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Telomerase activity in plant cells

Jiří Fajkus*, Aleš Kovařík, Robert Královics

Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic

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Abstract Telomerase is a ribonucleoprotein enzyme which elongates the G-rich strand of telomeric DNA to compensate for the progressive reduction in its length due to incomplete replication of chromosome ends, which in human somatic cells leads to cell cycle arrest upon shortening of telomeres to a critical length. To examine the possible involvement of telomerase in metabolism of plant genetic material, we used cells of *Nicotiana tabacum* strain TBY-2, a stable long-term culture which has kept a constant pattern of restriction fragments from chromosome termini during its 6 month period of cultivation in our laboratory. In a direct assay for telomerase, a 5′ end-labeled plant telomeric oligonucleotide $^{5'}(TTTAGGG)_6^{3'}$ was elongated in a TBY-2 cell extract, showing a pausing pattern which is a characteristic feature of telomerases from other organisms. The elongation was inhibited by RNase A pretreatment of the extract. We conclude that plant cells possess telomerase which is used for maintenance of their telomeres.

Key words: Plant telomerase; Telomeric DNA elongation

1. Introduction

Telomeres are nucleoprotein structures at the ends of eukaryotic chromosomes which are necessary for chromosome stability. Their DNA component is composed of direct tandem repeats of a short guanine-rich sequence, which is associated with various telomere-binding proteins including histones and with the nuclear matrix (for review see [1]). Each round of DNA replication results in shortening of the telomeres due to incomplete replication of the 3′ end of the parental strand; hydrolysis of the RNA primer and the absence of replication of the nucleotides upstream of its 5′ end result in a newly synthesized DNA strand that is shorter than the parental strand at its 5′ end [2]. In the absence of telomere-elongating activity the number of cell doublings is limited, as observed in human somatic cells (e.g. [3]).

Germline and tumor cells overcome this limitation by the expression of telomerase, a ribonucleoprotein complex which catalyzes the elongation of telomeric DNA by a process of reverse transcription. Its substrate is the overhanging G-rich telomeric DNA strand, and the template region is provided by the RNA component of telomerase [3,4]. In addition to telomerase, alternative mechanisms of telomere maintenance have been described such as retrotransposition of HET-A and TART elements present at *Drosophila* chromosome ends [5], and recombination by the gene conversion mechanism [6]. Telomerase activity was first detected in *Tetrahymena* [4] and subsequently in a variety of different organisms from the animal kingdom and in yeast (see [7] for a review).

In plants, the telomeric DNA sequence motif $^{5'}(TTTAGGG)^{3'}$ was first characterized in *Arabidopsis thaliana* [8] and was subsequently found at chromosome ends in several other plant species including *Nicotiana tabacum* and related species [9,10]. Each of the *Nicotiana* species studied shows a specific pattern of terminal restriction fragments, reflecting distinct telomere lengths in individual chromosomes [11]. As far as we are aware, there have been no reports of the detection of telomerase activity in plant cells although its presence would be anticipated in view of their totipotent character and their capacity for long-term propagation in culture. It has been suggested that telomerase is involved in the addition of the telomeric sequences to the ends of broken wheat chromosomes [12]. A protein which binds specifically to plant telomeric DNA in vitro has been found in *A. thaliana*, but its binding to DNA is not RNA-dependent as would be expected for telomerase [13]).

We show here, using a direct assay for telomerase activity and a well characterized, fast-growing tobacco cell line TBY-2 [14] which multiplies 80–100-fold in 1 week [15], that plant cells possess telomerase for maintenance of their telomeres.

2. Materials and methods

2.1. Tobacco BY-2 cell line (TBY-2)

The TBY-2 cell line was kindly provided by Dr. Dennis Francis, Cardiff, UK, and was propagated in modified Linsmaier and Skoog medium [16] in which the concentrations of KH_2PO_4 and thiamine HCl were increased to 370 and 1 mg/l, respectively, and sucrose and 2,4-dichlorophenoxyacetic acid were added to 3% and 0.2 mg/l, respectively [15]. Every week 2 ml of stationary phase cells were transferred to 100 ml of fresh medium and cultured on a rotary shaker at 130 rpm at 27°C in the dark. For extraction of telomerase activity, cells at the end of the exponential phase of growth (5 days) were used. High molecular weight DNA was prepared from cells cultured for 1 week.

2.2. Terminal restriction fragment patterns (TRFs) of chromosomes

Protoplasts were prepared from TBY-2 cells by treatment with 0.1% Pectolyase Y23 and 1% Cellulase YC (both from Seishin Pharmaceutical Co.) in 0.4 M D-mannitol (pH 5.5) at 30°C for 1–2 h [15]. The protoplasts were embedded in low melting point agarose and high molecular weight DNA was obtained by deproteinization and digested with frequently cutting restriction endonucleases as described previously [10,11]. The DNA fragments were then separated by pulsed field gel electrophoresis (PFGE) using a CHEF-DRII system (Bio-Rad) under the following conditions: 1% agarose gel, 0.5×TBE, 190 V, pulse time ramped from 1 to 20 s during 20 h, 15°C. The gel was alkali-blotted onto a HybondN+ membrane (Amersham) and TRFs were visualized by hybridization of the blot with the 5′ end-labeled $^{5'}(TTTAGGG)_6^{3'}$ probe (62°C, 0.25 M sodium phosphate, pH 7.5, 7% SDS, 100 μg/ml BSA) and autoradiography.

2.3. Preparation of cell extracts

Cells were collected by centrifugation at 2000×*g* for 5 min and washed once with phosphate-buffered saline and once in ice-cold wash buffer (10 mM HEPES-KOH, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol [17]). The resulting pellet (10^8 cells) was ground with a pestle and mortar in 4 ml of ice-cold extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5%

*Corresponding author. Fax: (42) (5) 41211293.
E-mail: fajkus@zoncna.ibp.cz

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CHAPS (Sigma), 10% glycerol [17]). The suspension was incubated on ice for 30 min, centrifuged at 45 000×*g* for 30 min at 4°C, and the supernatant was removed, quick-frozen in liquid nitrogen, and stored at −70°C. The protein concentration was approx. 5 mg/ml.

2.4. Telomerase assay

Telomerase activity was assayed using 14 μl of TBY-2 extract (representing 3.5×10^5 cells), 4 μl of $(TTTAGGG)_6$ oligonucleotide primer (1 μM) 5′ end-labeled with [γ-^{32}P]ATP using polynucleotide kinase (NEB), and 6 μl of 4 μl reaction buffer for *Tetrahymena* telomerase [18] to provide final concentrations of 500 μM each of dATP, dGTP and dTTP, 50 mM Tris-acetate pH 8.5, 100 mM potassium acetate, 1 mM spermidine, 5 mM β-mercaptoethanol, 2.5 mM $MgCl_2$. To inhibit RNA degradation and to improve the progress of the reaction, RNasin (Promega, 10 U/ml), and T4g32 protein (Boehringer, 20 μg/ml) were added to the reaction buffer. In control reactions the cell extracts were pretreated with RNase A (Boehringer, 100 μg/ml) for 20 min at 25°C prior to addition of primer and reaction buffer. Reactions were incubated at 30°C for 1 h, then terminated by addition of 1 vol. of stop solution (25 mM Tris-HCl (pH 7.5), 50 mM EDTA), extracted with phenol/chlorophorm and precipitated with 0.1 vol. of 3 M sodium acetate, 5 μg tRNA, and 3 vols. ethanol at −20°C. The precipitates were collected by centrifugation, resuspended in formamide loading buffer, denatured at 95°C for 5 min, and loaded on a 10% denaturing polyacrylamide gel.

3. Results

3.1. TRF lengths in TBY-2 cells

The lengths of telomeres evaluated by PFGE of TRFs ranged from 20 to 130 kb (Fig. 1A,B). These lengths are similar to those observed in tobacco leaf cells (40–160 kb, Fig. 1C; see also [10,11]), the only significant difference being an overrepresentation of shorter TRFs in TBY-2 cells where their average length is about 50 kb, in comparison to tobacco leaf cells where most TRFs fall between 90 and 130 kb. The TRF lengths were stable over the 6 month period of culture (Fig. 1B), demonstrating the considerable genomic stability of the TBY-2 cell line and the presence of a telomere-maintenance mechanism.

3.2. Detection of telomerase activity

The results of assays for telomerase activity are shown in Fig. 2. The substrate oligonucleotide $(TTTAGGG)_6$ (lanes 1) was elongated progressively in a TBY-2 cell extract (panel A, lane 2). The reaction products showed a pausing pattern similar to that produced by telomerases from animal sources. However, certain multiples of the length of the telomeric DNA repetitive unit are overrepresented (products of 49, 70 and 84 nt corresponding to $(TTTAGGG)_7$, $(TTTAGGG)_{10}$ and $(TTTAGGG)_{12}$), while the others are underrepresented in the resulting pattern. This may be a consequence of the ability of reaction products of certain size to fold into a stable secondary structure which is resistant to nuclease cleavage. During the telomerase reaction, the primer oligonucleotide and the reaction products undergo degradation from the 3′ end (see Fig. 2B); such cleavage activity is an intrinsic feature of telomerases and its proposed role is to provide a proofreading function or to improve their processivity [7]. Control reactions in which TBY-2 extract was pretreated with 100 μg/ml of RNase A showed that while specific elongation was suppressed (panel A, lane 3), cleavage of the primer oligonucleotide from its 3′ end was only partially reduced (panel B). The cleavage patterns revealed a regular 7 bp periodicity with double maxima corresponding to TTTAGGG positions.

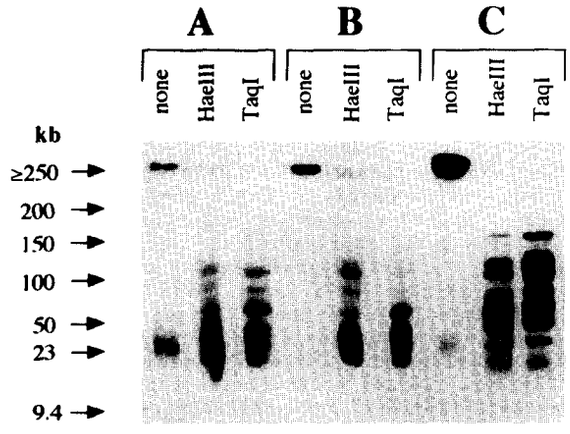

Fig. 1. Telomere lengths in TBY-2 cells compared with tobacco leaf cells. Terminal restriction fragments (TRFs) were obtained by cleavage of agarose-embedded high molecular weight DNA of TBY-2 cells from the initial culture (A) and after a 6 month period of continuous cultivation (B) with restriction endonuclease *Hae*III or *Taq*I, as indicated. Tobacco leaf TRFs are shown for comparison (C). After separation by PFGE and blotting, TRFs were visualized by hybridization with a 5′ end-labelled $^{5'}(TTTAGGG)_6^{3'}$ probe and autoradiography.

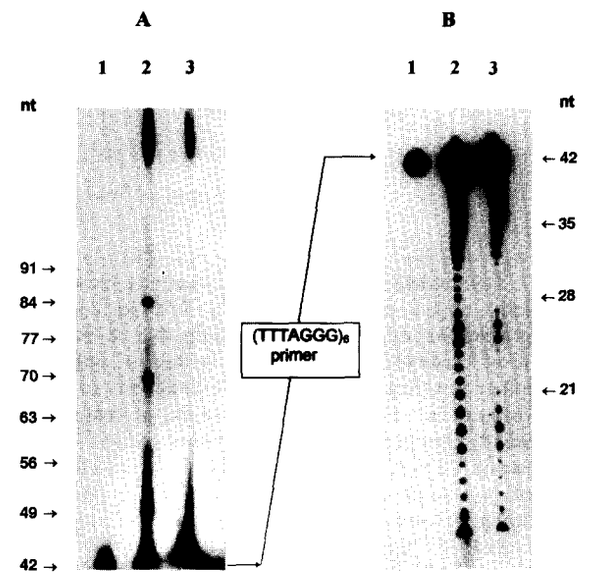

Fig. 2. Telomerase activity (A) and primer cleavage (B) in a TBY-2 cell extract. (A) Long exposure (7 days) of the upper part of the gel shows the 5′ end-labelled $^{5'}(TTTAGGG)_6^{3'}$ primer (lane 1) which was elongated in a TBY-2 cell extract (lane 2), and elongation was supressed by pretreatment of the extract with RNase A (lane 3). (B) Short exposure (12 h) of the lower part of the gel (B) shows a periodic cleavage pattern of the primer from its 3′ end, which is only partially prevented by RNase A pretreatment of the extract. The size scale is given in nucleotides (nt).

4. Discussion

The observed stability of TRF lengths over 6 months of continuous cultivation (Fig. 1A,B) demonstrates that a telomere maintenance mechanism is involved in the long-term survival of TBY-2 cells at a high growth rate (the average generation time of the cell line established in 1972 is about 16 h [15]). In comparison to the TRF pattern of tobacco leaf cells, there is an overall shift to shorter lengths in the undifferentiated state in spite of the observed telomerase activity.

This is in contradiction to recently published data on TRF lengths in differentiated and undifferentiated tissues of barley [19], but the interpretation of these data is questionable due to the high degree of nonspecific degradation of the substrate DNA which resulted in the absence of discrete bands on autoradiograms of PFGE blots of TRFs. Although the idea that telomerase is active in the dedifferentiated state and that its activity decreases during differentiation is plausible [20], the relationship between telomerase activity and telomere lengths in not simple and other factors, namely mitotic activity, must also be considered. Recent data support a model in which telomerase, once activated during the telomere shortening process, neither restores the original length of telomeres nor elongates them above the original length, but rather that it stabilizes their current lengths [21]. Our data on TBY-2 and leaf TRFs are consistent with this model.

The main conclusion of this work is that telomerase activity can be detected in plant cells using a direct assay (10^5 cells per assay are necessary). Studies of the primer specificity of plant telomerase are now in progress, which should allow us to design a non-telomeric primer suitable for a telomeric repeat amplification protocol (TRAP) analogous to that used for detection of telomerase activity in human cells [17]. The availability of telomerase-containing TBY-2 cell extracts and of the direct method for detection of plant telomerase reported here will enable a plant TRAP to be established which will increase the sensitivity of detection of telomerase by several orders of magnitude.

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