Expression of the Telomeric Repeat Binding Factor Gene NgTRF1 Is Closely Coordinated with the Cell Division Program in Tobacco BY-2 Suspension Culture Cells*

Seong Wook Yang, Dong Hyun Kim, Jai Jin Lee, Yoon Joo Chun, Jae-Hyeok Lee, Yun Ju Kim, In Kwon Chung, and Woo Taek Kim‡

From the Department of Biology, College of Science, Yonsei University, Seoul 120-749, Korea

Telomeres are vital for preserving chromosome integrity during cell division. Several genes encoding potential telomere-binding proteins have recently been identified in higher plants, but nothing is known about their function or regulation during cell division. In this study, we have isolated and characterized a cDNA clone, pNgTRF1, encoding a putative double-stranded telomeric repeat binding factor of Nicotiana glutinosa, a diploid tobacco plant. The predicted protein sequence of NgTRF1 (M r = 75,000) contains a single Myb-like domain with significant homology to a corresponding motif in human TRF1/Pin2 and TRF2. Gel retardation assays revealed that bacterially expressed full-length NgTRF1 was able to form a specific complex only with probes containing three or more contiguous telomeric TTAGGG repeats. The Myb-like domain of NgTRF1 is essential, but not sufficient, to bind the telomeric repeat sequence. The glutamine-rich extreme C-terminal region, which does not exist in animal proteins, was additionaly required to form a specific telomere-protein complex. The dissociation constant (K d) of the Myb motif plus the glutamine-rich domain of NgTRF1 to the two-telomeric repeat sequence was evaluated to be 4.5 ± 0.2 × 10⁻⁹ M⁻¹, which is comparable to that of the Myb domain of human TRF1. Expression analysis showed that NgTRF1 gene activity was inversely correlated with the cell division capacity of tobacco root cells and during the 9-day culture period of BY-2 suspension cells, while telomerase activity was positively correlated with cell division. In synchronized BY-2 cells, NgTRF1 was selectively expressed in G₁ phase, whereas telomerase activity peaked in S phase. These findings suggest that telomerase activity and NgTRF1 expression are differentially regulated in an opposing fashion during growth and cell division in tobacco plants. The possible physiological functions of NgTRF1 in tobacco cells are also discussed.

Telomeres are specialized nuclear protein complexes located at the ends of linear eukaryotic chromosomes. They are essential and functional components for preserving chromosome integrity and for protection from exonucleolytic degradation and end-to-end fusion with other chromosomes (1, 2). Telomeres are also crucial for chromosome organization in the nucleus, especially during cell division. The DNA sequence of telomeres is highly conserved in most eukaryotes and consists of tandem repeats of short, G-rich sequence elements, such as TTAGGG in vertebrates (3) or TTTAGGG in higher plants (4). This G-rich strand extends beyond the complementary C-rich strand and terminates as a single-stranded 3’-overhang in many divergent organisms (5–7). Telomeres are synthesized and maintained by telomerase, a ribonucleoprotein complex that contains a specialized reverse transcriptase activity that uses its own RNA subunit as a template (5, 8, 9). In mammalian cells, telomerase activity is tightly associated with cell proliferation, de-differentiation, senescence, and immortalization. For example, in humans, telomerase is highly expressed in tumor cells and germ-line and embryonic cells, but is not detected in most normal somatic tissues (10). Lack of telomerase activity in human somatic cells results in telomere shortening during differentiation and aging, while stable maintenance of telomere length occurs in germ-line and tumor cells, which have unlimited cell division capacity (10, 11). These observations suggest that telomerase activity and telomerase-mediated stabilization of telomere length are intimately tied to the proliferative abilities of cells (12, 13).

In addition to telomerase activity, associations between the telomere repeat sequences and specific binding proteins appear to be required for the integrity and proper functions of telomeres. Studies of telomere chromatin structure have suggested that telomeres are packaged into specialized nucleoprotein complexes (14–16). Protein components of the telomere complex have been identified and characterized in several organisms, including ciliates, yeast, and humans. The telomere-binding proteins have been divided into two distinct groups. Members of the first group bind specifically to the single-stranded 3’-extension at the extreme termini of telomeres, which are necessary for chromosome capping and telomerase regulation (17–19). The other group of proteins interacts with the double-stranded telomeric repeats. For example, Rap1p from Saccharomyces cerevisiae specifically binds to yeast duplex telomeric DNA and plays a role in the regulation of telomere length maintenance (20, 21). Taz1p was identified in Schizosaccharomyces pombe in a one-hybrid screen using double-stranded telomeric DNA as a target and found to be involved in telomere length regulation, repression of telomere adjacent genes, and the interactions between telomeres and the spindle pole body.
during meiotic phase (22–24). In humans, two distinct Myb-related proteins, TRF1 and TRF2, have been identified as double-stranded telomeric DNA-binding proteins (25–27). TRF1 is a suppressor of telomere elongation and involved in the negative feedback mechanism that stabilizes telomere length by inhibiting telomerase at the ends of individual telomeres (28). A dominant-negative allele of TRF2 induced end-to-end chromosome fusions in metaphase and anaphase cells, indicating that TRF2 plays a key role in the protecive activity of telomeres in human cells (29). Recently, it has been reported that TRF2, along with TRF1, acts as a negative regulator of telomere length (30). Another telomeric protein, Pin2, was identified in HeLa cells (31). Pin2 is identical in sequence to TRF1, except for an internal deletion of 20 amino acids, suggesting that TRF2, along with TRF1, acts as a negative regulator of telomerase activity (32). A dominant-negative allele of TRF2 induced end-to-end chromosome fusions in metaphase and anaphase cells, indicating that TRF2 plays a key role in the protective activity of telomeres in human cells (29). Recently, it has been reported that TRF2, along with TRF1, acts as a negative regulator of telomere length (30). Another telomeric protein, Pin2, was identified in HeLa cells (31). Pin2 is identical in sequence to TRF1, except for an internal deletion of 20 amino acids, suggesting that TRF2, along with TRF1, acts as a negative regulator of telomerase activity (32). A dominant-negative allele of TRF2 induced end-to-end chromosome fusions in metaphase and anaphase cells, indicating that TRF2 plays a key role in the protective activity of telomeres in human cells (29). Recently, it has been reported that TRF2, along with TRF1, acts as a negative regulator of telomere length (30). Another telomeric protein, Pin2, was identified in HeLa cells (31). Pin2 is identical in sequence to TRF1, except for an internal deletion of 20 amino acids, suggesting that TRF2, along with TRF1, acts as a negative regulator of telomerase activity (32).
fore loading, gel were prerun at 10 V/cm for 30 min, and electrophoresis was performed in 0.5× TBE (50 mM Tris base, pH 8.3, 1 mM EDTA) for 2.5 h. For the competition experiments, varying amounts of cold competitor molecules were preincubated with NgTRF1 before the addition of radiolabeled probe. The gel was dried and autoradiographed. Binding activity was quantified with a PhosphorImager (Fuji). Determination of the dissociation constants (Kd) was carried out by incubating a fixed amount of 12 kDa protein (2.0×10−8 M) with increasing amounts of probes (NgTR-2, M4, and M11) under the standard binding conditions. Probes were used at concentrations between 0.8×10−8 and 4.5×10−8 M depending on the protein-probe combination. After gel electrophoresis, bound and free probes were quantified with a scintillation counter (Beckman) and PhosphorImager. Two quantification methods were used: one involving a calibration curve with a known DNA concentration, and the other determining dissociation constants as described previously (44). For the reversible binding reaction of a 12-kDa NgTRF1 polypeptide (the Myb motif and the C-terminal glutamine-rich domain) to the two-telomeric repeat DNA site is K−[P][D]/[DP], where [DP] is the concentration of DNA-protein complex, [P] that of unbound protein in the solution and [D] that of unbound DNA. The corresponding equilibrium dissociation constants were estimated from the slope of a Scatchard plot of the results.

Subcellular Localization of NgTRF1—The termination codon of the green fluorescent protein (GFP) cDNA was removed using PCR. The resulting fragment was then fused in frame to the full-length pNgTRF1 construct to create a truncated pNgTRF1. Transformation of this construct into tobacco cells was carried out as described previously (43). Fluorescence photographs of onion cells were taken using a Zeiss (Jena, Germany) Axioship fluorescence microscope fitted with fluorescein isothiocyanate filters (excitation filter, 450–490 nm; emission filter, 520 nm; dichroic mirror, 510 nm) and Fuji 400 color film. The optimal exposure time was 1 s.

Isolation of Genomic DNA and Southern Blot Analysis—N. glutinosa leaf genomic DNA was isolated as described previously (43) with modifications. Each gram of N. glutinosa leaf pulverized under liquid nitrogen was suspended in 2.5 ml of extraction buffer (8.0 M urea, 50 mM Tris base, pH 7.2, 3% SDS, 0.1% mercuric acetate) for 2.5 h. After centrifugation at 13,000 rpm for 5 min at 4 °C, the supernatant was either used immediately for the telomerase activity assay or stored at −80 °C until use.

Telomerase Activity Assay—Telomerase activity was monitored using the telomere repeat amplification protocol (TRAP). The 184-bp DNA fragment derived from the multicloning site region of Bluescript SK was used for an internal standard (IS). The TRAP assay was conducted in 40 μl of reaction mixture composed of 50 mM Tris acetate (pH 8.3), 50 mM potassium glutamate, 0.1% Triton X-100, 1 mM spermidine, 1 mM dithiothreitol, 50 μM each dNTP, 5 mM MgCl2, 10 mM EGTA, 100 μg/ml bovine serum albumin, and 0.5 units of Taq polymerase (Promega). After the addition of tobacco cell extracts containing 1 μg of total proteins and 50 ng of GG (21) forward primer (CATTCTAAGCTTCCTG), the telomerase reaction was allowed to proceed at 24 °C for 45 min. For a negative control of the telomerase assay, some samples were pretreated with 10 ng of RNase A (Sigma) or heated to 95 °C for 10 min. 50 ng of (C5TA3)2 reverse primer (CCCTAACCTTTAACCTAAAA) and 1.5 ng of IS DNA containing GG (21) and (C5TA3)2 primer sequences at its 5’- and 3’-end, respectively, were then added, and the reaction mixture was covered with 50 μl of paraffin oil and heated to 94 °C. The products of the telomerase reaction were amplified with 30 cycles of PCR, each consisting of 30 s at 94 °C, 30 s at 65 °C, and 90 s at 72 °C, with an additional 5 min at 72 °C in an automatic thermal cycler (PerkinElmer Life Sciences). PCR products were separated on a 10% nondenaturing polyacrylamide gel, and the gel was stained in 0.1% SYBR Green I for 45 min and scanned on a FluorImager (Fuji). Three independent first-round PCR products were subcloned with the cloning primer up to below the IS band. Telomerase activities are expressed as the ratio of the intensity of the TRAP ladder to the IS signal.

RESULTS

Isolation and Characterization of NgTRF1 cDNA—As a first step to gain insight into the regulation of telomere-binding protein genes during the cell cycle, we proceeded to isolate cDNAs encoding homologs of the Myb-related double-stranded telomeric DNA-binding proteins that have been identified in plants and mammals. Poly(A)+ RNA was isolated from mature leaves of N. glutinosa L. Following the synthesis of the first strand cDNA from 1 μg of poly(A)+ RNA, PCR was carried out with mixed oligonucleotides corresponding to the amino acid sequence KKVRDD for the upstream primer, KDKWKT for the downstream primer (see “Experimental Procedures” for sequences), and the first strand cDNA as the template. These primer amino acid sequences are highly conserved in rice RTBP1 and Arabidopsis AtTBP1 and AtTRP1 (40–42). Total PCR products of about 500 bp were radioactively labeled and used as probes to screen an N. glutinosa leaf cDNA library under low stringency hybridization and washing conditions (43). Four putative clones containing inserts of about 0.7–2.5 kb in size were isolated. Subsequent restriction enzyme mapping and DNA sequencing analyses revealed that these clones represent a single group of overlapping sequences. Fig. 1A shows the restriction enzyme map of pNgTRF1 that contains the longest insert among isolated clones. The pNgTRF1 clone (GenBank™ accession no. AF543195) is 2459 bp long and comprises a 99-bp 5’-untranslated region, a 2046-bp coding region encoding 682 amino acids, and a 314-bp 3’-untranslated region. The predicted molecular mass of the polypeptide encoded by pNgTRF1 is 75 kDa, which is slightly larger than those of other putative plant telomere-binding proteins, including rice RTBP1 (70 kDa) and Arabidopsis AtTBP1 (70.6 kDa), and AtTRP1 (65 kDa) (40–42). Isolation of the complete pNgTRF1
sequence allowed us to compare it to the telomeric DNA-binding proteins of other organisms and to analyze their structural relationships. Despite apparently conserved functions, telomere-binding proteins show a relatively low degree of sequence similarity. Tobacco NgTRF1 shares 35–44% identity with other putative plant telomere-binding proteins, with AtTRP1 being

**Fig. 1.** Sequence analysis of tobacco NgTRF1. A, restriction enzyme map analysis of the *N. glutinosa* NgTRF1 cDNA clone. Solid bar depicts the coding region. Solid lines represent 5' and 3' untranslated regions. The position of the hybridization probe is indicated. The sequence of pNgTRF1 has been deposited in the GenBank database under accession number AF543195. B, comparison of the derived amino acid sequence of tobacco NgTRF1 with the double-stranded telomeric DNA-binding proteins from *Arabidopsis* (AtTBP1, AtTRP1, and a protein encoded by the expressed sequence tag clone NP190243), rice (RTBP1), and humans (TRF1 and TRF2). Amino acid residues that are conserved in at least four of the seven sequences are shaded, while amino acids identical in all seven proteins are shown in bold. Box A depicts the putative nuclear localization signal. Two serine residues at positions 347 and 349, which are possible CDK1 phosphorylation sites, are marked by asterisks in box B. Boxes C and D refer to the putative ubiquitin domain and Myb-like motif, respectively. The conserved glutamine residues found at the C-terminal region of the plant proteins are indicated by dots. The arrows represent the primer amino acid sequences for RT-PCR. Dashes show gaps in the amino acid sequences introduced to optimize alignment.

Tobacco NgTRF1 shares 35–44% identity with other putative plant telomere-binding proteins, with AtTRP1 being...
Cell Division Regulation of NgTRF1 in Tobacco

| Table I | Telomeric DNA probe and competitors |

| Probe name | Sequence |
|------------|----------|
| NgTR-1     | 5’T TAGGG |
| NgTR-2     | 3’AATCCC |
| NgTR-3     | 5’T TAGGGTTAGG |
| NgTR-4     | 3’AAATCCCAATCCC |
| NgTR-1      | 5’T TAGGGTTAGGTTAGG |
| NgTR-4      | 3’AAATCCCAATCCC |
| HTR-4      | 5’T TAGGGTTAGGTTAGGTTAGG |
| CTR-4      | 3’AATCCC |
| NS-4       | 5’T TAGGGTTAGGTTAGGTTAGG |
| NS-4*      | 3’AAATCCCAATCCC |

* NS, nonspecific DNA.

the most closely related, while NgTRF1 has a limited homology to human TRF1 (17% identity) and TRF2 (14% identity) (Fig. 1B). NgTRF1 is also homologous (44%) to a protein encoded by an Arabidopsis-expressed sequence tag clone (GenBank accession number NP190243) whose function has not been addressed. As was found in other double-stranded specific telomere-binding proteins, NgTRF1 possesses a single Myb-like domain near the C-terminal region and one potential nuclear localization signal. The Myb-like domain of NgTRF1 is strongly conserved (85–90%) compared with that of rice and Arabidopsis proteins, and is 27 and 24% identical to its corresponding domains in TRF1 and TRF2, respectively (Fig. 1B), indicating by dots). Taken together, these structural conservations imply that NgTRF1 may play a role in telomere function in tobacco plants.

NgTRF1 binds specifically to double-stranded telomeric DNA sequences in vitro—To explore whether NgTRF1 can bind telomeric DNA, we expressed NgTRF1 in E. coli, and the purified protein was used in a gel retardation assay with a 32P-labeled NgTR-4 (see Table I) possessing four plant duplex telomeric DNA repeats, (TTTAGGG)₄. The full-length 75-kDa NgTRF1 gave rise to a single, discrete DNA-protein complex that migrated more slowly than the free probe (Fig. 2A). The intensity of this shifted band increased upon the addition of increasing amounts (0.5–1 μg) of NgTRF1. The DNA binding specificity of NgTRF1 was also confirmed by competition binding experiments, which showed that a 25-fold excess of cold NgTR-4 was enough to displace the labeled probe (Fig. 2A). However, telomeric repeats of other organisms, such as human (HTR-4) and Caenorhabditis elegans (CTR-4), as well as non-specific DNA (NS), were not capable of interacting with NgTRF1 (Fig. 2A). In addition, a 50-fold molar excess of these unrelated, non-specific cold DNAs failed to compete with the labeled NgTR-4, implying that NgTRF1 indeed binds specifically to double-stranded plant telomeric DNA in vitro. In contrast, the expressed NgTRF1 did not exhibit any detectable DNA-binding capacity to single-stranded telomeric repeats (data not shown). To assess the minimum number of telomeric repeats required for binding to NgTRF1, we next performed gel shift assays using probes (NgTR-1, NgTR-2, NgTR-3, and NgTR-4) with different numbers of telomeric DNA repeats (Table I). As shown in Fig. 2B, full-length NgTRF1 can form complexes only with the probes comprising three or more contiguous TTTAGGG repeats. Thus, under our experimental conditions, the minimum length of a telomeric DNA bound by NgTRF1 in vitro spans at least three repeats. There was no significant difference in the complex formation pattern between NgTR-3 and NgTR-4 (Fig. 2B). This may indicate that NgTRF1 requires three TTTAGGG repeats for efficient complex formation. However, we could not rule out the possibility that probes with more than five TTTAGGG repeats have an enhanced binding activity to NgTRF1.

The isolated Myb motif of TRF1 binds specifically to human telomeric DNA (46). As the C-terminal Myb-like region of NgTRF1 is 27 and 24% identical to its corresponding domains of TRF1 and TRF2, respectively (Fig. 1B), we investigated whether it is responsible for the DNA binding specificity. Various NgTRF1 deletion mutants were expressed in E. coli, and the isolated proteins were tested for their DNA binding properties. The 67-kDa mutant, which lacks the C-terminal Myb-like domain, demonstrated complete absence of binding activity to NgTR-4, confirming that this motif is essential for effective complex formation (Fig. 2C). The gel shift analysis, however, revealed that the Myb-like motif of NgTRF1 is not sufficient to form the telomere-protein complex, as evidenced by the results that neither the Myb domain (8 kDa) nor a GST-Myb fusion protein (34 kDa) interacted with NgTR-4 (Fig. 2C). In contrast, the truncated mutants composed of the Myb motif plus the extreme C-terminal region (the 38- and 12-kDa proteins in Fig. 2C) retained full DNA binding activities. As removal of the C-terminal portion abrogated the DNA binding activity, it seems most likely that, in addition to the Myb motif, this domain is also required for optimal binding to telomeric DNA. This extreme C-terminal polypeptide is rich in glutamine residues (Fig. 1B, indicated by dots) and found only in the plant telomere-binding proteins. Its precise role, however, remains to be elucidated.

Fig. 2C also shows that polypeptides containing the Myb motif and the C-terminal glutamine-rich domain produced more than one specific complex when interacting with NgTR-4. This led us to repeat the gel mobility shift assay using various DNA sequences carrying one to four contiguous copies of the TTTAGGG repeat and the isolated DNA-binding domain of NgTRF1 to identify the recognition site within the telomeric sequence at which DNA-protein interactions occur. The results showed that a 26-kDa protein containing the Myb motif plus the glutamine-rich domain generated a single retarded band with the two-repeat sequence (NgTR-2) and two bands with the three-repeat sequence (NgTR-3), while it exhibited no DNA binding ability with the one-repeat sequence (NgTR-1) (Fig. 3A). When the NgTR-4 probe possessing four telomeric repeats was tested, a third, additional complex of weaker intensity and slower migration was resolved. These results indicate that the recognition site of the NgTRF1 DNA-binding domain resides within the two-telomere repeat sequence TTTAGGGTTTAGGG. To further specify the target sequence recognized by NgTRF1, we synthesized a series of telomeric repeat mutants and analyzed them for their binding activities to the isolated 26-kDa NgTRF1 polypeptide. 14 single-base mutants of the 14-bp two-telomeric repeat sequence, TTTAGGGTTTAGGG, were prepared (Fig. 3B). Gel retardation analysis revealed that...
substitutions of any of the first 3 nucleotides (M1, M2, and M3 probes) or the terminal 3 nucleotides (M12, M13, and M14 probes) did not significantly affect the binding ability of the protein, while A→T substitutions in the 4th and 11th positions, respectively, reduced the binding activity by ~50% (Fig. 3B). In contrast, the DNA binding capacity was almost completely diminished with the M5, M6, M7, M8, M9, and M10 probes, which contained single-base mutations in the internal GGGTTT sequence (Fig. 3B). These results suggest that the internal GGGTTT sequence in the two-telomeric repeat is crucial for binding of the Myb plus the glutamine-rich domains of NgTRF1.

Since the results described in Fig. 3 suggest that the internal GGGTTT core sequence is critical for binding of NgTRF1, we next went on to estimate its DNA binding affinity with different DNA substrates. The dissociation constant for binding of the 12-kDa protein containing the Myb plus glutamine-rich domains (Fig. 2) to the two-telomeric repeat sequence was calculated from a quantitative gel mobility shift assay (Fig. 4) as described under “Experimental Procedures.” The plot in Fig. 4B depicts the concentration of DNA-protein complex plotted against the concentration of complex divided by the concentration of free DNA (NgTR-2), and the dissociation constant was evaluated from the slope to be 4.5 ± 0.2 × 10^{-9} M. This value is slightly higher than that of the Myb-like domain of human TRF1 (3.2 ± 0.5 × 10^{-9} M) (46). To show that the affinity is specific for the plant telomeric repeat, we carried out a quantitative gel retardation analysis using M4 and M11, which contain A→T substitutions in the 4th and 11th positions of the two-telomeric repeat, respectively (Fig. 3B). Fig. 4 shows that the dissociation constants of the 12 kDa protein to M4 and M11 are 1.3 ± 0.3 × 10^{-8} M and 8.4 ± 0.6 × 10^{-9} M, respectively, indicating that M4 and M11 are still able to interact with the protein although they possess significantly lower affinity compared with NgTR-2. In contrast, as shown in Fig. 3B, the 12-kDa polypeptide failed to bind the DNA substrates (M5–M10), which have mutations in the internal GGGTTT core sequence, with any concentrations of those DNAs (data not shown). Thus, the results presented in Figs. 3 and 4 indicate that the internal GGGTTT sequence in the two-telomeric repeat site is indeed important, and are consistent with the recent model that base-specific contacts between telomere-binding proteins and telomeric repeat sequences are made within the internal core sequence in both humans (46) and yeast (47).

**Targeting of NgTRF1 to the Nucleus**—Since the predicted sequence of NgTRF1 contains a Myb-like domain as well as a putative nuclear localization signal (KRRK, Fig. 1B), the protein is expected to localize to the nucleus. To confirm this, we performed an in vivo targeting experiment that employed an NgTRF1-fused green fluorescent protein (GFP) as a fluorescent marker in a transient transfection assay. The GFP gene was

![Image](http://www.jbc.org/)

**Fig. 2.** Sequence-specific binding activity of NgTRF1 to plant double-stranded telomeric DNA. A, gel retardation assay showing full-length NgTRF1 binding to NgTR-4. An indicated amount (0–1.0 μg) of full-length NgTRF1 was added to each reaction mixture. Lanes 1–3, radiolabeled NgTR-4 probe; lanes 4–6, radiolabeled human (HTR-4), C. elegans (CTR-4), and nonspecific DNA (NS) probes, respectively; lanes 7 and 8, titration with cold NgTR-4 as a competitor; lanes 9–14, titration with cold human (HTR-4), C. elegans (CTR-4), and nonspecific DNA (NS) as competitors. B, gel retardation assay showing an indicated amount (0–1.0 μg) of full-length NgTRF1 binding to the different repeats of telomeric DNA. Lanes 1–3, radiolabeled NgTR-1; lanes 4–6, radiolabeled NgTR-2; lanes 7–9, radiolabeled NgTR-3; lanes 10–12, radiolabeled NgTR-4. C, gel retardation assays were performed with various deletion mutants of NgTRF1 and radiolabeled NgTR-4 probe. 1 μg of each construct was added to the reaction mixture. The shaded boxes refer to the GST polypeptide, while black boxes depict the Myb-like domain of NgTRF1. The coding region outside the Myb-like domain of NgTRF1 is shown in open boxes. The molecular mass of each mutant polypeptide is indicated.
fused to the 5’-end of the pNgTRF1 coding region in-frame under the control of the cauliflower mosaic virus 35S promoter (Fig. 5A), and the resulting construct was introduced into onion epidermal cells by the particle bombardment method (48). Localization of the fusion protein was then determined by visualization with a fluorescence microscope. As shown in Fig. 5B, the control GFP was uniformly distributed throughout the cell (panel a), while the GFP-NgTRF1 fusion protein was localized to the nucleus (panel b). This subcellular localization pattern was essentially identical to that of the GFP-NgTRF1 1–360 mutant protein in which the C-terminal-half was truncated (panel c). In contrast, the GFP-NgTRF1 361–681 protein, which lacked the N-terminal 360 amino acid residues, exhibited uniform accumulation inside the cell (panel d). These observations support the notion that the putative nuclear targeting sequence of NgTRF1 is sufficient and that no additional post-translational modification is necessary for the NgTRF1 protein to be targeted to the nucleus.

Organization and Expression of the NgTRF1 Gene—From the results described above, it appears that NgTRF1 is a nuclear protein that specifically binds plant telomeric DNA in vitro. We therefore wanted to characterize the NgTRF1 gene in more detail at the molecular level. To assess the NgTRF1 gene copy number in the tobacco genome, genomic Southern blot analysis was carried out using pNgTRF1 as a probe. The genomic DNA isolated from mature leaves of N. glutinosa (diploid, 2n) and N. tabacum cv. Samsun NN (amphidiploid, 4n) was digested with EcoRI, HindIII, or XbaI, and hybridized to the 32P-labeled EcoRI/BamHI fragment of pNgTRF1 under normal stringency conditions (Fig. 1A). This hybridization detected only one clear band in N. glutinosa, whereas two major hybridizing bands were observed in N. tabacum (Fig. 1A). No additional fragments were visible in any of the digests, even with low stringency hybridization or longer exposure of the blot to x-ray film (data not shown). These results imply that the NgTRF1 gene is present in a single copy per haploid tobacco genome.

To examine the spatial and temporal expression pattern of the NgTRF1 gene, we measured the level of corresponding mRNA in different parts of tobacco plants by Northern blot analysis. Total RNAs isolated from leaves, roots, stems, and flowers of N. glutinosa were hybridized to the 32P-labeled EcoRI/BamHI fragment of pNgTRF1 under high stringency conditions. A substantial level of the 2.5-kb transcript was detected in every tissue examined, indicating that NgTRF1 is constitutively expressed in mature tissues of tobacco plants (Fig. 6B). These results are in line with the previous observations that rice RTBP1 and Arabidopsis AtTBP1 are ubiquitously present in various parts of plants (40, 42). To investigate whether NgTRF1 expression is correlated with tissue development, roots were serially dissected according to different developmental stages (sections 1–4), and total RNAs from each section were subjected to RNA gel blot analysis. Interestingly, the abundance of the message varied depending on the sections; the maximum amount of NgTRF1 transcript was found in the fully differentiated region (section 1 in Fig. 6C), but expression gradually decreased acropetally, with only background levels detectable in section 4, where actively dividing...
meristemetic cells are present (Fig. 6C). Thus, these results suggest that the expression of \( \text{NgTRF1} \) is differentially regulated in different stages of root development, raising the possibility that the tobacco telomere-binding protein gene is subject to control by a development-specific mechanism.

**Cell Division-dependent Regulation of \( \text{NgTRF1} \) — Telomerase**

Telomerase is a specialized RNA-directed DNA polymerase and is responsible for the synthesis of telomeric repeat DNA (49). As found in animal tissues, the telomerase activity and expression of mRNA for the telomerase reverse transcriptase subunit, AtTERT, are closely linked to cell division in Arabidopsis; the \( \text{AtTERT} \) transcript is abundantly present in callus and shoot apical meristems, but not in mature leaf tissue (50, 51). Likewise, a high level of telomerase activity was detected in tobacco BY-2 suspension culture cells and roots, both of which contained a high proportion of actively dividing cells, while very low levels of activity were found in other mature tobacco tissues, including stems, leaves, and flowers (52). In synchronized BY-2 cells, telomerase activity is low during most phases of the cell cycle, but is strongly induced at the onset of S phase, indicating that it is regulated in a cell cycle-dependent fashion (53, 54).

Because \( \text{NgTRF1} \) has a distinct expression pattern in each stage of root development (Fig. 6C), we considered the possibility that, along with telomerase activity, \( \text{NgTRF1} \) expression is also mediated in a cell division-specific manner in tobacco cells. To address this possibility, we monitored the change in steady-state \( \text{NgTRF1} \) transcript levels as well as telomerase activity during a 9-day culture period in asynchronous tobacco BY-2 suspension cells. The results showed that telomerase activity, as measured by TRAP assay, was relatively low on day 1. This low, basal expression of activity was markedly enhanced on the second day, and reached a maximum at day 4 (Fig. 7B). This time point corresponded to the middle of the exponential growth phase of the cultures (Fig. 7A). Subsequently, telomerase activity declined, resulting in negligible activity in 9-day-old culture cells. In contrast, \( \text{NgTRF1} \) showed a remarkably different expression pattern. The amount of \( \text{NgTRF1} \) mRNA was very low during the logarithmic phase of growth in tobacco suspension cultures (Fig. 7C). The transcript began to accumulate at day 7, and attained a maximal level.

**Fig. 4. Determination of the dissociation constant for the Myb- and glutamine-rich domains of \( \text{NgTRF1} \) to two-telomeric repeat DNA sites.** A, quantitative binding assay of the 12-kDa protein (the Myb motif and the C-terminal glutamine-rich domain) to the wild-type and mutant two-telomeric repeat DNA sites (NgTR-2 and M4). The protein concentration is \( 2.0 \times 10^{-8} \text{ M} \), while the DNA concentrations in lanes 1–7 are \( 4.5 \times 10^{-9}, 3.2 \times 10^{-9}, 2.5 \times 10^{-9}, 1.7 \times 10^{-9}, 1.2 \times 10^{-9}, 1.0 \times 10^{-9}, \text{and} 0.8 \times 10^{-9} \text{ M} \) in gel (a), and \( 4.5 \times 10^{-8}, 3.5 \times 10^{-8}, 2.7 \times 10^{-8}, 2.0 \times 10^{-8}, 1.4 \times 10^{-8}, 1.2 \times 10^{-8}, \text{and} 1.0 \times 10^{-8} \text{ M} \) in gel (b). B, plot of bound DNA [DP_{n}] versus [DP_{m}] using data from A. The dissociation constant \( K_{d} \) was calculated from the slope and the concentration of active protein as the ordinate intercept of the linear regression line. C, \( K_{d} \) values of the 12-kDa \( \text{NgTRF1} \) polypeptide to NgTR-2, M4 and M11.

**Fig. 5. Subcellular localization of the \( \text{NgTRF1} \) gene products.** A, the \( \text{GFP} \) coding region was fused in-frame to the full-length \( \text{pNgTRF1} \) coding region or to truncated \( \text{pNgTRF1} \) mutants. Constructs were introduced into onion epidermal cells by the particle bombardment method and expressed under the control of the CaMV \( ^{\text{35S}} \) promoter. B, expression of the introduced genes was viewed after 12 h by fluorescence microscopy under dark field or light field.
during days 8 and 9, when BY-2 cells have no capacity for proliferation. The CYM gene, which belongs to the family of mitotic cyclins, was included in the RNA expression experiments as a positive control (55), and its mRNA was specifically expressed only in actively dividing cells (i.e. within the exponential growth phase) (Fig. 7C). Therefore, telomerase activity and NgTRF1 expression levels demonstrated positive and negative correlations, respectively, with the program of cell proliferation in BY-2 suspension cultures.

To obtain additional evidence supporting the cell division-dependent expression of NgTRF1, telomerase activity and NgTRF1 transcript levels were measured at different time points in BY-2 cell suspension cultures following exposure to 10 mM hydroxyurea, which arrests the cell cycle at S phase (56). The proportion of synchronized cells was estimated by mitotic index. The maximum mitotic index was measured to be about 70% 12 h after release from hydroxyurea-induced S phase arrest (Fig. 8A). RNA gel blot analysis revealed that CYM mRNA, a marker for M phase (55), was barely detectable until 10 h after S phase blocking, accumulated to a high level at 12–16 h, and started to decrease at the 18 h point, suggesting that partially synchronous cell division occurred during 12–16 h (Fig. 8A). By contrast, the NgTRF1 transcript accumulated only at low levels during the progression through S and M phase. Subsequently, the level increased gradually, and a peak was observed 24 h after release from S phase, a time point corresponding to late G1 phase (Fig. 8A). Consistent with previous results (54), the S phase-specific telomerase activity rapidly disappeared as the cell cycle progressed in BY-2 cells (Fig. 8B). Thus, these results suggest that NgTRF1 mRNA, in addition to telomerase activity, is expressed in a cell cycle-dependent fashion in tobacco BY-2 cells, with NgTRF1 being activated specifically in the cells that exit mitosis and enter interphase.

The prostaglandin inhibiting drug indomethacin was previously shown to arrest the cell cycle progression at G1/S phase of tobacco BY-2 cells (57). To address cell cycle-dependent expression of NgTRF1 in more detail, 10 μM indomethacin was added to the actively dividing 3-day-old BY-2 culture cells, after which the levels of both NgTRF1 and CYM mRNAs were examined at different time points. The expression of the M phase marker CYM showed a G1/S block in indomethacin-treated cells; its expression began to decline at 8 h, and thereafter much lower level of transcript was detected (Fig. 9). In contrast, induction of NgTRF1 mRNA was clearly detected at 8-h treatment, and this G1 phase-specific expression of the transcript continued and increased in the presence of indomethacin for at least 16 h after treatment (Fig. 9). These results suggest that the NgTRF1 gene is activated in the G1 phase during the cell division of BY-2 cells. Such a finding, in conjunction with the data presented in Figs. 6C, 7, and 8, is consistent with the hypothesis that there exists a tight regulatory mechanism by which the activities of telomerase and telomere-binding protein are inversely modulated during tobacco cell division. On the other hand, it is worth noting that the relative abundance of the G1-specific NgTRF1 mRNA was significantly lower than that in culture cells at the stationary growth phase (compare lanes 9 and 10 in Fig. 9) and that in various mature organs (data not shown). This may indicate that the NgTRF1 gene is more abundantly expressed in quiescent cells than in cycling cells of tobacco plants.

**DISCUSSION**

In humans, telomeres and telomerase have attracted much interest, since their structures and activities are closely associated with the program of cellular proliferation, differentiation, aging, and tumor growth. Although far less is known about plant telomeres and telomerase compared with animal systems, it is becoming increasingly apparent that telomere structure and patterns of telomerase regulation are largely conserved between animals and higher plants (33, 34).
Recent research on the organization of telomeric chromatin indicates that telomeres are packaged into specialized nucleo-protein complexes and that non-histone proteins are an integral component of and are responsible for the integrity and proper functioning of telomeres (14–16). Telomere-binding proteins have been extensively characterized in several organisms, such as ciliates, yeast, and humans, and are classified into two functional groups; the single-stranded binding proteins that interact with the 3′/H11032 extension of the extreme termini of telomeres (17–19), and the double-stranded-specific binding proteins (20–27). Although these telomere-binding proteins exhibit a limited amino acid sequence identity, they share a unique domain that resembles the DNA-binding motif present in the vertebrate c-Myb family of transcriptional activators (46, 58). For instance, yeast Rap1p contains two Myb-like domains that coordinate specific binding to DNA in a tandem orientation (59), whereas the DNA-binding domains of Taz1p, TRF1/ Pin2, and TRF2 possess a single Myb motif at their C-terminal ends (26, 60, 61). The isolated Myb motifs of TRF1 and Taz1p were found to bind specifically to duplex telomeric DNA, indicating that the Myb-like domain is responsible for specific telomeric DNA recognition (46, 60, 61).

In the present study, we have isolated and characterized a cDNA clone, pNgTRF1, that encodes a putative telomere-binding protein in N. glutinosa, a diploid tobacco plant. As expected, NgTRF1 contains a single C-terminal Myb-like domain that is
significantly similar to its corresponding domains in rice and Arabidopsis proteins as well as human TRF1/Pin2 and TRF2, suggesting that it may function as a DNA-binding motif on its telomeric recognition site in tobacco cells (Fig. 1). Gel retardation analysis reveals that the expressed Myb-like domain of NgTRF1 is essential, but not sufficient, for specific binding to the plant double-stranded telomeric repeat sequence (Fig. 2). The extreme C-terminal region of NgTRF1, which consists of eight glutamine residues, is also required for telomere-protein complex formation. This glutamine-rich region is highly homologous among plant proteins, but is not found in human telomeric proteins (Fig. 1). In addition, the N-terminal acidic region, dimerization domain, and D-like motif, which are apparent in TRF1/Pin2 (31), do not exist in plant telomeric proteins, while all of the plant proteins identified to date contain a putative UBD, a possible interacting motif with the 26 S proteasome (Fig. 1). These different architectural properties raise the possibility that the mechanism by which the telomeric repeats and telomere-binding proteins form specific complexes might be different between plants and animals. Since none of the plant telomeric proteins have yet been localized to telomeres in vivo, further studies on the mode by which the telomeric repeat-binding proteins recognize telomeric DNA and on their physiological relevance in plant cells are required.

The enzyme telomerase is one of the most important components of the telomere complex. Although plants show developmental differences and a more plastic pattern of differentiation, several lines of evidence indicate that the expression pattern of telomerase activity in higher plants is similar to that of animals (33, 34). As in mammalian cells, high levels of telomerase activity have been detected in actively dividing tissues, including young immature embryos, dedifferentiated callus, root tips, and immature floral buds of various plant species, but the activity is very low or undetectable in most mature vegetative tissues (52, 62–65). Recently, regulation of the gene encoding the telomerase reverse transcriptase subunit has been studied on a molecular level in Arabidopsis (50, 51). The AtTERT mRNA highly accumulates in callus and shoot apical meristems, as opposed to mature leaf tissue, and this expression pattern of AtTERT parallels the activity of telomerase. In Arabidopsis telomerase-null mutants generated by a T-DNA insertion, telomere length decreased by about 500 bp per generation (51). Although these telomerase-deficient Arabidopsis plants could survive up to 10 generations, the last five generations showed severe developmental defects in both vegetative and reproductive organs, confirming that telomerase function is essential for the maintenance of telomere integrity in higher plants (66). By using synchronized tobacco BY-2 suspension culture cells as a model system, we have recently shown that telomerase activity is tightly coordinated with cell cycle progression. The amount of telomerase activity strikingly increases at early S phase, and this activity was further induced by auxin, a cell division promoting hormone, and inhibited by abscisic acid, a phytohormone known to induce cyclin-dependent protein kinase inhibitors (54). Based on these findings, we were tempted to presume that, in addition to telomerase activity, the expression of telomere-binding protein genes is also associated with the program of cell division in tobacco plants. This hypothesis was particularly interesting because nothing was known about the regulation of telomeric protein genes during cell division in higher plants. Our present data demonstrate that the activity of the NgTRF1 gene is inversely linked to the capacity of cell division in tobacco roots; high amount of its mRNA is present in the fully differentiated region but gradually declines acropetally, with the lowest level occurring in the root tips, which are primarily populated with actively dividing cells (Fig. 6C). Our results further show that, during the 9-day culture period of BY-2 cells, the NgTRF1 transcript appears in cells within the stationary growth phase, during which telomerase activity is negligible (Fig. 7). Thus, the opposite pattern of expression between the cell division-associated telomerase activity and NgTRF1 gene has been demonstrated in roots and during the growth phase of BY-2 suspension cells of tobacco plants.

In human HeLa cells, Pin2 protein levels are highly regulated during the cell cycle, with increased expression during G2.

Fig. 9. Induction of NgTRF1 and suppression of CYM by indomethacin in 3-day-old BY-2 cells. The actively dividing 3-day-old BY-2 suspension culture cells were incubated with or without 10 μM indomethacin, which arrests the cell cycle progression at G1/S phase, then the mRNA levels of NgTRF1 and CYM were measured at different time points by Northern analysis as described in Fig. 6. The expression levels of transcripts in 9-day-old stationary growth phase cells were included for comparison. The equivalence of RNA loading among lanes was demonstrated by ethidium bromide staining of RNA on the gel. The relative levels of gene expression are shown as the ratio of intensities of corresponding mRNA and rRNA bands measured by PhosphorImager.
and M phase and decreased expression during G1 phase (31). Moreover, overexpression of Pin2 resulted in an accumulation of HeLa cells in G2 + M phase, suggesting that the Pin2 protein level may help regulate cell cycle progression (31). These results prompted us to examine if the activity of NgTRF1 fluctuates at distinct stages of the cell cycle in synchronized BY-2 cells. We found that NgTRF1 expression is tightly controlled in a cell cycle-dependent manner, with its mRNA level being selectively expressed only in G1 phase, in contrast to the S phase-specific telomerase activity and M phase-specific CYM mRNA expression (Figs. 8 and 9). Taken together, all the results presented in Figs. 5–9 led us to propose that the regulatory mechanisms of telomerase activity and NgTRF1 gene are inversely associated by an intimate signaling network of as-yet unidentified cellular factors during cell division progression.

The critical question that remains to be unraveled is whether NgTRF1 indeed plays a physiological role in tobacco cells. The ability of NgTRF1 to specifically bind to double-stranded plant telomeric repeat sequences in vitro (Figs. 2–4) as well as its nuclear localization (Fig. 5) suggest that it may be involved in telomere function in vivo. However, localization of NgTRF1 to telomeres in vivo must be confirmed. Telomeric proteins have been shown to negatively regulate telomere length. Such proteins include Rap1p and Tax1p in yeast (20–24) and TRF1/Pin2, and a cell cycle-dependent change in the level of Pin2 protein is a signal for cells to stop dividing and go into the G0 phase (31). Intriguingly, NgTRF1 has been recently reported that telomere architecture is developmentally controlled at the post-transcriptional level, possibly through a D-like motif related to the destruction box that mediates degradation of many mitotic proteins (31). Thus, it is attractive to speculate that NgTRF1 participates in telomere length regulation by affecting telomerase activity during the tobacco cell division. Conversely, NgTRF1 may serve as a regulatory marker in cell cycle progression; its accumulation is required for tobacco cells to exit M phase and enter G2 phase (Figs. 8 and 9). In this regard, a high concentration of NgTRF1 could be a signal for cells to stop dividing and go into the interphase.

Both human TRF1 and Pin2 are phosphoproteins in vivo, and a cell cycle-dependent change in the level of Pin2 protein is controlled at the post-transcriptional level, possibly through a D-like motif related to the destruction box that mediates degradation of many mitotic proteins (31). Intriguingly, NgTRF1 contains putative CDK1-dependent phosphorylation sites as well as UBD, a potential binding domain for the 26 S proteasome (Fig. 1). Thus, further experiments are needed to define the possible role of phosphorylation and control of the NgTRF1 protein level in the course of the cell division in tobacco plants. By using PENT (primer extension/nick translation) assay, it has been recently reported that telomere architecture is developmentally different in all telomerase-negative tissues of the dicot plants Silene latifolia and A. thaliana; the fraction of telomeres with detectable G-overhang structures is substantially changed from 50% in seedlings to 35% in leaves (68). These results, along with the growth- and cell division-dependent expression of NgTRF1 and telomerase activity, are consistent with the idea that plant telomere structure is developmentally dynamic (34). Further functional studies of NgTRF1 and characteristics of the proteins it interacts with will be critical to the understanding of the relationship between cell cycle progression and such a dynamic function of telomeres and telomerase activity in higher plants.
57. Ehsan, H., Roef, L., Witters, E., Reichheld, J.-P., Bockstaele, D. V., Inze, D., and Ossckelen, H. V. (1999) *FEBS Lett.* **458**, 349–353

58. Bilaud, T., Koering, C. E., Binet-Brasselet, E., Anselin, K., Pollice, A., Gasser, S. M., and Gilson, E. (1996) *Nucleic Acids Res.* **24**, 1294–1303

59. Konig, P., Giraldo, R., Chapman, L., and Rhodes, D. (1996) *Cell* **85**, 125–136

60. Spink, K. G., Evans, R. J., and Chambers, A. (2000) *Nucleic Acids Res.* **28**, 527–533

61. Vassetzky, N. S., Gaden, F., Brun, C., Gasser, S. M., and Gilson, E. (1999) *Nucleic Acids Res.* **27**, 4687–4694

62. Fitzgerald, M. S., McKnight, T. D., and Shippen, D. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14422–14427

63. Heller, K., Killan, A., Piatyszek, M. A., and Kleinhofs, A. (1996) *Mol. Gen. Genet.* **252**, 342–345

64. Killan, A., Heller, K., and Kleinhofs, A. (1996) *Plant Mol. Biol.* **37**, 621–628

65. Riha, K., Fajkus, J., Siroky, J., and Vyskot, B. (1998) *Plant Cell* **10**, 1691–1698

66. Riha, K., McKnight, T. D., Griffith, L. R., and Shippen, D. E. (2001) *Science* **291**, 1797–1800

67. Fulneckova, J., and Fajkus, J. (2000) *FEBS Lett.* **467**, 305–310

68. Riha, K., McKnight, T. D., Fajkus, J., Vyskot, B., and Shippen, D. E. (2000) *Plant J.* **23**, 633–641
Expression of the Telomeric Repeat Binding Factor Gene \textit{NgTRF1} Is Closely Coordinated with the Cell Division Program in Tobacco BY-2 Suspension Culture Cells

Seong Wook Yang, Dong Hyun Kim, Jai Jin Lee, Yoon Joo Chun, Jae-Hyeok Lee, Yun Ju Kim, In Kwon Chung and Woo Taek Kim

\textit{J. Biol. Chem.} 2003, 278:21395-21407.
doi: 10.1074/jbc.M209973200 originally published online March 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M209973200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 68 references, 20 of which can be accessed free at http://www.jbc.org/content/278/24/21395.full.html#ref-list-1