Interaction of an *Arabidopsis* RNA-Binding Protein with Plant Single-Stranded Telomeric DNA Modulates Telomerase Activity*

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SUMMARY

Telomeres are the specialized structures at the end of linear chromosomes and terminate with a single-stranded 3’ overhang of the G-rich strand. The primary role of telomeres is to protect chromosome ends from recombination and fusion and from being recognized as broken DNA ends. This protective function can be achieved through association with specific telomere-binding proteins. While proteins that bind single-stranded G-rich overhang regulate telomere length and telomerase activity in mammals and lower eukaryotes, equivalent factors have yet to be identified in plants. Here we have identified proteins capable of interacting with the G-rich single-stranded telomeric repeat from the *Arabidopsis* extracts by affinity chromatography. MALDI-TOF mass spectrometry analysis indicates that the isolated protein is a chloroplast RNA-binding protein (and a truncated derivative). The truncated derivative, which we refer to as STEP1 (single-stranded telomere-binding protein 1), binds specifically the single-stranded G-rich plant telomeric DNA sequences but not double-stranded telomeric DNA. Unlike the chloroplast-localized full-length RNA-binding protein, STEP1 localizes exclusively to the
nucleus, suggesting that it plays a role in plant telomere biogenesis. We also demonstrated that
the specific binding of STEP1 to single-stranded telomeric DNA inhibits telomerase-mediated
telomere extension. The evidence presented here suggests that STEP1 is a telomere-end binding
protein that may contribute to telomere length regulation by capping the ends of chromosomes
and thereby repressing telomerase activity in plants.

INTRODUCTION

Telomeres are the specialized nucleoprotein complexes of eukaryotic chromosome ends that
are essential for chromosome stability and integrity (1-3). Telomeres enable cells to distinguish
chromosome ends from double-strand breaks in the genome. Without functional, intact
telomeres, the chromosomes are prone to nucleolytic degradation leading to apoptosis and cell
death (4-6). Alternatively, the chromosome ends may rearrange by recombination or end-to-end
fusion. Dividing cells show progressive loss of telomeric DNA during successive rounds of
replication because the lagging strand of DNA synthesis is unable to replicate the extreme 3’ end
of the chromosome (7). Thus, telomere shortening has been proposed as a regulatory mechanism
that controls the replicative capacity of primary cells and cellular senescence by acting as a mitotic clock (8). The loss of telomeric sequences can be avoided by the reactivation of telomerase, the ribonucleoprotein enzyme that carries out telomere elongation and thus stabilizes the telomere length (9). Telomeres in most eukaryotes are composed of tandem repeats of short sequence elements, which are five to eight nucleotides long (10). The G-rich strands of the repeated sequences terminate as a single-stranded 3’ overhang (11, 12). In vivo, it is thought that telomeres form a large folded loop (t-loop) formed by the invasion of the 3’ overhang into a duplex region of telomeric repeats (13, 14).

Telomere maintenance is achieved through association with specific binding proteins. Telomere proteins that specifically interact with double-stranded telomeric DNA include budding yeast Rap1p (15), fission yeast Taz1p (16), human TRF1 and TRF2 (17, 18), and plant RTBP1 and AtTB1 (19, 20). Several proteins specific to single-stranded telomeric G-rich overhangs have been reported to be involved in telomeric end protection and length maintenance. In Saccharomyces cerevisiae, Cdc13p and Est1p are single-stranded telomeric DNA binding proteins required for chromosome end protection and telomere replication (21). Cdc13p mediates telomerase access to chromosome termini by a direct interaction with Est1, a component of telomerase that interacts with telomerase RNA (22). The fission yeast Pot1p plays a direct role in protecting the ends of chromosomes (23). Deletion of pot1 gene affects chromosome stability, causing rapid loss of telomeric DNA and circularization. In unicellular organisms, several
telomere end-binding proteins, such as αβ protein from *Oxytricha* (24), TBP from *Euplotes* (25), TEP and TGP from *Tetrahymena* (26, 27), GBP from *Chlamydomonas* (28), have been characterized. These proteins protect single-stranded overhangs from nucleolytic degradation and chemical modification, and thus confer chromosome stability. In mammals, members of hnRNP family have been reported to associate with single-stranded G-rich extensions and to have roles in telomere and telomerase regulation (29). HnRNP A1/UP1 protein is the first single-stranded binding protein shown to be directly involved in mammalian telomere biogenesis (30). Short telomeres in hnRNP A1-deficient mouse cell line can be elongated by restoring A1 expression. Since expressing UP1, the proteolytic fragment of hnRNP A1, does not affect alternative splicing (but increases telomere length in hnRNP A1-deficient cells), the effect of hnRNP A1 on telomere length regulation is not due to A1-induced alternative splicing. HnRNP A2/B1 and D have also been reported as functional G-rich single-stranded DNA binding proteins (31, 32).

Telomeric DNA in plants consists of tandem arrays of TTAGGG repeats (33). Plants share many common features in the regulation of telomere length and telomerase activity with mammals (34-36). The dicot plants, *Silene latifolia* and *Arabidopsis thaliana* telomeres carry G-rich overhangs longer than 20-30 nucleotides (37); however, in contrast to mammalian telomeres, only half of the telomeres possess detectable G-rich overhangs, implying that two distinct telomere architectures may exist in plants. Nevertheless, the presence of G-rich overhangs in plant telomeres suggests that single-stranded telomere-binding activity plays an
important role in telomere function. In a search for proteins capable of interacting with the G-rich single-stranded telomeric DNA, we isolated several polypeptides from *Arabidopsis* extracts by affinity chromatography. MALDI-TOF MS analysis allowed us to identify three proteins that turned out to be multiple isoforms encoded by a chloroplast RNA-binding protein gene (38, 39). One of the naturally occurring isoforms, which we refer to as STEP1 (single-stranded telomere-binding protein 1), lacks a chloroplast transit peptide and acidic domain but contains RBDs (RNA-binding domains). Here we show that STEP1 binds specifically the single-stranded G-rich plant telomeric DNA sequences but not double-stranded telomeric DNA. STEP1 binding to single-stranded telomeric DNA inhibits telomerase-mediated telomere extension. We also demonstrate that STEP1 is targeted to the nucleus. Thus, our results suggest that STEP1 could function *in vivo* to protect the ends of chromosomes and modulate telomere replication in plants.
EXPERIMENTAL PROCEDURES

Preparation of Plant Extracts—Four week-grown Arabidopsis whole plants were ground in a mortar and pestle under liquid nitrogen, suspended in buffer A (25 mM Tris-HCl, pH 8.5, 10 mM MgCl₂, 0.25 M dextrose, 0.25 M sucrose, and 10 mM β-mercaptoethanol), filtered through several layers of cheese cloth, and centrifuged at 10,000 x g at 4°C for 10 min. The pellet was washed twice with buffer A and resuspended with buffer B (50 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, and 0.1 mM PMSF). Nonidet P-40 was immediately added to a final concentration of 1% and mixed for 30 min at 4°C. To extract proteins, one-tenth volume of 5 M NaCl was slowly added and mixed for 60 min at 4°C with constant stirring. The extracts were then subjected to centrifugation at 20,000 x g for 30 min at 4°C. The supernatant were extensively dialyzed against buffer B and stored at -80°C until used. Protein concentrations in extracts ranged from 1 to 2 mg/ml.

Affinity Chromatography—Streptavidin immobilized on agarose (Sigma) was activated by incubation with the 3’ biotinylated oligodeoxyribonucleotide (TTTAGGG)₆. For protein purification, the activated resin (1 ml) was packed in a column and extensively washed with binding buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT, and 5% Glycerol).
Arabidopsis extracts containing 10 mg of protein were preincubated with 100 µg of nonspecific single-stranded oligodeoxyribonucleotide (Table I) to reduce nonspecific DNA-protein binding and then loaded onto the column. After washing the column with 20 ml binding buffer, the bound proteins were eluted with 0.1% SDS in binding buffer.

Mass Spectrometry and Protein Identification— The gel pieces corresponding to protein bands were excised from the polyacrylamide gel, crushed, and destained by washing with 50% acetonitrile in 25 mM NH₄HCO₃. After washing with 100% acetonitrile, the gel pieces were dried and incubated in trypsin digestion solution containing 10 µg/ml of sequencing grade trypsin (Promega) in 25 mM NH₄HCO₃ at 37°C for 16 hr. Peptides were extracted by incubating the gel pieces with 50% acetonitrile and 0.1% trifluoroacetic acid at 37°C for 1 hr. The extraction was repeated three times, and the extracted solutions were pooled and evaporated in a SpeedVac vacuum centrifuge. The dried peptides were resuspended in 0.1% trifluoroacetic acid and treated with ZipTips containing C18 resin (Millipore) according to the manufacturer’s instructions. The washed peptides were eluted with matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid and 50% acetonitrile), applied to the sample plate and then dried. Peptides molecular weights were measured on a MALDI-TOF mass spectrometer (Voyager-DE STR; Applied Biosystems, Inc.). For interpretation of the mass spectra, we used the MS-Fit program available on the web site (prospector.ucsf.edu).
Production and Purification of Recombinant Proteins—The plasmid vector, pGEX-6p-1 (Amersham Pharmacia Biotech) was used for the expression of full length and various deletion proteins. Recombinant proteins were expressed in Escherichia coli BL21 (DE3). Fusion proteins were purified by affinity chromatography using glutathione Sepharose 4B (Amersham Pharmacia Biotech) as manufacturer’s instructions. To obtain the proteins lacking the GST moiety, the bound proteins were treated with Prescision Protease (Amersham Pharmacia Biotech), and the released proteins were collected.

Electrophoretic Mobility Shift Assay (EMSA)—DNA probes and competitors used for EMSA are described in Table I. DNA probes were end-labeled with γ-[32P]-ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England Biolabs), and purified by free nucleotide removal spin column (Qiagen). The recombinant proteins were preincubated with 0.5 µg of poly (dI-dC) and 0.5 µg of nonspecific single-stranded DNA oligonucleotide in 20 µl of a binding buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 5% glycerol) for 15 min on ice to reduce nonspecific DNA-protein binding. End-labeled DNA probe (0.3 ng) was then added to the reaction mixtures and incubated for 10 min at room temperature. The mixtures were loaded on an 8% nondenaturing polyacrylamide gel, and the binding activity was quantified with an image analyzer (BAS 2500; Fuji Photo Film). For competition experiments, various competitors were preincubated with proteins before the addition of labeled DNA probe.
Telomerase Assays—Telomerase activity was detected by a modified version of the TRAP assay (35). Whole plant extracts were prepared by grinding of 7 day-grown Arabidopsis in CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 5 mM β-mercaptoethanol, 10% glycerol, and 0.5% CHAPS). The GG substrate primer (5’-CACTATCGACTACGCGATCGG-3’) and the antisense telomeric repeat primer (5’-CCCTAAACCCTAAACCCTAA-3’) were used as forward and reverse primers, respectively. Telomerase extension reactions were carried out in TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween-20, and 1 mM EGTA) containing 200 nM GG primer, and 100 µM concentrations of each dNTP. Extracted plant protein (1.5 µg) was added to the reaction mixture and incubated at room temperature for 45 min. The extended products were extracted with phenol/chloroform and precipitated with ethanol. The DNA samples were amplified by PCR using 200 nM forward primer and 200 nM reverse primer for 30 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, with an additional 10 min extension step at the end. The amplified products were resolved by 10% nondenaturing polyacrylamide gel and visualized by staining with SYBR Green (Molecular Probes). The signal intensity was quantified with an image analyzer (LAS-1000 Plus; Fuji Photo Film). To test RNA-dependent extension, RNase A (0.25 mg/ml) was added to the extract before incubation with the GG primer.

Nuclear Localization Analysis of STEP1—The green fluorescent protein (GFP) expression
vector pBI221-GFP was generated by replacement of GUS region of pBI221 vector (BD Biosciences Clontech) with GFP. The coding sequence of STEP1 was inserted into pBI221-GFP to be fused in frame to the N-terminus of GFP coding sequence. The fusion construct was introduced into onion epidermal cells using a helium biolistic particle delivery system according to the manufacturer’s instructions (BioRad). After incubation for 24 hr at 22°C, the subcellular distribution of GFP was examined by fluorescence microscopy (Zeiss). A pBI221-GFP construct without STEP1 was used as a control.

RESULTS

Identification of Arabidopsis Proteins That Specifically Bind to the Single-Stranded G-Rich
Telomeric Repeats—Proteins that bind single-stranded telomeric overhang play an important role in chromosome capping and telomere length regulation (21, 30, 40). Such proteins have not yet been identified and characterized in plants. To identify telomere end-binding proteins in plants, affinity chromatography using single-stranded (TTAGGG)_6 oligodeoxyribonucleotide was applied to Arabidopsis extracts. The eluted fraction was subjected to SDS-PAGE and stained with Coomassie blue (Fig. 1). The electrophoretic pattern of the proteins recovered by affinity chromatography shows two major bands, three intermediate bands, and six faint bands having apparent molecular masses ranging from 20 to 64 kDa (Lane 2). No stainable protein was retained when streptavidin-agarose conjugate resins were not functionalized (Lane 3). Since Arabidopsis extracts were preincubated with nonspecific single-stranded oligonucleotide as a competitor DNA, the retained proteins should show high specificity for the single-stranded G-rich telomeric sequence. To identify the protein components with the telomere binding activity, each protein band was excised from the gel and digested with trypsin, and the recovered peptide mixtures were directly subjected to MALDI-TOF MS analysis. The obtained mass data were fitted by MS-fit data base search analysis. Although there were some unidentified bands that had no matching proteins in the database (or the amount of protein was too little to be precisely identified), this method allowed us to identify nine polypeptides. Table II shows the peptide mass fingerprint results, which contain NCBI accession number, theoretical and experimental molecular weights, the number of matching peptides, and the amino acid coverage of the obtained
peptides. Bands 6 and 7 were identified as a chloroplast RNA-binding protein (cp31) that contains four structural domains: a chloroplast transit peptide (TP) in the N-terminus, followed by an acidic domain (AD) and a tandem pair of RNA-binding domains (RBD1 and RBD2) in the C-terminus (Fig. 2A) (38, 39). Band 11 was identified as a truncated derivative (RNA binding protein 3) lacking the N-terminal chloroplast transit peptide and acidic domain. It was previously reported that the single chloroplast RNA-binding protein gene generates multiple messages that potentially encode two distinct proteins (38). While the larger messages encode a full-length protein that functions in chloroplast, the shorter messages encode a truncated derivative (named STEP1 in this study) that is likely to function in cellular compartments other than chloroplast due to the absence of a chloroplast transit peptide (38).

Binding of STEP1 to Single-Stranded Telomeric Repeats—STEP1 contains an RNA-binding domain and is a member of the hnRNP family. The hnRNPs have been implicated to have roles in telomere and telomerase regulation (29, 30). Therefore, we sought to examine whether STEP1 interacts directly with the single-stranded G-rich telomeric repeat. Recombinant forms of full-length cp31, STEP1, N-terminal half (NT), RBD1, and RBD2 were expressed in bacteria (Fig. 2A and 2B) and used in gel shift assays with a labeled PT4 oligonucleotide having four plant telomeric repeats. As shown in Fig. 2C, cp31 gave rise to a discrete protein/DNA complex that migrated more slowly than the free probe. Intensities of shifted bands increased upon the addition of increasing amounts of the protein. Likewise, STEP1 bound slightly more efficiently to PT4
than cp31. In contrast, NT containing the transit peptide (TP) and acidic domains (AD) did not show any binding activity under the conditions used in these experiments. We also tested the binding activity of each RBD. No binding activity was observed in RBD1 or RBD2.

Sequence Specificity of STEP1 Binding to Single-Stranded Telomeric Repeats—To determine the relative binding specificity of STEP1 to plant telomeric sequence, we performed various competition experiments. The complex detected in STEP1 was significantly reduced in the presence of 10-fold excess of unlabeled PT4 oligonucleotide and completely disappeared by 50-fold excess (Fig. 3A). When the human telomeric repeats were substituted for the plant telomeric repeats, HT4 competed less efficiently than its counterpart for the PT4-binding activity. In contrast, the signals were not diminished by the addition of the same molar excess of unlabeled *C. elegans* telomeric repeats. To see whether STEP1 binds to double-stranded telomeric DNA, a duplex DNA that contains 10 copies of the telomeric repeats was prepared. Even when excess amounts of double-stranded telomeric DNA were used as competitor DNA, the binding activity remained unaffected (Fig. 3A).

We next determined the sequence specificity of the STEP1 binding activity at nucleotide level. A series of mutant oligonucleotides were synthesized and assayed for their ability to bind STEP1 (see Table I for the sequences of mutant oligonucleotides). Each oligonucleotide contained a single nucleotide transition at the same position in all four repeats of TTTAGGG. Gel shift assays with STEP1 revealed that A4 shifted efficiently than PT-4, whereas G5 and G7 were
slightly weaker binders. However, T1, T2, T3, and G6 were incapable of significant binding (Fig. 3B). These results indicate that the binding between STEP1 and plant telomeric repeats is very specific for the first 3 bases and the sixth base of the TTTAGGG repeats, with some additional preference for a T over an A in position 4 of the repeats. The minimal number of the telomeric repeats needed for binding was determined using oligonucleotides containing different copy numbers of the telomeric repeats. STEP1 did not bind two-copies of TTTAGGG, but it efficiently bound three-copies of TTTAGGG, although the binding affinity is slightly lower than four-copies of TTTAGGG (Fig. 3C). These results indicate that the minimal length of the DNA probe required for complex formation is at least three contiguous TTTAGGG repeats.

**STEP1 Inhibits Telomerase-Mediated Extension**—To examine whether STEP1 could affect the ability of telomerase to extend telomeric DNA and to gain some insight into the possible function of the protein, recombinant STEP1 lacking the GST moiety was added to the telomerase fraction extracted from *Arabidopsis*. After the telomerase reaction, the extended telomeric DNA was purified to avoid *Taq* polymerase inhibitors contained in the extracts and to exclude the effect of supplemented STEP1 on PCR, and amplified by the TRAP method. The amplified products were analyzed on a nondenaturing polyacrylamide gel (Fig. 4). No telomere elongation was detected in the absence of telomerase extract or when the extract was treated with RNase to inactivate the telomerase activity. In the absence of STEP1, telomerase activity was readily detected, indicative of addition of multiple repeats to the substrate primer. As shown in Fig. 4A,
the supplemented STEP1 inhibited telomerase extension in a dose-dependent manner. Note that inhibition by STEP1 reduced the overall rate of telomerase reaction, but not the relative length of DNA products. To allow quantification, the band intensities of extension products were analyzed, and dose-response curves were generated (Fig. 4C). Addition of 0.6 µM STEP1 reduced the formation of the telomerase products by 64% when compared with telomerase activity in the absence of STEP1. To examine whether STEP1 binding to the extended telomerase substrates is required for the inhibition of telomerase activity, telomerase reaction mixtures were mixed with either NT, RBD 1, or RBD 2 (which do not bind to single-stranded telomeric repeats). None of these proteins inhibited telomerase extension (Fig. 4B and 4C). Because STEP1 binds to the human telomeric repeats although its binding is weaker than the plant telomeric repeats, we examined whether STEP1 affects the activity of human telomerase. Addition of increasing amounts of STEP1 led to a gradual reduction in telomerase activity (Fig. 4D). Taken together, our results suggest that stable STEP1 binding to telomeric DNA inhibits telomerase activity.

*Nuclear Localization of STEP1—* Since STEP1 lacks a chloroplast transit peptide and binds specifically to telomeric repeats, it could function in nucleus. To address this issue, we determined whether **STEP1 is targeted to the nucleus**. The coding sequence of STEP1 was fused in frame to the N-terminus of GFP coding sequence using a conventional GFP fusion recombinant plasmid. The gene encoding STEP1-GFP was introduced into onion epidermal cells using a helium biolistic particle delivery system. The onion epidermal cells expressing the STEP1-GFP
fusion protein were examined with a fluorescence microscope. Fluorescence was detected as a dense glittering spot which corresponds well with the position of the nucleus (Fig. 5). For comparison, GFP alone was used as a control for subcellular localization. GFP control was distributed in both cytoplasm and nucleus. Unlike the chloroplast-localized full-length cp31 protein, our observations suggest that STEP1 is capable of localizing exclusively to the nucleus and is involved in plant telomere biogenesis.

DISCUSSION

In most species examined to date, telomeres are highly conserved structures that terminate with a single-stranded overhang of the G-rich strand (11, 12). The fact that disruption of the G-
strand overhang results in loss of telomere protection suggests that the 3’ overhang plays an essential role in telomere function (18, 41). The primary role of telomeres is to protect chromosome ends from recombination and fusion and from being recognized as broken DNA ends. This protective function of telomeres can be achieved through association with specific telomere-binding proteins. In this study, we have identified proteins capable of interacting with the G-rich single-stranded telomeric repeat from the Arabidopsis extracts by affinity chromatography. MALDI-TOF MS analysis indicates that the isolated proteins with the telomere-binding activity are chloroplast RNA-binding protein cp31 and its shortened derivative STEP1. We have shown that STEP1 binds to the single-stranded G-rich plant telomeric repeat and that this binding is sequence specific. We also have examined the effect of STEP1 on the process that is relevant to telomere function. The binding of STEP1 to telomeric repeat prevents in vitro telomere extension by telomerase. Because STEP1 is a naturally occurring isoform of chloroplast RNA-binding protein (38) and is targeted to the nucleus, its interaction with G-rich telomere overhang may contribute to telomere stabilization and influence telomere lengths by modulating the telomerase activity.

The cp31 protein contains conserved RBD structural motifs as hnRNP A1/UP1, A2/B1, and D proteins which have been previously reported to specifically bind to G-rich telomeric repeat sequences (30-32). The cp31 gene encodes multiple transcripts with different 5’ ends possibly due to multiple transcription start sites (38). While the larger messages encode the full-length
protein that functions in chloroplast, the shorter messages encode STEP1. Although STEP1 contains a RBD1 lacking the N-terminal 17 amino acids and a complete RBD2, it binds to telomeric repeat with a slightly higher affinity than the full-length protein. In contrast, each RBD alone is not sufficient for binding to telomeric DNA sequences, suggesting the requirement of cooperativity between two RBDs. These findings are consistent with a previous observation indicating that both RBDs of hnRNP A1/UP1 are required for binding to telomeric DNA. The co-crystal structure of UP1 bound to telomeric DNA revealed that UP1 binds as a dimer to two strands of DNA, and that each strand contacts RBD1 of one monomer and RBD2 of the other (42). Thus, we compared the conservation of amino acid residues between cp31 and UP1 at positions involved in contacting telomeric DNA. Thirty two amino acid residues of UP1 are involved in direct contacts with telomeric repeats (42). An alignment of the RBDs of cp31 and UP1 revealed that 21 of 32 (66%) of the contact residues are conserved, while 54% overall sequence similarity was observed between cp31 and UP1 (Fig. 6). This sequence conservation suggests the ability of STEP1 to interact with telomeric DNA in a way that mirrors UP1. However, in contrast to co-crystal structure of UP1, the recent biochemical assays indicated that RBD1 alone is sufficient for strong and specific binding to telomeric repeat in vitro, and that UP1 does not simultaneously interact with two telomere strands (43, 44). Therefore, it is of immediate interest to determine the binding mode of STEP1 to telomeric repeat.

The critical question that remains to be answered is whether STEP1 binds the plant telomeric
repeat in vivo. The homology displayed by STEP1 to the RBDs of other telomere end-binding proteins suggests that these proteins are functionally related. Proteins that bind single-stranded telomeric sequences have been shown to regulate telomere lengths and telomerase activity (21, 30, 40). Such proteins include Est1p and Cdc13p in Saccharomyces cerevisiae. EST1 mutant leads to progressive telomere shortening and eventual loss of viability (45). Est1p was subsequently shown to bind the single-stranded G-rich overhang (46) and to be associated with telomerase via an RBD that is able to directly bind the RNA component of the enzyme (47). Cdc13 also binds the single-stranded G-rich telomeric sequences and apparently caps the telomere, protecting it from nuclease digestion (21, 48). In mammals, the interaction of hnRNP A1/UP1 with telomeric single-stranded overhang protects telomeric DNA from nuclease attack (43). This observation suggests that hnRNP A1/UP1 may play a crucial role in vivo in telomere protection. Based on the evolutionary conservation of telomere sequences and functions, the functional homology between STEP1 and other RBD-containing proteins raises the interesting possibility that STEP1 may be the important component of protective capping structure associated with telomere function in plants. This hypothesis is further supported by the observation that STEP1 is localized exclusively to the plant nucleus.

In plants, as in mammals, the G-rich overhangs serve as substrates for extension by telomerase. The G-rich overhangs longer than 20-30 nucleotides are present at the ends of
chromosomes (37). The human hnRNP A1/UP1 (43) and the *Oxytricha* telomere end-binding proteins (49) inhibit telomerase extension *in vitro* by rendering the telomeric DNA inaccessible. Likewise, the interaction of STEP1 with plant single-stranded telomeric repeat inhibits telomerase extension. Our data show that the inhibition by STEP1 reduces the overall rate of telomerase reaction but does not affect the relative length of DNA products. This suggests that STEP1 does not change the processivity of the enzyme but alters the accessibility of telomeric DNA, thereby helping maintain telomere length. Consistent with our results, telomere-binding proteins that can inhibit telomerase activity have been detected in plant cell nuclei (50). Recently, the telomeric DNA from the higher order plant *Pisum sativum* (garden pea) has been reported to be arranged into t-loops as shown in mammals (51). This observation suggests that t-loop structure is highly conserved as a mechanism to protect chromosome ends. The t-loop seems likely to inhibit telomeric elongation by telomerase. However, telomerase must gain access to a 3’ overhang at some point in the cell cycle. During S phase, t-loops might be disassembled by the DNA replication machinery. The newly formed 3’ overhang may remain free for a sufficient time to provide telomerase access to the 3’ end (52). Thus, it is possible that the binding of STEP1 to newly formed telomere extensions plays a role in the maintenance of 3’ overhangs in plants. Although the interaction of STEP1 with single-stranded overhangs remains to be determined *in vivo*, the evidence presented here suggests that STEP1 may contribute to telomere
length regulation by capping the ends of chromosomes and thereby repressing telomerase activity in plants.

REFERENCES

1. Blackburn, E. H. (1999) *Nature* **350**, 569–573

2. Greider, C. W. (1996) *Annu. Rev. Biochem.** 65, 337–365

3. Muniyappa, K., and Kironmai, K. M. (1998) *Crit. Rev. Biochem. Mol. Biol.* **33**, 297–336
4. Blackburn, E. H. (2000) *Nature* **408**, 53-56

5. Gasser, S. M. (2000) *Science* **288**, 1377-1379

6. Kim, S. H., Kaminker, P., and Campisi, J. (2002) *Oncogene* **21**, 503-511

7. Lingner, J., Cooper, J. P., and Cech, T. R. (1995) *Science* **269**, 1533-1534

8. Harley, C. B. (1991) *Mutat. Res.* **256**, 271-282

9. Blackburn, E. H. (1992) *Annu. Rev. Biochem.* **61**, 113-129

10. Zakian, V. A. (1995) *Science* **270**, 1601-1606

11. Makarov, V. L., Hirose, Y., and Langmore, J. P. (1997) *Cell* **88**, 657-666

12. Wright, W. E., Tesmer, V. M., Huffman, K. E., Levene, S. D., and Shay, J. W. (1997) *Genes Dev.* **11**, 2810-2821

13. Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999) *Cell* **97**, 503-514

14. Greider, C. W. (1999) *Cell* **97**, 419-422

15. Lustig, A. J., Kurtz, S., and Shore, D. (1990) *Science* **250**, 549-553

16. Cooper, J. P., Nimmo, E. R., Allshire, R. C., and Cech, T. R. (1997) *Nature* **385**, 744-747

17. van Steensel, B., and de Lange, T. (1997) *Nature* **385**, 740-749

18. van Steensel, B., Smogorzewska, A., and de Lange, T. (1998) *Cell* **92**, 401-413

19. Yu, E. Y., Kim, S. E., Kim, J. H., Ko, J. H., Cho, M. H., and Chung, I. K. (2000) *J. Biol. Chem.* **275**, 24208-24214
20. Hwang, M. G., Chung, I. K., Kang, B. G., and Cho, M. H. (2001) FEBS Lett. 503, 35-40

21. Nugent, C. I., Hughes, T. R., Lue, N. F., and Lundblad, V. (1996) Science 274, 249-252

22. Evans, S. K., and Lundblad, V. (1999) Science 286, 117-120

23. Baumann, P., and Cech, T. R. (2001) Science 292, 1171-1175

24. Raghuraman, M. K., and Cech, T. R. (1989) Cell 59, 719-728

25. Price, C. M. (1990) Mol. Cell. Biol. 10, 3421-3431

26. Schierer, T., and Henderson, E. (1994) Biochemistry 33, 2240-2246

27. Sheng, H., Hou, Z., Schierer, T., Dobbs, D. L., and Henderson, E. (1995) Mol. Cell. Biol. 15, 1144-1153

28. Petracek, M. E., Konkel, L. M. C., Kable, M. L., and Berman, J. (1994) EMBO J. 13, 3648-3658

29. Ford, L. P., Wright, W. E., and Shay, J. W. (2002) Oncogene 21, 580-583

30. LaBranche, H., Dupuis, S., Ben-David, Y., Bani, M. R., Wellinger, R. J., and Chabot, B. (1998) Nat. Genet. 19, 199-202

31. Kamma, H., Fujimoto, M., Fujiwara, M., Matsui, M., Horiguchi, H., Hamasaki, M., and Satoh, H. (2001) Biochem. Biophys. Res. Commun. 280, 625-630

32. Eversole, A., and Maizels, N. (2000) Mol. Cell. Biol. 20, 5425-5432

33. Richards, E. J., and Ausubel, F. M. (1988) Cell 53, 127-136

34. Riha, K., Fajkus, J., Siroky, J., and Vyskot, B. (1998) Plant cell 10, 1691-1698
35. Fitzgerald, M. S., McKnight, T. D., and Shippen, D. E. (1996) Proc. Natl. Acad. Sci. U. S .A. 93, 14422-14427
36. Kilian, A., Heller, K., and Kleinhofs, A. (1998) Plant Mol. Biol. 37, 621-628
37. Riha, K., McKnight, T. D., Fajkus, J., Vyskot, B., Shippen, D. E. (2000) Plant J. 23, 633-641
38. Cheng, S. H., Cline, K., and DeLisle, A. J. (1994) Plant Physiol. 106, 303-311
39. Ohta, M., Sugita, M., and Sugiura, M. (1995) Plant Mol. Biol. 27, 529-539
40. Grandin, N., Reed, S. I., and Charbonneau, M. (1997) Genes Dev. 11, 512-527
41. Gravel, S., Larrivee, M., Labrecque, P., and Wellinger, R. J. (1998) Science 280, 741-744.
42. Ding, J., Hayashi, M. K., Zhang, Y., Manche, L., Krainer, A. R., and Xu, R. M. (1999) Genes Dev. 13, 1102-1115.
43. Dallaire, F., Dupuis, S., Fiset, S., and Chabot, B. (2000) J. Biol. Chem. 275, 14509-14516.
44. Fiset, S., and Chabot, B. (2001) Nucleic Acids Res. 29, 2268-2275.
45. Lundblad, V., and Szostak, J. W. (1989) Cell 57, 633-643.
46. Virta-Pearlman, V., D. K. Morris, and V. Lundblad. (1996) Genes Dev. 10, 3094-3104.
47. Zhou, J., Hidaka, K., and Futcher, B. (2000) Mol. Cell. Biol. 20, 1947-1955.
48. Lin, J. J., and Zakian, V.A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13760-13765.
49. Froelich-Ammon, S. J., Dickinson, B. A., Bevilacqua, J. M., Schultz, S. C., and Cech, T. R. (1998) Genes Dev. 12, 1504-1514.
50. Fulneckova, J., and Fajkus, J. (2000) FEBS Lett. 467, 305-310.
51. Cesare, A. J., Quinney N., Willcox, S., Subramanian D., and Griffith J. D. (2003) *Plant J.* **36**, 271-279.

52. Collins, K., and Mitchell, J. R. (2002) *Oncogene* **21**, 564-579.

53. Kim, J. H., Kim, J. H., Lee, G. E., Lee, J. E., and Chung I. K. (2003) *Mol. Pharmacol.* **63**, 1117-1124.

**FIGURE LEGENDS**

**FIG. 1.** Analysis of proteins recovered by affinity chromatography. Telomere-binding proteins were isolated from *Arabidopsis* extracts by affinity chromatography using single-stranded DNA oligonucleotide (TTTAGGG)$_6$. The eluted proteins were separated on an 8% SDS-PAGE and stained with Coomassie blue. *Lane 1*, molecular weight markers; *lane 2*, affinity chromatography recovered sample; *lane 3*, sample recovered from the non-functionalized resin. The two major bands (*closed triangles*), three intermediate bands (*open circles*), and six faint bands (*closed circles*) are indicated. *Arrows* indicate eleven polypeptides identified by MALDI-TOF MS analysis.

**FIG. 2.** Binding of STEP1 to single-stranded telomeric repeats. A, domain structure of the chloroplast RNA-binding protein (cp31). The positions of four functional domains are depicted:
a chloroplast transit peptide (TP), an acidic domain (AD), and a tandem pair of RNA-binding domains (RBD1 and RBD2). B, recombinant GST fusion proteins were purified on glutathione-Sepharose, separated on SDS-PAGE, and stained with Coomassie Blue. C, gel shift assays were performed with the labeled PT4 probe. Recombinant proteins were used at concentrations of 2 and 5 µM as indicated. Complexes were resolved on an 8% non-denaturing polyacrylamide gel. The positions of complexes and free PT4 probe are shown. The first lane contained free probe alone.

FIG. 3. Sequence specific binding of STEP1 to single-stranded telomeric repeats. A, competition assay for STEP1 binding to the PT4 probe. Labeled PT4 was incubated with STEP1 (5 µM) in the presence of either unlabeled PT4, HT4, CT4, or PT10(ds) as a competitor. The excess molar amounts of the competitors are indicated above each lane. The first lane contained free probe alone, and the second lane contained STEP1 (5 µM) without competitor. B, gel shift assay of STEP1 to the mutated telomeric repeats. Each probe contained a single nucleotide transition at the same position in all four repeats of TTTAGGG. Gel shift assays were carried out using STEP1 (5 µM) and labeled PT4 or mutated probes as indicated above each lane. The first lane contained free PT4 probe alone, and the second lane contained PT4 and STEP1. C, gel shift assays were performed with labeled PT4, PT3, or PT2 and STEP1. The first lane contained PT4 probe alone.
FIG. 4. **Inhibition of telomerase-mediated extension by STEP1.** A, telomerase extension assays were performed in an *Arabidopsis* extract using the GG substrate primer. STEP1 at concentrations of 0.2, 0.4, and 0.6 μM were added to the GG primer before incubation in the *Arabidopsis* mixture. The first lane contained no extract, and the second lane contained RNase A-treated extract. B, NT, RBD1, and RBD2 at concentrations of 0.2, 0.4, and 0.6 μM were added to the GG primer before incubation in the *Arabidopsis* mixture as indicated. C, the intensities of telomerase extension products in the presence of supplemented proteins was normalized to the intensities of extension products in the control without protein and plotted against the concentration of protein. D, telomerase assays were carried out in a telomerase-enriched extract prepared from HeLa cells using the TS primer as previously described (53). The first lane contained no extract, and the second lane contained RNase A-treated extract.

FIG. 5. **Nuclear localization of STEP1.** GFP fusion constructs were introduced into onion epidermal cells using a helium biolistic particle delivery system. A construct encoding only GFP was used as a control. GFP was fused in-frame to the C-terminus of the open reading frame of STEP1. The top row depicts the onion cell structure observed by light microscopy, and the bottom row depicts the subcellular localization of GFP (cytoplasmic and nuclear) and STEP1-GFP fusion protein (nuclear) observed by fluorescence microscopy. Nuclei are indicated by arrows.
FIG. 6. Amino acid sequence alignment of cp31 and hnRNP UP1. The regions corresponding to RBD1, RBD2, and spacer are shown. Sequence identity and similarity between the two RBDs are indicated by vertical lines and colons, respectively. The residue numbers are shown at the beginning of each line. UP1 residues critical for interaction with the telomeric repeat are shown in red. The STEP1 region of cp31 is shown in blue.

TABLE I
Oligonucleotides used in this study
| Oligonucleotide<sup>a</sup> | Sequence ($5'\rightarrow3'$) |
|--------------------------|-----------------------------|
| PT4                     | (TTTAGGG)$_4$               |
| HT4                     | (TTAGGG)$_4$                |
| CT4                     | (TTAGGC)$_4$                |
| T1                      | (ATTAGGG)$_4$               |
| T2                      | (TATAGGG)$_4$               |
| T3                      | (TTAAGGG)$_4$               |
| A4                      | (TTTTGGG)$_4$               |
| G5                      | (TTTACGG)$_4$               |
| G6                      | (TTTAGCG)$_4$               |
| G7                      | (TTTAGGC)$_4$               |
| NS                      | TAAGCTTTCTAGATTTAGGCTATCAGA |
| PT10<sup>(ds)</sup>     | (TTTAGGG)$_{10}$/ (AAATCCC)$_{10}$ |

<sup>a</sup>PT, plant G-rich telomere; HT, human telomere; CT, *C. elegans* telomere; NS, nonspecific sequence; ds, double-stranded.
| Band No. | Identified protein                              | NCBI Accession No. | Theor. MW | Ex. MW | Peptide matches | Coverage % |
|---------|------------------------------------------------|--------------------|-----------|--------|----------------|------------|
| 1       | Unidentified                                   | -                  | -         | -      | -              | -          |
| 2       | 3-methylcrotonyl-CoA carboxylase non-biotinylated subunit | 18418356           | 64,013    | 63,000 | 12             | 26         |
| 3       | Catalase 3                                      | 18394888           | 56,696    | 56,000 | 6              | 17         |
| 4       | Unidentified                                   | -                  | -         | -      | -              | -          |
| 5       | Chloroplast RNA binding protein (cp33)         | 681912             | 35,057    | 35,000 | 8              | 31         |
| 6       | Chloroplast RNA binding protein (cp31)         | 681908             | 33,346    | 33,000 | 5              | 27         |
| 7       | Chloroplast RNA binding protein (cp31)         | 681908             | 33,346    | 32,000 | 6              | 36         |
| 8       | myb-related transcription factor MYB52         | 11358541           | 28,935    | 28,000 | 3              | 15         |
| 9       | DNA-binding protein                             | 99684              | 27,172    | 27,000 | 4              | 17         |
| 10      | DNA-binding protein p24-related                | 15223748           | 29,058    | 24,000 | 8              | 43         |
| 11      | RNA binding protein 3 (designated as STEP1)    | 475720             | 18,319    | 20,000 | 4              | 50         |
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