Extended DNA Binding Site in Pot1 Broadens Sequence Specificity to Allow Recognition of Heterogeneous Fission Yeast Telomeres*†

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The Pot1 (protection of telomeres) protein binds to single-stranded telomeric DNA and is essential for the protection of chromosome ends from degradation and end-to-end fusions. The Pot1 amino-terminal DNA binding domain, Pot1N, adopts an oligonucleotide/oligosaccharide binding fold and binds GGTTAC motifs cooperatively and with exceptionally high sequence specificity. We have now examined DNA binding to naturally occurring telomeric substrates based on the analysis of 100 cloned chromosome ends and in the context of the full-length Pot1 protein. Here, we describe several important differences between Pot1 and Pot1N with apparent consequences for chromosome end protection. Specifically, full-length Pot1-DNA complexes are more stable, and the minimal binding site for a Pot1 monomer is extended into two adjacent telomeric repeats. We provide evidence that Pot1 contains a second DNA binding motif that recognizes DNA with reduced sequence specificity compared with the domain present in Pot1N. The two DNA binding motifs cooperate, whereby the amino-terminal oligonucleotide/oligosaccharide binding fold determines the registry of binding, and the internal DNA binding motif stabilizes the complex and expands the protected region toward the 3′-end. Consistent with a role in chromosome end capping, Pot1 prevents access of telomerase to the 3′-end and protects against exonucleolytic degradation.

Telomeres comprise the ends of eukaryotic chromosomes and ensure their protection and complete replication (1). In most eukaryotes, telomeric DNA consists of tandem arrays of short repetitive sequences, with a guanine-rich strand running 5′ to 3′ toward the chromosome end. These sequences are either composed of direct perfect repeats, such as TTAGGG in vertebrates, or show some sequence heterogeneity, for example G1–8TTACA0–1C0–1 in Oxytricha nova (9, 10). The protein is comprised of α and β subunits that form a ternary complex with telomeric DNA (16). Structural analysis revealed the presence of three oligonucleotide/oligosaccharide binding folds (OB-folds) in the α subunit and one in the β subunit (17). Telomeric DNA is deeply embedded in a cleft between the α and β subunits, explaining the high affinity and efficient protection of the DNA 3′ terminus within the complex. The α subunit can also homodimerize and may mediate telomere-telomere associations by forming a complex with two chromosome ends (10, 18).

In S. cerevisiae, genetic studies revealed the critical role of Cdc13 in chromosome end protection (12, 13). Further studies demonstrated that Cdc13 plays multiple roles in the protection of telomeres as well as in the regulated recruitment of telomerase and the coordinated synthesis of G-rich and C-rich strands (13, 19–22). To accomplish these functions, Cdc13 interacts with several distinct protein complexes including the telomerase holoenzyme, polymerase α, and a protective complex comprised of Stn1 and Ten1 (20, 22–24). Like ciliate TEBPs, purified Cdc13 binds to G-rich telomeric DNA with high affinity (13, 19, 25). Although Cdc13 shares no apparent sequence similarity with the ciliate proteins, the NMR structure of the Cdc13 DNA binding domain revealed the presence of an OB-fold that is structurally similar to the amino-terminal OB-fold of the TEBP α subunit (26).

Similarity with the amino-terminal OB-fold of ciliate TEBPs is also observed in Pot1 proteins (14). Initial characterization of fission yeast, human, and chicken Pot1 demonstrated that they bind to the cognate G strand of telomeric DNA and play a critical role in telomere length regulation and end protection (14, 27–29). Deletion of the fission yeast pot1+ gene results in rapid loss of telomeric and subtelomeric DNA, chromosome end fusions, and segregation defects (14). Most cells die as a consequence of this chromosomal instability. However, in a subset of cells, random fusion of chromosome ends results in the circularization of all three chromosomes. Cells with circular chromosomes divide readily and form survivor strains, which subsequently lack a requirement for factors involved in chromosome end maintenance (14, 30, 31).

Consistent with a role in binding to the 3′-overhang at the ends of telomeres in vivo, Pot1 binds to the G-rich strand of fission yeast telomeric DNA but not to the C-rich strand or double-stranded telomeric DNA (14). The DNA binding domain

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† The abbreviations used are: TEBPs, telomere end-binding proteins; OB-fold, oligonucleotide/oligosaccharide binding fold; Pot1, protection of telomeres.
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Pot1 has been mapped to include the 185 amino-terminal residues of Pot1. This fragment, here referred to as Pot1N, retains the binding specificity of the full-length protein, and the high-resolution crystal structure of Pot1N in complex with DNA has been solved (32). Remarkably, the DNA in the complex adopts a folded conformation involving base stacking and two unusual G-T bp. Any sequence change in the DNA prevents this structure from forming, providing an explanation for the high-sequence specificity in DNA binding.

Here we describe the purification of full-length Pot1 protein and characterize its interactions with a variety of DNA sequences based on the analysis of 100 cloned telomeres. A comparison between full-length Pot1 and Pot1N revealed that protein sequences beyond the NH2-terminal 185 amino acids present in Pot1N contribute to DNA binding by the naturally occurring Pot1 protein. These additional DNA contacts increase the half-life of the protein-DNA complex considerably and explain why Pot1N is a poor competitor for chromosome ends in vivo. Surprisingly, we found that the high cooperativity and sequence specificity observed with Pot1N are relaxed in the context of the full-length protein. These differences are likely to impact on telomerase regulation as well as the distinction between chromosome ends and DNA double-stranded breaks.

MATERIALS AND METHODS

Expression and Purification of Recombinant Pot1—The full-length Pot1 cDNA was cloned downstream of a 6-histidine affinity tag and 3C protease cleavage site to generate plasmid pPB460. pPB460 was transformed into Rosetta-Gami (DE3) pLysS Escherichia coli cells (Novagen) and plated onto Luria Broth plus 50 µg/ml carbenicillin and 34 µg/ml chloramphenicol agar plates. Several colonies were selected to inoculate 10 ml of tryptone phosphate medium (20 g of tryptone, 15 g of yeast extract, 8 g of NaCl, 2 g of Na2HPO4, 1 g of KH2PO4, and 2 g of dextrose/liter) plus carbenicillin and 50 µg/ml bovine serum albumin, 250 µg/ml denatured herring sperm DNA, and 5 mM magnesium chloride (Invitrogen) for sequence analysis. PCR products were gel purified and cloned into pCR4blunt-TOPO plasmid vector (Invitrogen) for sequencing.

Expression and Purification of Recombinant Pot1 Protein—Pot1 and Pot1N were expressed in E. coli Boli550, and Taq Plus Long polymerase mix (Stratagene). PCR products were gel purified and cloned into pCR4blunt-TOPO plasmid vector (Invitrogen) for sequence analysis.

Integrative DNA Technologies. For electrophoretic mobility shift assays, oligonucleotide substrates were purified by PAGE and 5′-end labeled with [γ-32P]ATP and polynucleotide kinase (New England Biolabs). Oligonucleotide substrates (250 mM) were bound by the indicated amounts of protein, and 6 × loading buffer (50 mM Tris-HCl, pH 7.5, 50% glycerol (v/v), 0.1 mM/ml bromphenol blue) was added just prior to loading onto Tris borate 4–20% gradient gels, run at 4 °C for 160 V for 80 min. Gels were dried onto DE81 (Whatman paper) and subjected to phosphorimaging analysis.

Pot1N was purified from 1 liter of TB2 buffer plus 1M KCl. The peak fraction (3 µl) was removed and placed in an Eppendorf tube containing cold competitor DNA at the indicated molar excess of telomeric repeats. For the competitor-free control, an equivalent volume of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was substituted for the competitor DNA. The samples were incubated in the presence of competitor for the indicated times and analyzed as described above.

Enzyme Protection Assays—DNA substrates (100 µM) were incubated in 10-µl reactions with 280 µM Pot1 or 0.56 µM Pot1N in 20 mM Tris-acetate, pH 7.5, 50 mM NaCl, and 2 mM MgCl2 by heating to 95 °C followed by slow cooling to room temperature. To attach PBoli548 to the 3′-end of telomeres covalently, 1 µM of telomere was added to a 100-µl reaction containing 10 units of T4 DNA ligase (New England Biolabs) and the manufacturer’s supplied buffer at 16 °C for 16 h. Telomeres ligated to PBoli548 were amplified by PCR with PBoli434 (GTGGTAGGATCTATGGTGA), PBoli550, and Taq Plus Long polymerase mix (Stratagene). PCR products were gel purified and cloned into pCR4blunt-TOPO plasmid vector (Invitrogen) for sequencing.

DNA Substrates—All oligonucleotide substrates were synthesized by Integrated DNA Technologies. For electrophoretic mobility shift assays, oligonucleotide substrates were purified by PAGE and 5′-end labeled with [γ-32P]ATP and polynucleotide kinase (New England Biolabs). Oligonucleotide substrates (250 mM) were bound by the indicated amounts of protein, and 6 × loading buffer (50 mM Tris-HCl, pH 7.5, 50% glycerol (v/v), 0.1 mM/ml bromphenol blue) was added just prior to loading onto Tris borate 4–20% gradient gels, run at 4 °C for 160 V for 80 min. Gels were dried onto DE81 (Whatman paper) and subjected to phosphorimaging analysis.

RESULTS

Expression and Purification of Recombinant Pot1 Protein—Pot1 and Pot1N were expressed in E. coli, and soluble extracts of several clones were analyzed for expression by Western blotting. Differences in expression levels among clones were found to be negligible (data not shown), and a number of colonies were pooled for large scale inductions. Extracts derived from ~360 g of cell pellet (40 liters of culture) were fractionated by a combination of affinity and classical chromatography, including Talon metal affinity, Q-Sepharose, and Affi-Blue (Fig. 1, A and B). Difficulty arose in separating the
highly labile Pot1 protein from the products of proteolysis which occurred during the initial steps of purification. These Pot1 fragments were strikingly similar in their chromatographic behavior on a variety of resins but were successfully separated from full-length Pot1 by gel filtration (Fig. 1C). Fractions 12 and 13 were used in the binding experiments presented here. From 40 liters of bacterial culture, ~400 μg of purified Pot1 protein was obtained (Fig. 1D, lane 2). Pot1N was expressed at much higher levels, and essentially homogeneous protein was obtained after only two chromatographic steps on nickel affinity and Q-Sepharose resin (Fig. 1D, lane 3). The yield was ~50 mg/liter of culture and hence 5,000-fold higher than for Pot1.

**Pot1 Has an Extended DNA Binding Site**—The minimal binding site for Pot1N has been determined by filter binding assay as GGTTAC (33). For simplicity, we will refer to the GGTTAC motif as Tel, followed by single letter abbreviations for additional nucleotides present in certain DNA substrates. A stable complex between the Tel hexamer and Pot1N could only be visualized by electrophoretic mobility shift assay when the protein was present in large excess (supplemental Fig. 1, I). When the protein:DNA ratio was below 10:1, a smear or no shift at all was observed, presumably because of the dissociation of complexes during electrophoresis. Single nucleotide permutations of the Tel sequence in either direction significantly reduced Pot1N binding, in accord with the high sequence specificity of Pot1N (Ref. 33 and supplemental Fig. 1, II and VI). A stable Pot1N-DNA complex was also observed with a TelA substrate, a sequence motif that commonly occurs in fission yeast telomeres (Fig. 2A, lane 3). The stability of the Pot1N-DNA complex diminished as further nucleotides, corresponding to part of an additional telomeric repeat, were added to the 3'-end (Fig. 2A, lanes 4–12). These observations are in agreement with previous data, where nontelomeric 3'-extensions also had a deleterious effect on Pot1N binding (33).

Under the conditions used here, full-length Pot1 also forms a complex with the GGTTACA substrate, which partially dissociates during electrophoresis, as suggested by a smeary band shift (Fig. 2B, lane 2). However, unlike Pot1N, single nucleotide additions to the 3'-end increased Pot1-DNA complex stability.
Pot1 binding to natural telomeres

A. Mushegian and P. Baumann, unpublished data.

**Fig. 2.** Pot1 has an extended DNA binding site and binds in a unique registry. A, binding of 560 nM and 2.8 μM Pot1N to 100 nM telomeric oligonucleotides in 40 mM Tris acetate, pH 7.5, 100 mM NaCl, and 4 mM MgCl2 was analyzed by electrophoretic mobility shift assay. Tel corresponds to the GGTTAC sequence. B, binding of 560 nM Pot1 to the same DNA substrates used in A, C, binding of 140 nM Pot1 to 15-mer oligonucleotide substrates with the indicated point mutations (100 nM). Samples were run on a 4–20% TBE polyacrylamide gel, dried onto DE81 paper (Whatman), and exposed to a phosphorimaging screen.

To characterize the additional protein-DNA contacts, we generated a number of oligonucleotide substrates composed of two tandem heptameric repeats with mutations in either the first or the second repeat. The mutations within each repeat were chosen to eliminate binding by Pot1N. We found that binding of full-length Pot1 to these DNA substrates was highly dependent on the integrity of the first heptameric repeat (Fig. 2C, lane 2). Consistent with the first OB-fold binding the first GGTTACA motif in the same manner as observed with Pot1N, single nucleotide changes of G2 to C or T3 to A dramatically reduced Pot1 binding (lanes 4 and 8). In contrast, the same mutations in the second repeat had little effect on complex stability (lanes 6 and 10).

Taken together our data suggest that full-length Pot1 has an extended DNA binding site compared with Pot1N. Sequence specificity largely depends on the contacts made by the amino-terminal OB-fold. A predicted second OB-fold stabilizes the Pot1-DNA complex and extends the binding site toward the 3′-end.

Sequence Analysis of Fission Yeast Telomeres—We speculated that the relaxed sequence specificity in the second DNA binding domain may allow the protein to bind and protect different naturally occurring telomeric sequences. Although previous analysis revealed considerable heterogeneity surrounding each GGTTAC core motif, this conclusion was largely based on only five cloned chromosome ends (3). In addition, the procedure used to generate these clones removed the single-stranded 3′-overhang from each telomere and hence did not include the region bound by Pot1. To examine telomeric repeat heterogeneity from a larger data set, we have now cloned and analyzed 100 individual chromosome ends from a culture of S. pombe cells using a method that retains the naturally occurring 3′-overhang.

Individual telomere length varied between 70 and 354 nucleotides, with a mean length of 188 ± 55 nucleotides. The GGTTAC sequence clearly emerged as the most common motif, occurring a total of 2,196 times or nearly 22 times/telomere. Although 14% of these core hexamers occurred in tandem, the rest are separated by 1–20 nucleotides of spacer sequences (Fig. 3A). Although longer spacers were sporadically encountered, 84% of GGTTAC repeats are separated by 0–3 nucleotides. The average spacer length for all repeats is 2.3 nucleotides. Two intervening sequences (GT and AA) are present once in each telomere, localizing to the proximal and largely variable region. By far the most common sequences separating two core hexamers are a single adenine (29%) and adenine followed by a variable number of guanines (31%; Fig. 3B). Indeed, 81% of all core hexamers were followed by an adenine (Fig. 3C), making direct heptameric GGTTACA repeats almost twice as common as tandem GGTTAC motifs. Guanines were the most common nucleotide encountered in the second to fourth positions between repeats (Fig. 3C). Spacer sequences and length were very similar between the eight most distal and internal repeats, suggesting that heterogeneity arises as telomeres are synthesized by telomerase (data not shown).

The GGTTAC core motif is unusual among telomeric repeats, in that it only contains two consecutive guanines as opposed to three or more found in the telomeric repeats of most other species. Our analysis of 2,196 GGTTAC repeats revealed that spacer sequences add additional guanines to 31% of the core hexamers (Fig. 3D). This form of repeat heterogeneity may be particularly important because the major telomere-binding protein in fission yeast, Tel1, requires three consecutive guanines for stable binding (35).3

Cooperativity in Binding to Longer Oligonucleotide Substrates—Based on the observations described above, a variety of oligonucleotide substrates were synthesized and analyzed for binding by Pot1 and Pot1N in electrophoretic mobility shift experiments. A DNA substrate consisting of four perfect GGTTAC repeats, referred to as (Tel)4, whereas three consecutive GGTTACAC repeats are abbreviated as (TelAC)3. Longer naturally occurring sequences with variable spacer sequence are listed as TelN followed by the number of core repeat units. The actual sequences for these DNA substrates are listed in the respective figure legends.

To explore the possibility that spacer sequences between GGTTAC repeats affect binding, we compared two DNA substrates of identical length, (Tel)4 and (TelAC)3. Both DNA substrates were readily bound by Pot1 and Pot1N (Fig. 4A). The complex formed between Pot1N and (TelAC)3 had a slower electrophoretic mobility than the Pot1N-(TelAC)3 complex (compare lanes 2 and 3), suggesting that an additional Pot1N

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2 A. Mushegian and P. Baumann, unpublished data.

3 J. Cooper, personal communication.
FIG. 3. Analysis of S. pombe telomere sequences. Sequences of 100 telomeres (18,818 nucleotides (nts) combined) were processed into GGTTAC repeats and intervening sequences. The latter were analyzed for length and sequence content. A, graphical representation of the frequency at which spacers of different length occur between two consecutive GGTTAC repeats. B, frequency at which different spacer sequences occur. C, frequency distributions for each nucleotide for the first four positions following GGTTAC repeats. Guanines were not counted when they were part of the next GGTTAC motif. D, frequency distribution for the number of consecutive guanines preceding the TTAC sequence.

FIG. 4. Reduced cooperativity in DNA binding by Pot1. A, Pot1N binds cooperatively to adjacent telomeric repeats. Oligonucleotide substrates of equal length, but differing in the number of core repeating units (250 nM) were compared for binding by the indicated amounts of Pot1 or Pot1N. B, Pot1 and Pot1N were incubated with longer oligonucleotide substrates (250 nM) to assess cooperativity in binding. Sequences used: (TelN)₆, GGTTAC AC GGTTAC A GGTTAC A GGTTAC A GGTTAC A GGTTAC A; (TelN)₇, GGTTAC AC GGTTAC A GGTTAC A GGTTAC A GGTTAC A GGTTAC A GGTTAC; (TelN)₈, GGTTAC AC GGTTAC A GGTTAC A GGTTAC A GGTTAC A GGTTAC A GGTTAC A GGTTAC. C, 1.4 μM Pot1 was bound to (TelN)₇, followed by the addition of a 170-fold molar excess of cold competitor DNA for the times indicated. NC represents samples incubated for 90 min in the absence of competitor.
molecule was bound to (Tel)_4. The binding stoichiometry of Pot1N is hence dependent on the number of GGTTAC units, not on substrate length. In contrast, the Pot1-(Tel)_4 and Pot1-(TelA)_3 complexes migrated to the same position in the gel, consistent with the same number of Pot1 molecules being bound to both DNA substrates (Fig. 4A, lanes 6 and 7).

In agreement with previous observations (33), Pot1N bound cooperatively to longer DNA substrates, and only one protein-DNA complex was detected with oligonucleotides containing up to eight TelN or TelA repeats (Fig. 4B and data not shown). In contrast, multiple protein-DNA complexes were detected with full-length Pot1 on longer DNA substrates (Fig. 4B). The fact that slower migrating complexes became more abundant as additional Pot1 protein was added suggests that the high cooperativity in binding observed with Pot1N is diminished in the context of the full-length protein (Fig. 4B, e.g. compare lanes 12–14). When Pot1 complexes were formed with radiolabeled (TelN)_6 followed by the addition of excess cold competitor, the slowest migrating complexes appear to be converted to the faster migrating forms prior to release of the free DNA substrate (Fig. 4C). These results may reflect a hierarchy in the stability of different conformations of Pot1-(TelN)_7 complexes. However, as the faster migrating complexes appear to be intermediates in the formation as well as in the dissociation of slower migrating forms, the simplest explanation is that the complexes reflect differences in binding stoichiometry, suggesting that the high cooperativity in DNA binding observed with Pot1N is not a feature of the full-length protein. An apparent lack of cooperativity in DNA binding has also been reported for the Pot1 homolog from chicken (29).

**Increased Stability of Pot1-DNA Complexes—** We next wanted to compare the stability of complexes formed by Pot1 and Pot1N with DNA. Each protein was allowed to bind to radiolabeled DNA (TelN)_6*, followed by the addition of a 170-fold molar excess of cold competitor (TelN)_6. Samples were taken at different time points and analyzed by gel electrophoresis. The Pot1-(TelN)_6* complex proved to be quite stable, and only 2% of the bound fraction dissociated during the first minute (Fig. 5A, I). During the same time, 68% of Pot1N bound (TelN)_6* was released, and after 5 min virtually no Pot1N-(TelN)_6* complex was observed (Fig. 5A, II). In contrast, 36% of Pot1-(TelN)_6* persisted even after 90 min.

The substantial difference in turnover rate suggests that Pot1N would be a poor competitor for binding to chromosome ends when expressed in the presence of endogenous full-length protein. To test this hypothesis, Pot1 and Pot1N were placed under the control of the inducible no-message-in-thiamine (nmt1) promoter and introduced into *S. pombe*. Both proteins were expressed with Pot1N accumulating to at least 10-fold higher levels than Pot1 (Fig. 5B). We were unable to detect endogenous Pot1 under these conditions, but estimate that it was at least 500-fold less abundant than Pot1N. If Pot1N displaced Pot1 from telomeres, we expect a “dominant negative” effect, as the truncated protein would fail to provide any “active” effect, as the truncated protein would fail to provide any

We conclude that the high off-rate of Pot1N allows endogenous Pot1 to compete efficiently for binding sites, preventing a dominant negative phenotype despite high level expression of Pot1N. In addition, the Pot1 COOH-terminal region may mediate protein-protein interactions that further stabilize the naturally occurring protein at the telomere.

**Accessibility of Telomeric DNA to Exonuclease Activity—** The terminal unpaired nucleotides of a telomere are of particular importance because they constitute the substrate for telomerase. Equally, they may serve as the entry site for exonucleolytic degradation or homologous recombination and form the target for end joining activities. To examine the accessibility of different 3'-end sequences in the presence of Pot1N and Pot1, a set of 15 DNA substrates was generated, starting at five TelA repeats and increasing in single nucleotide increments to seven TelA repeats. These were radiolabeled at their 5'-ends, and *E. coli* exonuclease I was titrated to degrade >98% of each substrate DNA within 5 min in the absence of Pot1 or Pot1N (Fig. 6A). In the presence of Pot1N, a distinct pattern of protection was observed. Oligonucleotides ending with a complete repeat (GGTTAC or GGTTACA) were up to 40-fold less accessible to exonuclease I than the unprotected DNA (Fig. 6, A and B). Because GGTTAC constitutes the minimal binding site for Pot1N, single nucleotide additions to the 3'-end were not protected up until a new Tel repeat was complete (Fig. 6A, VII). Consistent with an additional Pot1N molecule binding this new repeat, a shift in the Pot1N-DNA complex was observed (data not shown).

At the Pot1N concentrations used in this experiment, a portion of the DNA substrates was subject to partial degradation during the 5-min incubation with exonuclease I. The pattern of

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4 N. Bae and P. Baumann, unpublished data.
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Relaxed Sequence Specificity for Pot1 in DNA Binding—The Pot1N-DNA co-crystal structure provided a fascinating insight into how DNA self-recognition forms a basis for the high specificity with which Pot1N recognizes the GGTTAC sequence (32). Highly selective binding to the cognate telomeric sequence is an important feature of telomere-capping proteins because it prevents promiscuous binding to single-stranded DNA that arises at other sites in the genome during replication, recombination, and repair. We were hence surprised to find that the high specificity for the GGTTAC sequence observed with Pot1N is not maintained in the context of the full-length protein. Specifically, Pot1 bound to other G-rich substrates, including mutant GGATCA repeats (Fig. 7A, lanes 9 and 10) and telomeric sequences from Tetrhyymena thermophila (Fig. 7B, lanes 4 and 5), O. nova (data not shown), and S. cerevisiae (Fig. 7C, lanes 3 and 4). Efficient binding to the budding yeast sequence (SC1) was perhaps most surprising, as the sequence consists of seven TG1 repeats and hence bears the least resemblance to the GGTTAC unit found in fission yeast telomeres. When Pot1 was present in a 3.5-fold excess over the SC1 primer, more than 90% of the DNA was shifted (Fig. 7C, lane 4). In contrast, incubation with a 76-fold excess of Pot1N did not result in stable complex formation (lane 6).

To address the possibility that a nonspecific DNA binding activity contaminates our Pot1, but not Pot1N preparations, a 130-fold excess of boiled herring sperm DNA was added as nonspecific competitor. In addition, we examined binding of Pot1 and Pot1N to several other DNA substrates. Consistent with earlier results, neither Pot1 nor Pot1N bound to the C-rich strand or double-stranded telomeric DNA from fission yeast (supplemental Fig. 2A). Binding of Pot1 was also abolished when other mutations were introduced into TelA repeats. Specifically, no complex formation was observed when G2 was changed to C or T3 to A (supplemental Fig. 2A). Taken together these results suggest that the electrophoretic mobility shifts observed in Fig. 7 are indeed the result of a relaxed sequence specificity of full-length Pot1 compared with the isolated Pot1N domain.

Pot1 Inhibits Telomerase Activity in Vitro—Telomerase activity from S. pombe has been assayed following partial purification by ion exchange chromatography (36) and affinity purification (37). Both studies came to the surprising conclusion that S. cerevisiae primers were extended far more efficiently than primers derived from S. pombe telomeric repeat sequences. Because Pot1 bound efficiently to the S. cerevisiae telomeric sequence, we next asked whether Pot1 binding would affect telomerase activity. Anti-myc immunoprecipitations were performed on cell extracts from a strain expressing myc-tagged telomerase (Trt1-myc9), and robust telomerase activity was observed with the SC1 primer (Fig. 7D, lane 1). Preincubation of the primer with an excess of Pot1N as high as 76-fold had no effect on telomerase activity (Fig. 7D, lanes 4 and 5); however, addition of Pot1 resulted in a dosage-dependent inhibition of telomerase (Fig. 4A, lanes 2 and 3). At an equimolar Pot1:template ratio, telomerase activity was reduced by 5-fold, and when a 3.5-fold excess of Pot1 was added, telomerase activity was almost completely ablated. These results are consistent with Pot1 sequestering the DNA substrate and preventing access by telomerase.

DISCUSSION

The fission yeast Pot1 protein is critical for chromosome end protection, as evidenced by the rapid loss of telomeres when Pot1 function is lost. Recently, RNA interference-mediated knock-down of the human homolog, hPot1, revealed telomere instability phenotypes that are strikingly similar to the fission yeast knock-out, indicating that the function of telomere end protection is conserved evolutionarily (38). Overexpression of...
FIG. 7. Reduced sequence specificity in the context of full-length Pot1.
A, Pot1 and Pot1N binding was compared for an oligonucleotide containing six tandem natural telomeric repeats, and a corresponding oligonucleotide in which each tandem repeat has been mutated at the third position (both at 250 nM). B, Pot1 and Pot1N were tested for their ability to bind telomeric sequences from 250 nM T. thermophila. C, Pot1 and Pot1N were tested for binding to a telomeric sequence derived from S. cerevisiae (SC1) TGTG-TGTGGGTGTGG; 250 nM. D, telomerase activity was measured using SC1 as template in the presence or absence of the indicated amounts of Pot1 or Pot1N as described in Ref. 37. The lower panel represents an internal loading control. Samples were run on a 10% sequencing gel in TBE, dried onto Whatman paper, and subjected to phosphorimaging analysis.

hPot1, expression of a COOH-terminal fragment of hPot1 (hPot1OB), and moderate down-regulation of hPot1 by RNA interference all lead to telomere elongation in telomerase-positive cells in culture, indicating a role for hPot1 in telomere length regulation (27, 28, 39, 40).

The importance of Pot1 for chromosomal stability and for controlling access to the ends of telomeres provides impetus to examine its interactions with telomeric DNA closely. Here we have characterized the interactions of Pot1 with naturally occurring telomeric DNA substrates. Compared with the Pot1N fragment, Pot1 occupies a larger binding site and forms a more stable complex with DNA. Surprisingly, the high degree of sequence specificity observed with Pot1N is relaxed in the context of the full-length protein. We show that Pot1 binding to a telomeric primer prevents access by telomerase and protects telomeric DNA from exonucleolytic degradation. Interestingly, because Pot1 binds in a specific register with respect to the telomeric repeats, a subset of possible terminal sequences are protected, whereas others remain accessible. These findings may have important consequences for telomere end processing in vivo as well as for regulating access by telomerase.

Comparison of Pot1N and Pot1—There has been a long tradition of studying DNA binding domains of transcriptional regulators in isolation, and in many cases the properties of the isolated domain closely reflect the behavior of the naturally occurring protein. The success of this approach relies on the independent folding of structural domains and on defining the part of a protein that encompasses the entire DNA binding domain. Its limitations lie in the fact that inter- as well as intramolecular protein-protein interactions can affect affinity, specificity, and cooperativity of binding.

Pot1N was isolated among proteolytic fragments of Pot1 based on its high affinity for G-rich telomeric DNA (14). The boundaries were defined by mass spectrometry and amino-terminal sequencing, and the fragment was cloned, expressed in E. coli, and purified. The well behaved nature of Pot1N during purification further supported the notion that this part of the protein constitutes an independently folding domain, a supposition that was later confirmed by crystallographic analysis (32). Biochemical studies showed that Pot1N binds specifically and cooperatively to telomeric repeats, with each monomer occupying a hexameric binding site (Ref. 33 and Fig. 4).

In the context of full-length Pot1, the binding site is increased by 3–4 nucleotides, the affinity for DNA is higher, and the sequence specificity is reduced. These observations suggest that protein sequences outside of Pot1N contribute to Pot1-DNA interactions and are consistent with the presence of a second DNA binding domain downstream from Pot1N. The first OB-fold displays high specificity for the GGCTAC sequence and aligns Pot1 in a defined register and orientation. Additional DNA contacts are then provided on the 3′-site by the predicted second OB-fold, thereby increasing the stability of the complex and extending the protected region. We were initially surprised to find that the full-length protein displays reduced sequence specificity for pombe telomeric repeats compared with Pot1N. However, given the sequence variability in the spacer regions between repeats, relaxed sequence specificity of the second OB-fold may ensure that all single-stranded telomeric sequences are bound by Pot1. In this context, it is interesting to note that the GGCTAC motif occurred six times in our data set of telomeric sequences, whereas neither GGTTAC nor GGTGTAC sequences were present. Although an oligonucleotide containing the former motif is bound by Pot1 (Fig. 7A), neither of the two latter is (supplemental Fig. 2B). It appears that the selective advantage in Pot1 recognizing a broader spectrum of sequences compensates for the possibility of Pot1 mislocalizing to single-stranded DNA, which arises transiently at other sites in the genome.
Conservation among Chromosome End-capping Proteins—

TEBP{s} appear to be universal among eukaryotes, and related proteins have been identified in a variety of organisms ranging from yeast and ciliated protozoa to plants and vertebrates. Human and chicken Pot1 have been cloned and characterized biochemically (14, 29, 34, 41). The minimal DNA binding site for hPot1 has been defined as TAGGTTAG in the presence of nontelomeric 5'-sequence and as TTAGGGTTAG in isolation. Like fission yeast Pot1, the human homolog binds in a specific register, and on longer DNA substrates each hPot1 monomer could occupy two repeats (34). Chicken Pot1 does not form a stable complex unless two complete GTTATG repeats are present (29).

The recently solved crystal structure of hPot1 splice variant 2 bound to telomeric DNA revealed that the amino-terminal OB-fold interacts with the first 6 nucleotides and contributes most of the sequence specificity, with the second OB-fold adding additional DNA contacts and protecting the 3'-end (34). The crystal structure of Pot1N and our DNA binding data suggest a similar arrangement for fission yeast Pot1. Although the core telomeric repeat unit is similar between fission yeast and vertebrates, the sequences recognized by the respective amino-terminal OB-folds of Pot1 and hPot1 are permutations of each other (TTAGGG versus GTTAT). This difference may have evolved as a consequence of the respective repeat units synthesized by telomerase.

The realization that tandem OB-folds are involved in telomeric DNA binding, rather than a single OB-fold as initially suspected for fission yeast Pot1, adds to the apparent similarity for hPot1 has been defined as TTAGGGTTAG in the presence of the RNA subunit of telomerase. Such a role for Pot1 as an inhibitor of telomerase is consistent with the analysis of telomere sequences and Kalman Benesath for contributions during the early stages of this work. We also thank Chris Counter, Julie Cooper, and Tom Cech for sharing data prior to publication and Jerry Workman and Joan Conaway for critical reading of the manuscript.

Acknowledgments—We thank Earl Glyn for computational help with the analysis of telomere sequences and Kalman Benesath for contributions during the early stages of this work. What could reconcile these findings is the observation that the majority of hPot1 appears to localize to the telomeres via protein-protein interactions with Ptop/Pip1, which in turn binds to Tin2 forming a bridge with the Trf1- and Trf2-containing complexes bound to the double-stranded part of the telomere (40, 44, 45). Reducing the amount of Trf1, Tin2, or Ptp bound to chromosome ends all leads to telomere elongation, consistent with these proteins forming a higher order structure that impedes telomere elongation (40, 45–47). Pot1 may serve to sequester the single-stranded portion of the telomere into this complex and thereby transmit telomere length information to the terminus as suggested previously (27).

Inhibition and stimulation of telomerase by Pot1 need not be mutually exclusive as evidenced by telomere length regulation in budding yeast. Cdc13 negatively regulates telomerase length in a complex with Stn1 but actively recruits telomerase by interacting with the telomerase subunit Est1 (21, 48). Homologs of Est1 have been identified recently in fission yeast and human cells (49–51), making it an attractive hypothesis that the basic mechanism of telomere length regulation is also conserved.

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