. [PubMed: 23826127]
- (26). Gelinas AD, Paschini M, Reyes FE, Héroux A, Batey RT, Lundblad V, and Wuttke DS (2009) Telomere capping proteins are structurally related to RPA with an additional telomere-specific domain. *Proc. Natl. Acad. Sci. U. S. A* 106, 19298–19303. [PubMed: 19884503]
- (27). Sun J, Yu EY, Yang Y, Confer LA, Sun SH, Wan K, Lue NF, and Lei M (2009) Stn1-Ten1 is an Rpa2-Rpa3-like complex at telomeres. *Genes Dev* 23, 2900–2914. [PubMed: 20008938]
- (28). Bochkareva E, Korolev S, Lees-Miller SP, and Bochkarev A (2002) Structure of the RPA trimerization core and its role in the multistep DNA-binding mechanism of RPA. *EMBO J.* 21, 1855–1863. [PubMed: 11927569]
- (29). Fan J, and Pavletich NP (2012) Structure and conformational change of a replication protein A heterotrimer bound to ssDNA. *Genes Dev* 26, 2337–2347. [PubMed: 23070815]
- (30). Chen LY, Majerska J, and Lingner J (2013) Molecular basis of telomere syndrome caused by CTC1 mutations. *Genes Dev* 27, 2099–2108. [PubMed: 24115768]
- (31). Lue NF, Chan J, Wright WE, and Hurwitz J (2014) The CDC13-STN1-TEN1 complex stimulates Pol. *Nat. Commun* 5, 5762. [PubMed: 25503194]
- (32). Qi H, and Zakian VA (2000) The *Saccharomyces* telomerebinding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein. *Genes Dev* 14, 1777–1788. [PubMed: 10898792]
- (33). Wang F, Stewart JA, Kasbek C, Zhao Y, Wright WE, and Price CM (2012) Human CST Has Independent Functions during Telomere Duplex Replication and C-Strand Fill-In. *Cell Rep* 2, 1096–1103. [PubMed: 23142664]
- (34). Chen L-Y, Redon S, and Lingner J (2012) The human CST complex is a terminator of telomerase activity. *Nature* 488, 540–544. [PubMed: 22763445]

- (35). Gu P, and Chang S (2013) Functional characterization of human CTC1 mutations reveals novel mechanisms responsible for the pathogenesis of the telomere disease Coats plus. *Aging Cell* 12, 1100–1109. [PubMed: 23869908]
- (36). Stewart JA, Wang F, Chaiken MF, Kasbek C, Chastain PD, Wright WE, and Price CM (2012) Human CST promotes telomere duplex replication and general replication restart after fork stalling. *EMBO J.* 31, 3537–3549. [PubMed: 22863775]
- (37). Gu P, Min J-N, Wang Y, Huang C, Peng T, Chai W, and Chang S (2012) CTC1 deletion results in defective telomere replication, leading to catastrophic telomere loss and stem cell exhaustion. *EMBO J.* 31, 2309–2321. [PubMed: 22531781]
- (38). Kasbek C, Wang F, and Price CM (2013) Human TEN1 Maintains Telomere Integrity and Functions in Genome-wide Replication Restart. *J. Biol. Chem* 288, 30139–30150. [PubMed: 24025336]
- (39). Wang F, Stewart J, and Price CM (2014) Human CST abundance determines recovery from diverse forms of DNA damage and replication stress. *Cell Cycle* 13, 3488–3498. [PubMed: 25483097]
- (40). Anderson BH, Kashner PR, Mayer J, Szykiewicz M, Jenkinson EM, Bhaskar SS, Urquhart JE, Daly SB, Dickerson JE, O'Sullivan J, Leibundgut EO, Muter J, Abdel-Salem GMH, Babul-Hirji R, Baxter P, Berger A, Bonafé L, Brunstom-Hernandez JE, Buckard JA, Chitayat D, Chong WK, Cordelli DM, Ferreira P, Fluss J, Forrest EH, Franzoni E, Garone C, Hammans SR, Houge G, Hughes I, Jacquemont S, Jeannot P-Y, Jefferson RJ, Kumar R, Kutschke G, Lundberg S, Lourenço CM, Mehta R, Naidu S, Nischal KK, Nunes L, Öunap K, Philippart M, Prabhakar P, Risen SR, Schiffmann R, Soh C, Stephenson JBP, Stewart H, Stone J, Tolmie JL, van der Knaap MS, Vieira JP, Vilain CN, Wakeling EL, Wermenbol V, Whitney A, Lovell SC, Meyer S, Livingston JH, Baerlocher GM, Black GCM, Rice GI, and Crow YJ (2012) Mutations in CTC1, encoding conserved telomere maintenance component 1, cause Coats plus. *Nat. Genet* 44, 338–342. [PubMed: 22267198]
- (41). Polvi A, Linnankivi T, Kivelä T, Herva R, Keating JP, Mäkitie O, Pareyson D, Vainionpää L, Lahtinen J, Hovatta I, Pihko H, and Lehesjoki A-E (2012) Mutations in CTC1, Encoding the CTS Telomere Maintenance Complex Component 1, Cause Cerebroretinal Microangiopathy with Calcifications and Cysts. *Am. J. Hum. Genet* 90, 540–549. [PubMed: 22387016]
- (42). Walne AJ, Bhagat T, Kirwan M, Gitiaux C, Desguerre I, Leonard N, Nogales E, Vulliamy T, and Dokal IS (2013) Mutations in the telomere capping complex in bone marrow failure and related syndromes. *Haematologica* 98, 334–338. [PubMed: 22899577]
- (43). Chastain M, Zhou Q, Shiva O, Whitmore L, Jia P, Dai X, Huang C, Fadri-Moskwick M, Ye P, and Chai W (2016) Human CST Facilitates Genome-wide RAD51 Recruitment to GC-Rich Repetitive Sequences in Response to Replication Stress. *Cell Rep* 16, 1300–1314. [PubMed: 27487043]
- (44). Brosey CA, Yan C, Tsutakawa SE, Heller WT, Rambo RP, Tainer JA, Ivanov I, and Chazin WJ (2013) A new structural framework for integrating replication protein A into DNA processing machinery. *Nucleic Acids Res* 41, 2313–2327. [PubMed: 23303776]
- (45). Bhattacherjee A, Stewart J, Chaiken M, and Price CM (2016) STN1 OB Fold Mutation Alters DNA Binding and Affects Selective Aspects of CST Function. *PLoS Genet* 12, e1006342. [PubMed: 27690379]
- (46). Bieniossek C, Imasaki T, Takagi Y, and Berger I (2012) MultiBac: expanding the research toolbox for multiprotein complexes. *Trends Biochem. Sci* 37, 49–57. [PubMed: 22154230]
- (47). Trowitzsch S, Palmberger D, Fitzgerald D, Takagi Y, and Berger I (2012) MultiBac complexomics. *Expert Rev. Proteomics* 9, 363–373. [PubMed: 22967074]
- (48). Altschuler SE, Lewis KA, and Wuttke DS (2013) Practical strategies for the evaluation of high-affinity protein/nucleic acid interactions. *J. Nucleic Acids Invest* 4, 19–28.
- (49). Lloyd NR, Dickey TH, Hom RA, and Wuttke DS (2016) Tying up the Ends: Plasticity in the Recognition of Single-Stranded DNA at Telomeres. *Biochemistry* 55, 5326–5340. [PubMed: 27575340]
- (50). Kim C, Paulus BF, and Wold MS (1994) Interactions of human replication protein A with oligonucleotides. *Biochemistry* 33, 14197–14206. [PubMed: 7947831]

- (51). Altschuler SE, Dickey TH, and Wuttke DS (2011) Schizosaccharomyces pombe protection of telomeres 1 utilizes alternate binding modes to accommodate different telomeric sequences. *Biochemistry* 50, 7503–7513. [PubMed: 21815629]
- (52). Croy JE, Podell ER, and Wuttke DS (2006) A new model for Schizosaccharomyces pombe telomere recognition: the telomeric single-stranded DNA-binding activity of Pot11–389. *J. Mol. Biol* 361, 80–93. [PubMed: 16842820]
- (53). Anderson EM, Halsey WA, and Wuttke DS (2002) Delineation of the high-affinity single-stranded telomeric DNA-binding domain of Saccharomyces cerevisiae Cdc13. *Nucleic Acids Res* 30, 4305–4313. [PubMed: 12364610]
- (54). Hughes TR, Weilbaecher RG, Walterscheid M, and Lundblad V (2000) Identification of the single-strand telomeric DNA binding domain of the Saccharomyces cerevisiae Cdc13 protein. *Proc. Natl. Acad. Sci U. S. A* 97, 6457–6462. [PubMed: 10841551]
- (55). Wan B, Tang T, Upton H, Shuai J, Zhou Y, Li S, Chen J, Brunzelle JS, Zeng Z, Collins K, Wu J, and Lei M (2015) The Tetrahymena telomerase p75-p45-p19 subcomplex is a unique CST complex. *Nat. Struct. Mol. Biol* 22, 1023–1026. [PubMed: 26551074]
- (56). Mitton-Fry RM, Anderson EM, Hughes TR, Lundblad V, and Wuttke DS (2002) Conserved structure for single-stranded telomeric DNA recognition. *Science* 296, 145–147. [PubMed: 11935027]
- (57). Croy JE, Podell ER, and Wuttke DS (2006) A new model for Schizosaccharomyces pombe telomere recognition: the telomeric single-stranded DNA-binding activity of Pot11–389. *J. Mol. Biol* 361, 80–93. [PubMed: 16842820]
- (58). Fan J, and Pavletich NP (2012) Structure and conformational change of a replication protein A heterotrimer bound to ssDNA. *Genes Dev* 26, 2337–2347. [PubMed: 23070815]
- (59). Litman Flynn R, Chang S, and Zou L (2012) RPA and POT1: friends or foes at telomeres? *Cell Cycle* 11, 652–657. [PubMed: 22373525]
- (60). Dickey TH, McKercher MA, and Wuttke DS (2013) Nonspecific recognition is achieved in Pot1pC through the use of multiple binding modes. *Structure* 21, 121–132. [PubMed: 23201273]
- (61). Yu EY, Sun J, Lei M, and Lue NF (2012) Analyses of Candida Cdc13 orthologues revealed a novel OB fold dimer arrangement, dimerization-assisted DNA-binding, and substantial structural differences between Cdc13 and RPA70. *Mol. Cell. Biol* 32, 186–198. [PubMed: 22025677]
- (62). Jiang J, Chan H, Cash DD, Miracco EJ, Ogorzalek Loo RR, Upton HE, Cascio D, O'Brien Johnson R, Collins K, Loo JA, Zhou ZH, and Feigon J (2015) Structure of Tetrahymena telomerase reveals previously unknown subunits, functions, and interactions. *Science* 350, aab4070–aab4070. [PubMed: 26472759]
- (63). Conomos D, Stutz MD, Hills M, Neumann AA, Bryan TM, Reddel RR, and Pickett HA (2012) Variant repeats are interspersed throughout the telomeres and recruit nuclear receptors in ALT cells. *J. Cell Biol* 199, 893–906. [PubMed: 23229897]
- (64). Lee M, Hills M, Conomos D, Stutz MD, Dagg RA, Lau LMS, Reddel RR, and Pickett HA (2014) Telomere extension by telomerase and ALT generates variant repeats by mechanistically distinct processes. *Nucleic Acids Res* 42, 1733–1746. [PubMed: 24225324]

Figure 1. Domain map and expression and purification of the recombinant CST heterotrimer complex. (A) Domain map of the predicted OB folds of CTC1 (gray) and the known OB fold and winged helix–turn–helix (wHTH) domains of STN1–TEN1. (B) Purified recombinant 2x-FLAG CTC1, 6x-His-STN1, and 6x-His-TEN1 heterotrimer complex run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel.

Figure 2.

CST complex binds to (TTAGGG)₃ telomere sequence. Representative double-filter binding data (top) plotted as a function of fraction bound vs protein concentration of the CST complex in nanomolar and fitted to a two-state binding model. The $K_{D,app}$ value is the mean from at least three independent experiments as reported for Tables 1–3. The representative membranes (bottom) from a double-filter binding experiment are labeled as “bound” for nitrocellulose membrane and “free” for the Hybond filter.

Figure 3. Specificity profile for CST with respect to telomeric ssDNA (TTAGGG)₃ that reveals a strong preference for guanine nucleotides. The bar graph plots the AAG of binding, defined as $G = RT \ln[K_{D, \text{complement}}/K_{D(\text{TTAGGG})_3}]$, where $R = 9872 \text{ cal mol}^{-1} \text{ K}^{-1}$, $T = 277.15 \text{ K}$, and $K_{D(\text{TTAGGG})_3} = 21.6 \text{ nM}$. The error bars are standard errors of the mean from at least three independent experiments.

Table 1.Apparent K_D Values for Binding to Telomeric Sequences That Vary in Length

Oligonucleotide Name	18mer Oligonucleotide Sequence	$K_{D,app}$ (nM) ^a	Fold Change ^b
18mer	TTAGGG TTAGGG TTAGGG	21.6±1.0	
17mer 3'	TTAGGG TTAGGG TTAGG	39.9±3.2	1.8
16mer 3'	TTAGGG TTAGGG TTAG	55.3±4.1	2.6
15mer 3'	TTAGGG TTAGGG TTA	294.9±12.7	13.7
17mer 5'	TAGGG TTAGGG TTAGGG	20.2±2.8	0.9
16mer 5'	AGGG TTAGGG TTAGGG	46.9±1.9	2.2
15mer 5'	GGG TTAGGG TTAGGG	124.9±12.1	5.8

^a Apparent K_D values were obtained from at least $n > 3$ experiments. Errors are standard errors of the mean.^b The fold change is relative to the 18mer sequence.

Table 2.Specificity Profile K_D Values for CST Binding

Oligonucleotide Name	18mer Oligonucleotide Sequence			K_D (nM) ^a	G kcal/mol	Fold Change ^b
(TTAGGG) ₃	TTAGGG	TTAGGG	TTAGGG	21.6±1.0		
T1A	ATAGGG	TTAGGG	TTAGGG	27.1±0.4	0.12±0.01	1.3
T2A	TAAGGG	TTAGGG	TTAGGG	65.5±4.5	0.60±0.04	3.0
A3T	TTTGGG	TTAGGG	TTAGGG	45.7±4.1	0.40±0.05	2.1
G4C	TTACGG	TTAGGG	TTAGGG	67.6±3.5	0.62±0.03	3.1
G5C	TTAGCG	TTAGGG	TTAGGG	230.0±14.0	1.30±0.03	10.6
G6C	TTAGGC	TTAGGG	TTAGGG	36.4±3.2	0.30±0.04	1.7
T7A	TTAGGG	ATAGGG	TTAGGG	19.3±1.8	-0.07±0.05	0.9
T8A	TTAGGG	TAAGGG	TTAGGG	26.2±1.7	0.10±0.04	1.2
A9T	TTAGGG	TTTGGG	TTAGGG	23.8±0.3	0.05±0.01	1.1
G10C	TTAGGG	TTACGG	TTAGGG	78.7±6.1	0.71±0.04	3.6
G11C	TTAGGG	TTAGCG	TTAGGG	364.6±25.3	1.55±0.04	16.9
G12C	TTAGGG	TTAGGC	TTAGGG	261.8±19.5	1.37±0.04	12.1
T13A	TTAGGG	TTAGGG	ATAGGG	21.3±2.1	-0.02±0.06	1.0
T14A	TTAGGG	TTAGGG	TAAGGG	38.7±3.9	0.30±0.06	1.8
A15T	TTAGGG	TTAGGG	TTTGGG	29.9±3.8	0.16±0.07	1.4
G16C	TTAGGG	TTAGGG	TTACGG	221.5±12.6	1.27±0.03	10.3
G17C	TTAGGG	TTAGGG	TTAGCG	89.2±1.8	0.78±0.01	4.1
G18C	TTAGGG	TTAGGG	TTAGGC	31.4±1.9	0.20±0.04	1.5
Random TG	GTGGTG	TTGTGT	TGGTGT	78.3±6.3	0.70±0.05	3.6
Random ATCG	ATCTAT	TACGGT	TGGCAC	129.4±4.7	0.98±0.02	6.0
(TTTTTT) ₃	TTTTTT	TTTTTT	TTTTTT	NB		

^a Apparent K_D values were obtained from at least $n = 3$ experiments. Errors are standard errors of the mean. NB indicates no binding was observed.

^b The $K_{D,app}$ fold change is relative to the (TTAGGG)₃ sequence.

Table 3.Apparent K_D Values for Binding of CST to Altered Telomeric and Nonspecific Oligonucleotides

18mer Oligonucleotide Name	18mer Oligonucleotide Sequence			$K_{D,app}$ (nM) ^a	Fold Change ^b
(TTAGGG) ₃	TTAGGG	TTAGGG	TTAGGG	21.6±1.0	
(ATAGGG) ₃	ATAGGG	ATAGGG	ATAGGG	99.3±2.2	4.6
(TAAGGG) ₃	TAAGGG	TAAGGG	TAAGGG	70.1±5.8	3.2
(TTTGGG) ₃	TTTGGG	TTTGGG	TTTGGG	66.2±2.1	3.1
(TTACGG) ₃	TTACGG	TTACGG	TTACGG	422.3±59.5	19.6
(TTAGCG) ₃	TTAGCG	TTAGCG	TTAGCG	NB	
(TTAGGC) ₃	TTAGGC	TTAGGC	TTAGGC	785.4±64.6	36.4
(TTAAGG) ₃	TTAAGG	TTAAGG	TTAAGG	145.4±19.8	6.7
(TTAGAG) ₃	TTAGAG	TTAGAG	TTAGAG	96.5±4.1	4.5
(TTAGGA) ₃	TTAGGA	TTAGGA	TTAGGA	73.3±2.7	3.4
(GGGTTA) ₃	GGGTTA	GGGTTA	GGGTTA	120.6±15.0	5.6
(TTAGG) _{3,6}	TTAGG	TTAGG	TTAGG TTA	30.8±2.7	1.4
(TGTGTG) ₃	TGTGTG	TGTGTG	TGTGTG	52.0±3.3	2.4
(TATATA) ₃	TATATA	TATATA	TATATA	NB	

^a Apparent K_D values were obtained from at least $n = 3$ experiments. Errors are standard errors of the mean. NB indicates no binding was observed.^b Fold change is relative to the (TTAGGG)₃ sequence.