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Barley telomeres shorten during differentiation but grow in callus culture

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ABSTRACT **Eukaryotic chromosomes terminate with long stretches of short, guanine-rich repeats. These repeats are added *de novo* by a specialized enzyme, telomerase. In humans telomeres shorten during differentiation, presumably due to the absence of telomerase activity in somatic cells. This phenomenon forms the basis for several models of telomere role in cellular senescence. Barley (*Hordeum vulgare* L.) telomeres consist of thousands of TTTAGGG repeats, closely resembling other higher eukaryotes. *In vivo* differentiation and aging resulted in reduction of terminal restriction fragment length paralleled by a decrease of telomere repeat number. Dedifferentiation in callus culture resulted in an increase of the terminal restriction fragment length and in the number of telomere repeats. Long-term callus cultures had very long telomeres. Absolute telomere lengths were genotype dependent, but the relative changes due to differentiation, dedifferentiation, and long-term callus culture were consistent among genotypes. A model is presented to describe the potential role of the telomere length in regulation of a cell's mitotic activity and senescence.**

Telomeres are essential structural and functional components of eukaryotic chromosomes (reviewed in refs. 1 and 2). The most terminal fragment of the chromosome consists of tandem arrays of short, G-rich sequences referred to as telomeric repeats (TRs). There are several to thousands of TRs per chromosome end, added *de novo* by a specialized enzyme, telomerase (reviewed in ref. 1). Telomerase is a ribonucleoprotein polymerase that contains its own RNA template component, thus representing a unique reverse transcriptase.

Telomerase activity was first identified in the unicellular ciliate *Tetrahymena* (3). Its activity has also been reported in immortalized human (4, 5) and mouse (6) cell lines. Recently, telomerase activity was detected throughout oogenesis and embryogenesis in *Xenopus* (7). Telomerase is apparently inactive in differentiated human tissues (8, 9). Developmental inactivation of telomerase was suggested as the cause of telomere shortening in human somatic tissues compared to the germ line (9). Telomere shortening was predicted based on the characteristics of DNA polymerases (10, 11) and inferred as a potential cause of cellular senescence (10).

The role of telomere shortening in cellular senescence has attracted considerable attention and several speculative models have been proposed to explain the relationship (9, 12). An important question about the generality of this phenomenon remains. Telomere length is apparently not related to cellular senescence in unicellular eukaryotes like yeast (13) and *Paramecium* (14). It is possible that during the evolution of multicellular eukaryotes the telomere became a mitotic clock in differentiation (9). Data supporting the hypothesis that telomere length has a role in cellular aging is limited to humans. Mouse somatic cells do not show a decrease in telomere length compared to germ-line cells (15, 16).

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Although the first higher eukaryote telomere sequence to be cloned was from a plant, *Arabidopsis thaliana* (17), nothing is known about the developmental dynamics of telomere length in the plant kingdom. In this report telomere length was analyzed in barley (*Hordeum vulgare* L.) *in vivo* and in tissue culture cells. Differentiation and aging resulted in reduction of terminal restriction fragment (TRF) length paralleled by a decrease in the number of TRs. Dedifferentiation in callus culture resulted in an increase in the TRF length and in the number of TRs. A model is presented to describe the potential role of telomere length in regulation of a cell's mitotic activity and senescence.

MATERIALS AND METHODS

Plant Material. Plants used for DNA extraction were kept in growth cabinets at 18°C, with 16 hr of light. Immature embryos were isolated from two or three spikes representing a similar developmental stage. The youngest embryos amenable for analysis were <1 mm long. The oldest embryos analyzed were 2–2.5 mm long and close to maturity. Usually 30–40 embryos of similar size were bulked for one DNA extraction except for the youngest stage, where ≈100 embryos were combined. Immature inflorescences of 8–10 mm, ≈30 mm, and 55–60 mm were used for DNA extraction. The youngest inflorescences were bulked (3–5), while individual spikes at the older stages provided enough DNA for Southern and dot blot analyses. Leaves from 3- to 4-week-old plants were used for DNA extraction.

Callus Culture. Callus cultures were initiated from immature embryos (≈1.5 mm), cultured, and maintained on an autoclaved modified MS medium (P. Bregitzer, U.S. Department of Agriculture, Agriculture Research Station, Pacific West Area, Aberdeen, ID). The medium contained MS salts (18), maltose (30 g/liter), inositol (100 mg/liter), thiamine (1 mg/liter), nicotinic acid (0.5 mg/liter), pyridoxine HCl (0.5 mg/liter), Gelrite (3.5 g/liter) (Schweizer Hall, South Plainfield, NJ), and 2,4-dichlorophenoxyacetic acid (1.5 mg/liter). Cultures were incubated in the dark at 23°C and subcultured at 3- to 4-week intervals to fresh medium. Long-term cv. Golden Promise, Klages, Morex, and Steptoe callus cultures were from Phil Bregitzer and one cv. Golden Promise callus line was from Peggy Lemaux (University of California, Berkeley, Plant Gene Expression Center, Albany, CA).

DNA Extraction and Analysis. DNA from all tissues was extracted according to published methods (19). The integrity of DNA for Southern blot analysis was determined by pulsed-field gel electrophoresis using the CHEF DR III system (Bio-Rad) and the conditions recommended by the manufacturer for resolving λ concatamers. Only samples with the bulk of DNA running above 150 kb were used for Southern blot analysis. About 5 μg of genomic DNA was cut with *Mbo* I

Abbreviations: TR, telomeric repeat; TRF, terminal restriction fragment.

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restriction enzyme and resolved in 1% agarose gel using the CHEF DR III system and the conditions suggested by the manufacturer for resolving a 5-kb ladder, with the length of the run reduced from 11 to 8 hr. λ concatamers and a *Hin*dIII digest of λ DNA were used as markers. DNA in the gel was stained with ethidium bromide, depurinated by a 20-min incubation in 0.25 M HCl, washed with deionized water, and incubated for 20 min in transfer solution (0.4 M NaOH/0.6 M NaCl). DNA fragments were transferred to GeneScreen*Plus* nylon membranes (New England Nuclear) and hybridized with labeled TR primer $(CCCTAAA)_4$. T_4 polynucleotide kinase (BRL) was used to end-label 15 pmol of the primer with 50 μCi of [γ-^{32}P]ATP (specific activity, 6000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). Pre- and hybridization buffers were as described (19). Hybridizations and final washes were at 55°C. Two washes with 4× SSC/1% SDS were followed by the final wash with 2× SSC/1% SDS. Hybridizations with all other probes were according to published methods (19).

For dot blot analysis, ≈5 μg of barley DNA was denatured in 0.25 M NaOH/0.5 M NaCl for 10 min at room temperature, quenched on ice for 10 min, and blotted onto GeneScreen*Plus* membranes. Plasmid DNA from clone pT56 containing 25 barley TRs $[(CCCTAAA)_{25}]$ as determined by sequencing (data not shown) in pUC19 vector was included on each blot. DNA from pT56 (0, 1, 5, and 25 ng) was mixed with 5 μg of pUC19 plasmid and processed as barley genomic DNA. Hybridizations and washes were done as described for Southern blots.

Data Analysis. The membranes were exposed to x-ray film and scanned with an AMBIS radioanalytic imaging system. Quantitation of the signal was on AMBIS scans using the software provided by the manufacturer.

For TRF estimation, the middle 50% of each Southern blot lane (to avoid interference from neighboring lanes) was quantified and mean telomere length was calculated from the output as described (20). For quantitation of dot blots, the number of counts for pUC19 without pT56 was used as the background. To determine the number of TRs per sample, the signal obtained with TR primer was divided by the signal for the 5-ng pT56 sample and multiplied by 3.8×10^{10} (number of copies of the 7-bp TR sequence in 5-ng pT56 sample). Since 5 μg of barley DNA contains $\approx 10^6$ copies of the barley genome [based on genome size of 5×10^9 bp (21) and Avogadro's number], the number of TRs per haploid genome was obtained by dividing the number of TRs per sample by 10^6. The number of TRs per genome was converted to telomere length by multiplying the TR number by the repeat length (7 bp) and dividing by the number of telomeres (14) in a haploid genome.

A correction factor for the amount of DNA loaded was determined by averaging the signal with total genomic DNA probe from five very carefully quantitated DNA samples and dividing the signal for each sample by this value.

Telomere length estimates based on TRF analysis were often much smaller than those based on dot blot analysis (i.e., 75 kb versus >200 kb for long-term Klages cell cultures). The difference between these estimates was smaller for shorter telomeres—i.e., long-term Morex cell cultures (50 kb versus 60 kb). Random shearing during DNA extraction probably accounts for most of the differences since the telomere length estimated from dot blots was sometimes higher than the average length of undigested DNA. Reduced Southern transfer efficiency for high molecular weight DNA as well as target DNA quantitation for dot blots (both for barley genomic and plasmid pT56) may also contribute to the differences between the estimates. We want to stress, however, that in spite of the differences in absolute values, both methods gave very similar relative values for genotypes/tissues compared.

RESULTS

Telomeres Shorten During Differentiation. To determine the fate of telomeres during differentiation in barley, embryo and inflorescence tissues at different stages of development were analyzed. Strictly meristematic tissues are not available in barley in sufficient amounts for routine DNA extraction. However, growing embryos and inflorescences allowed clear separation of various developmental stages based on size measurements. Both TRF size and the number of TRs were determined.

TRF length decreased with increased differentiation and age in the cv. Golden Promise embryos (Fig. 1*A*). The oldest embryo telomeres were between 25 and 30 kb, ≈20 kb shorter than those from the youngest embryos. Telomeres of the oldest embryos were still ≈5 kb longer than the telomeres of fully expanded leaves that had a mean TRF around 23 kb (Fig. 1*A*). The quality of restriction digest and quantity of DNA loaded per lane did not influence the results significantly (Fig. 1*B*).

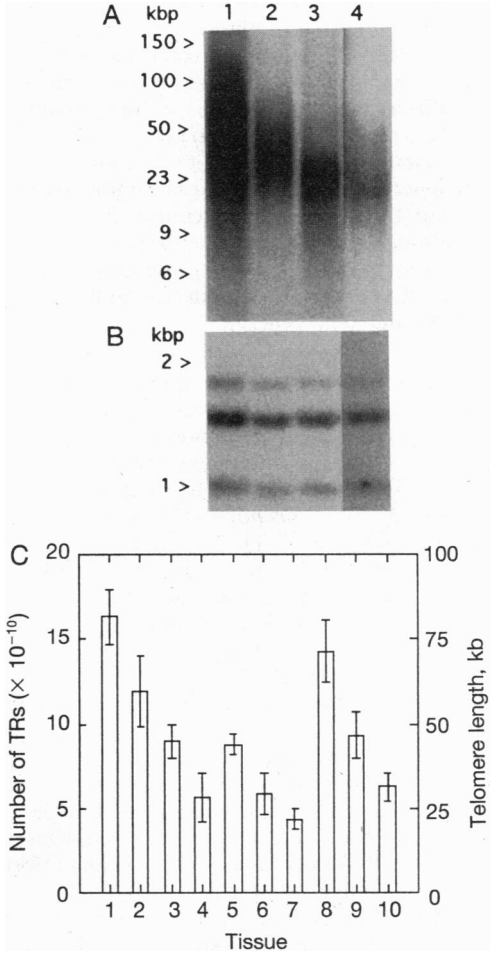

FIG. 1. Telomeres shorten during barley embryo development. *(A)* TRFs were resolved and detected as described from cv. Golden Promise immature embryos <1 mm (lane 1), ≈1.5 mm (lane 2), and 2–2.5 mm (lane 3) long. Leaf tissue from 3- to 4-week-old plants was included for comparison (lane 4). *(B)* Same blot stripped and hybridized with barley ADP-glucose pyrophosphorylase single copy clone besF2 to demonstrate restriction digest quality and to compare DNA quantity per lane. *(C)* TR number was determined from dot blots hybridized with a TR probe and scanned on an AMBIS radioanalytic imaging system. TR number and telomere length were calculated as described. Mean and standard error bars are based on approximately five independent extractions for each sample. Samples 1–4, as in *A*; samples 5–7, cv. Morex immature embryos 1–1.5 mm, 2–2.5 mm, and leaf tissue, respectively; samples 8–10, cv. Steptoe immature embryos 1–1.5 mm, 2–2.5 mm, and leaf tissue, respectively.

The dot blot method used to estimate TR number allowed good quantitation of the dynamics of telomere shortening, although between-sample variation was considerable. Some of this variation may be due to size and stage of development differences among the embryos bulked for analyses. In the cv. Golden Promise, the number of TRs per 5-μg sample in the youngest embryos analyzed was $\approx 16 \times 10^{10}$, corresponding to an average length of 80 kb for each telomere (Fig. 1*C*). In somewhat older embryos, the TR number decreased by 25% to 12×10^{10}, or $\approx$60 kb per average chromosome end. The oldest embryos analyzed had 9×10^{10} TRs, representing 56% of the youngest embryo value. DNA from leaves had 30% fewer TRs than the oldest embryos, $\approx$30 kb per average chromosome end.

Analysis of immature embryos and leaves from two other genotypes, Morex and Steptoe, showed a similar pattern of TR number reduction during differentiation (Fig. 1*C*). Interestingly, the number of TRs in all tissues of the cultivar Morex was significantly smaller than in Steptoe, but in both genotypes the number of TRs in leaves was about half that observed in very young embryos.

TRF length and TR number changes during development of cv. Golden Promise inflorescence showed a pattern similar to that observed with immature embryos. In the youngest inflorescence analyzed (8–10 mm), mean TRFs were 40–45 kb (Fig. 2*A*). In the oldest inflorescence (5 cm without awns), TRFs were shorter by $\approx$20 kb, having a length similar to that in leaves ($\approx$23 kb). Dot blot estimates of the number of TRs also showed a similar reduction with differentiation (Fig. 2*C*). The TR number decreased by nearly 50% between the youngest and the oldest inflorescences analyzed. Leaves had only slightly fewer TRs than the oldest inflorescences studied.

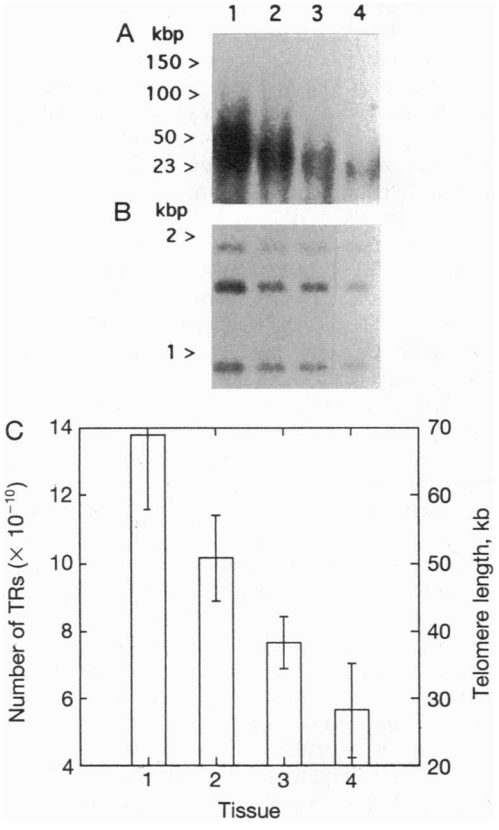

FIG. 2. Telomeres shorten during barley inflorescence development. *(A)* TRFs (see Fig. 1*A* legend) from cv. Golden Promise inflorescences 8–10 mm long (lane 1), $\approx$30 mm long (lane 2), 55–60 mm long (lane 3), and leaf tissue (lane 4). *(B)* Same blot stripped and hybridized with barley ADP-glucose pyrophosphorylase single copy clone besF2 to demonstrate restriction digest quality and to compare DNA quantity per lane. *(C)* TR number and telomere length (see Fig. 1*C* legend) for the same samples as in *A*.

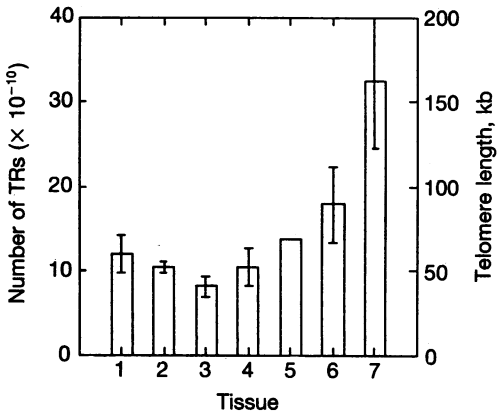

FIG. 3. Telomeres grow in barley callus culture. TR number and telomere length (see Fig. 1*C* legend) for cv. Golden Promise explant (1.5-mm immature embryos) (sample 1) and callus 2 (sample 2), 4 (sample 3), 6 (sample 4), 8 (sample 5), and 16 (sample 6) weeks, and >1 year (sample 7) in culture.

Telomeres Grow in Barley Callus Culture. To study the dynamics of telomere length *in vitro*, callus cultures were initiated from cv. Golden Promise immature embryos ($\approx$1.5 mm). The number of TRs was estimated at various time points after culture initiation. During the first 4 weeks a small decrease in TR number was observed (Fig. 3). Later, the trend reversed and, in the older cultures, very high TR numbers were observed. The average number of TRs in a year-old culture was 32×10^{10}, almost 3 times the number in the explant tissue (Fig. 3). Variation of the parameter studied increased along with the mean and the highest value was $>70 \times 10^{10}$, representing $\approx$350 kb of telomere. The TR number in shoots differentiating from young calli was about the same as in normal leaf tissues (data not shown).

TRF length and TR number determined for cv. Steptoe, Morex, and Klages long-term culture calli showed a dramatic increase when compared to leaf tissue (Fig. 4 *A* and *C*). This was particularly apparent in the TR number (Fig. 4*C*) for cv. Klages. The shortest telomeres, both TRF and TR assay, were observed for cv. Morex. This was in agreement with previous observations for cv. Morex immature embryos; however, the average TR number increase was $\approx$3-fold compared to leaf tissue, as in the other genotypes.

DISCUSSION

Telomeres Contract During Differentiation. TRF length and TR number decreased in barley embryos and inflorescences during differentiation (Figs. 1 and 2). Hybridization of the blots with several barley telomere-associated sequence probes (22) did not detect differences in the pattern or abundance of TR-proximal sequences in any of the tissues analyzed (data not shown). These observations support the conclusion that telomere shortening is due to reduction of TR number at the chromosome ends. Incomplete replication of DNA molecules at the ends of the chromosomes, probably due to limited telomerase activity, seems to be the most likely explanation for this phenomenon.

Precise quantitation of telomere shortening dynamics is difficult because of lack of information on the number of cell divisions separating the developmental stages analyzed. The number of cell divisions between zygote and fully expanded maize leaf was estimated to be $\approx$50 (23). Our estimates for barley, based on DNA yield from various stages/tissues, gave a similar value, with $\approx$15 cell divisions from the zygote to the youngest embryo analyzed and 35 from the youngest embryo

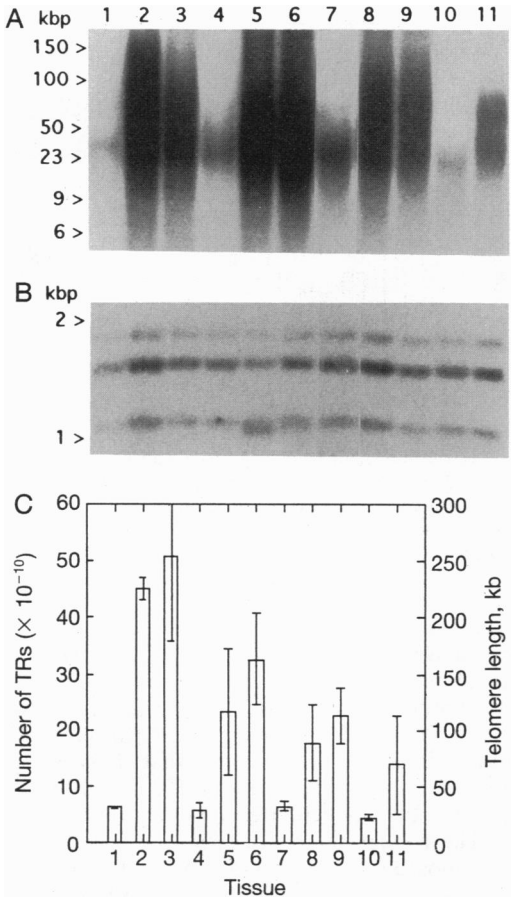

FIG. 4. Long-term callus cultures have very long telomeres. *(A)* TRFs (see Fig. 1*A* legend) from cv. Klages leaf (lane 1) and callus culture 6 months (lane 2) and 3 years (lane 3) old, cv. Golden Promise leaf (lane 4), callus culture 9 months (lane 5) and 14 months (lane 6) old, cv. Steptoe leaf (lane 7) and callus cultures 4 months (lane 8) and 6 months (lane 9) old, cv. Morex leaf (lane 10) and callus culture 6 months (lane 11) old. *(B)* Same blot stripped and hybridized with barley ADP-glucose pyrophosphorylase single-copy clone besF2 to demonstrate restriction digest quality. *(C)* TR number and telomere length (see Fig. 1*C* legend) for the same samples as in *A*.

to a fully expanded leaf. Since the telomeres of the youngest embryo were 25–50 kb longer than in the fully expanded leaf in cv. Golden Promise (based on TRF and TR data, respectively), the average reduction was estimated to be 700–1400 bp per chromosome end per cell division. This value is much higher than the 40–200 bp reported for human telomeres (20, 24). The rate of telomere shortening in humans was determined by using somatic cell lines in culture, where each cell has a similar chance of undergoing a mitotic division. In plants, where the mitotic activity is concentrated in discrete meristems (25), the assumption of exponential growth (synchronous cell divisions) is not fulfilled. As a result, the cell cycle number is probably underestimated.

Telomeres Expand During Undifferentiated Growth. TR number increased in barley cells during undifferentiated growth in cell culture (Fig. 3) and long-term cultures had very long TRFs and a large number of TRs (Fig. 4). This observation is in contrast with human cell cultures (20, 24). Human telomeres shorten until the culture reaches a crisis stage, when very rare immortalization events may occur, usually as a result of transformation by a DNA tumor virus (9, 26). Telomerase activation seems to be involved in immortalization events *in vitro* (20) and in malignant cells (5, 8, 26, 27). Telomerase may be activated in barley cell culture, since the telomere length increased soon after callus initiation (Fig. 3). Alternatively, a small portion of cells in the explant (immature embryo) expressing telomerase may have a selective advantage leading to their preferential growth in tissue culture and an apparent overall telomere length increase.

The reason for the very long telomeres in long-term barley callus culture cells is not clear. Interestingly, all of the long-term cultures analyzed were nonmorphogenic. The relationship between chromosome instability and the loss of regeneration potential in long-term callus cultures of barley has been established (28, 29). Two types of chromosomal abnormalities have been reported to be predominant in callus cells, chromosomal structural rearrangements (28) and polyploidy (28, 29). An apparent increase in telomere length in human cell lines stably transfected with plasmids containing TR sequences was attributed to chromosome truncation and seeding of new telomeres (30). In yeast, an increase in telomere length was caused by transformation with plasmids containing stretches of TRs (31). The increase of telomere length was proportional to plasmid copy number, suggesting an active role of TR binding protein(s) in controlling telomere length (31). Polyploidization in barley callus cultures may be analogous to yeast cells transformed with TR-containing plasmids since the number of telomeres (and TRs) per cell increases with the ploidy level. It is possible that an increase in titration of telomere binding protein(s) by the increased number of telomeres may trigger a growth of individual telomeres in barley as suggested for yeast (31).

The possibility of ploidy changes in long-term callus cultures means that we cannot calculate the TR number per genome for these samples. However, the number (and the length) of TRs per chromosome end is not affected by the ploidy changes since there is a fixed number of chromosomes (and telomeres) in a given amount of DNA. An increase in TR number can be due to increased telomere length or an increase in the number of telomeres per unit of DNA. The latter situation would occur only if the chromosomes became fragmented. This is not the usual phenomenon in barley callus culture. The presence of many short telomeres versus a few long telomeres in the long-term barley callus cultures is also excluded by the TRF length measurements, which parallel the telomere length estimates based on TR number.

Model for TR-Based Mitotic Clock. Telomere shortening during differentiation in barley is similar to processes observed in humans (9, 20, 32). The phenomenon of telomere shortening is presumed to be due to the absence of telomerase activity and forms the basis for a model of cellular senescence (9, 12). Deletion of critical genes in the course of telomere shortening, limiting the proliferative capacity of human somatic cells, was originally proposed as the reason for the senescence (9). Reversibility of cell mortality in culture prompted a new model, in which telomere positional effects regulate cellular senescence (12). In this model, genes repressing cellular senescence are presumed to be located in the telomeric regions of chromosomes and inactivated by telomere shortening. Recent information raises questions about this model. First, longer TR tracts in yeast were shown to increase rather than decrease transcriptional repression at the telomere (33). Second, telomere length did not affect expression of the SV*neo* gene in human and mouse cell lines in spite of a very small physical distance between this gene and the TR (30). Third, telomere-associated sequences just proximal to TRs are often highly repetitive and believed to form a gene-poor "buffering zone" for highly recombinogenic chromosomal ends (reviewed in refs. 2 and 34). Location of a developmentally important gene in such a "risky" environment would seem to be selectively disadvantageous.

Here we propose an alternative model based on the role of telomere binding protein(s) in repression/activation of genes controlling mitotic and metabolic activity of the cell. Two scenarios can be put forward. The first one invokes the gene

coding for the telomere binding protein that is positively autoregulated. In the germ line (long telomeres) a large proportion of the protein is bound to chromosomal termini. This reduces the interaction of the hypothetical protein with other proteins and/or TR-like sequences elsewhere in the genome, including the promoter of its own gene. During telomere shortening, more and more protein is liberated, increasing the level of its own expression. This positive feedback results in a high protein concentration in differentiated cells (short telomeres). Above a threshold level the protein induces genes for antimitotic proteins and/or represses genes involved in mitotic processes. Tumor suppressor genes encoding p53 and Rb may be targets for the hypothetical protein in a human system (35).

The second scenario is based on the assumption that telomerase downregulates a gene for telomere binding protein or suppresses the hypothetical protein's activity. Once telomerase is repressed by the cell's entry into a differentiation process, concentration and/or activity of the TR binding protein may increase with successive cell divisions up to a threshold level. This, again, may lead to the induction of antimitotic proteins' genes.

These two scenarios are not mutually exclusive but differ in the way the amount/activity of the protein relates to telomere length in the cells with a different "mitotic history." In the first scenario, telomere length has an active role in the senescence process; in the second, the telomere shortening is merely the result of telomerase repression and incomplete DNA replication. Both scenarios could be used as a possible mortality stage 1 (M1) mechanism in the model of reversible cellular senescence developed for mammalian *in vitro* systems (26). Also, both scenarios could be verified when the TR binding proteins are identified in organisms that exhibit telomere shortening during the differentiation process.

A number of TR binding proteins have been characterized (reviewed in ref. 2). The most relevant to our model is RAP1 protein, a very abundant (at least for a sequence-specific DNA-binding protein) repressor/activator with numerous functions in the yeast cell (reviewed in ref. 36). The RAP1 protein binding sites are found upstream of numerous genes encoding the components of protein synthesis and glycolytic enzymes. Interestingly, the RAP1 binding site was identified in the promoter of its gene, suggestive of autoregulation of *Rap1* (37). RAP1 protein appears to be a key element orchestrating transcription of genes regulated by growth rate, probably via a *ras* pathway through protein kinase A (38). Apart from activating and repressing metabolically important genes, RAP1 functions as a major telomere binding protein that may be involved in regulation of TR number (39, 40), telomere positional effects (33), or attaching telomeres to the nuclear envelope (41).

A higher eukaryote equivalent of the yeast RAP1 protein remains to be identified, but telomere repeat-like sequences (teloboxes) have been found in the 5′ regions of some *A. thaliana* genes (42). Four *A. thaliana* and one tomato gene encoding the translation elongation factor EF-1α contain a telobox at the same location within the promotor (42), while the yeast gene for the same protein has a RAP1 binding site in its promoter (43, 44). Functional importance of the teloboxes in plant genes remains to be established, but it is tempting to speculate that one (or more) telomere binding protein may control metabolic activity in higher eukaryotic cells like the RAP1 protein does in yeast cells. Cloning of the components of the system and reconstitution *in vitro* will be necessary for testing this hypothesis.

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