

Telomerase activity in ‘immortal’ fish

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Abstract Eukaryotic chromosome termini consist of telomeres, short sequence repeats. According to the telomere hypothesis, DNA replication leads to telomere shortening, resulting in a cellular mitotic clock. Telomerase resets it by telomere synthesis. In mammals with a limited growth phase, telomerase activity in somatic tissues is restricted to stem cell derivatives with high proliferation potential. But other animals, like some fish, grow throughout their life with little senescence. All somatic cells require a high proliferation capacity and telomerase should be active in all cells, irrespective of fish age. Indeed, we detected high telomerase activities in all analyzed organs of rainbow trout (*Oncorhynchus mykiss*).

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Key words: Indeterminate growth; Cell proliferation; Senescence; Telomerase assay; Rainbow trout

1. Introduction

Replication of linear chromosomes results in continuous loss of 5'-terminal segments [1,2]. Telomeres, repetitive sequences at chromosome termini, protect against deleterious effects, but cell proliferation leads to erosion of telomeres [3,4]. According to the telomerase hypothesis, telomere shortening acts like a mitotic clock, short telomeres lead to cell senescence and subsequent mortality. New telomeres are synthesized by telomerase [5]. Unicellular eukaryotes express high telomerase levels, for example ciliates [6–8] and yeasts [9,10]. Telomerase studies in metazoa have focused on mammals which grow only in the embryonic and juvenile, but not in the adult and senescent stages. In adult humans, telomerase activity in normal somatic tissues is limited to stem cell derivatives with high proliferation potential [5]. To test the telomerase hypothesis in a cellular model, telomerase was activated by transfection of telomerase-negative human somatic cells. Primary cells senesced, whereas transfected, telomerase-positive cells were immortalized [11]. In contrast to mammals, some animals grow throughout their life and show very slow senescence. Examples are several fish species [12], and cells in all organs require a high proliferation capacity. Extending the telomerase hypothesis to fully developed multicellular organisms, proliferation capacity would require high telomerase activities in all fish cells.

2. Materials and methods

2.1. Preparation of tissue extracts

Rainbow trout were obtained from local fish farms. Tissue samples were collected in 1.5 ml microcentrifuge tubes, flash-frozen on liquid nitrogen and stored at -70°C . Tissues were lysed in the standard CHAPS buffer [14]. Mechanic disruption was improved by grinding the samples with plastic micropestles [29], obtained from Eppendorf (Hamburg, Germany). Removal of cell debris and recovery of the supernatant lysate was done as described [14]. If appropriate, lysates were flash-frozen again and stored at -70°C . Protein concentrations were determined with the Bradford assay, using reagents supplied by Bio-Rad.

2.2. Telomerase assays

Telomerase activities were determined as described for human cell extracts and PCR amplifications were performed with an Eppendorf Mastercycler [14]. We used a 5'-fluorescein-labeled reverse primer CX-ext to obtain fluorescent-labeled PCR products. The inclusion of an internal amplification standard (ITAS) permits quantitative comparisons, and ITAS was prepared by PCR essentially as described [15]. As template we used the gene of the RNA component of *Thermus thermophilus* RNase P [30]. Primers were F-286 GCTTGC-CAATCCGTCGAGCAGAGTTCGGGATGGGCCGCTTGAGGC and R-376 GTGCCCTTACCCCTTACCCCTTACCCCTAACGCCTCGGGACGAGGCGTAAG. The primers include the sequences for TS and CX-ext, respectively (underlined). The PCR product was cloned by insertion into plasmid pCR2.1 (Invitrogen). Prior to its use in the TRAP assay, the resulting plasmid pITAS-P129 was digested with *EcoRI* to liberate the linear insert. Optimal amounts for a 50 μl reaction were in the range of 0.01–0.1 amol (10^{-18} mol) of digested plasmid pITAS-P129, which yields a 129 bp PCR product in the TRAP assay. Aliquots of the TRAP assay products (2 μl) were analyzed by capillary electrophoresis (ABI prism 310) as described [14]. Integrated values were added up for all telomerase products containing five (one repeat beyond primer dimer size) to 17 telomeric hexamer repeats and calibrated by dividing by the value for ITAS (see Fig. 1). The values shown in Fig. 2 were obtained by comparison with a calibration curve for extracts (0.04–2 μg) from the Hodgkin's disease human tumor cell line L-428 [14], available from DSMZ, Braunschweig, Germany (DSM ACC 197). Fish extracts with different protein concentrations (prepared from at least two fish of each age group) were analyzed and all assays were performed at least in triplicate; standard deviations are indicated (Fig. 2). For ribonuclease inactivation, extracts (containing 1 μg protein) from different organs were preincubated with 100 ng RNase A for 10 min at 30°C .

3. Results

Chromosomes from all vertebrates, including fish, contain the same telomeric repeat sequence TTAGGG [13]. Therefore, we could apply our recent modification of the TRAP assay, initially developed for the determination of human telomerase activity [14]. The inclusion of an internal amplification standard permitted semiquantitative determination [15]. Telomerase activities were determined in extracts from several organs of rainbow trout. Irrespective of age and size, we found high telomerase levels in liver, skin, heart, even in muscle and brain tissue (Fig. 1, samples B–G). For a convenient direct comparison, all analyses shown in Fig. 1 were prepared with highly

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We dedicate this publication to E.S. Quabius. This study would have been impossible without her initiating ideas.

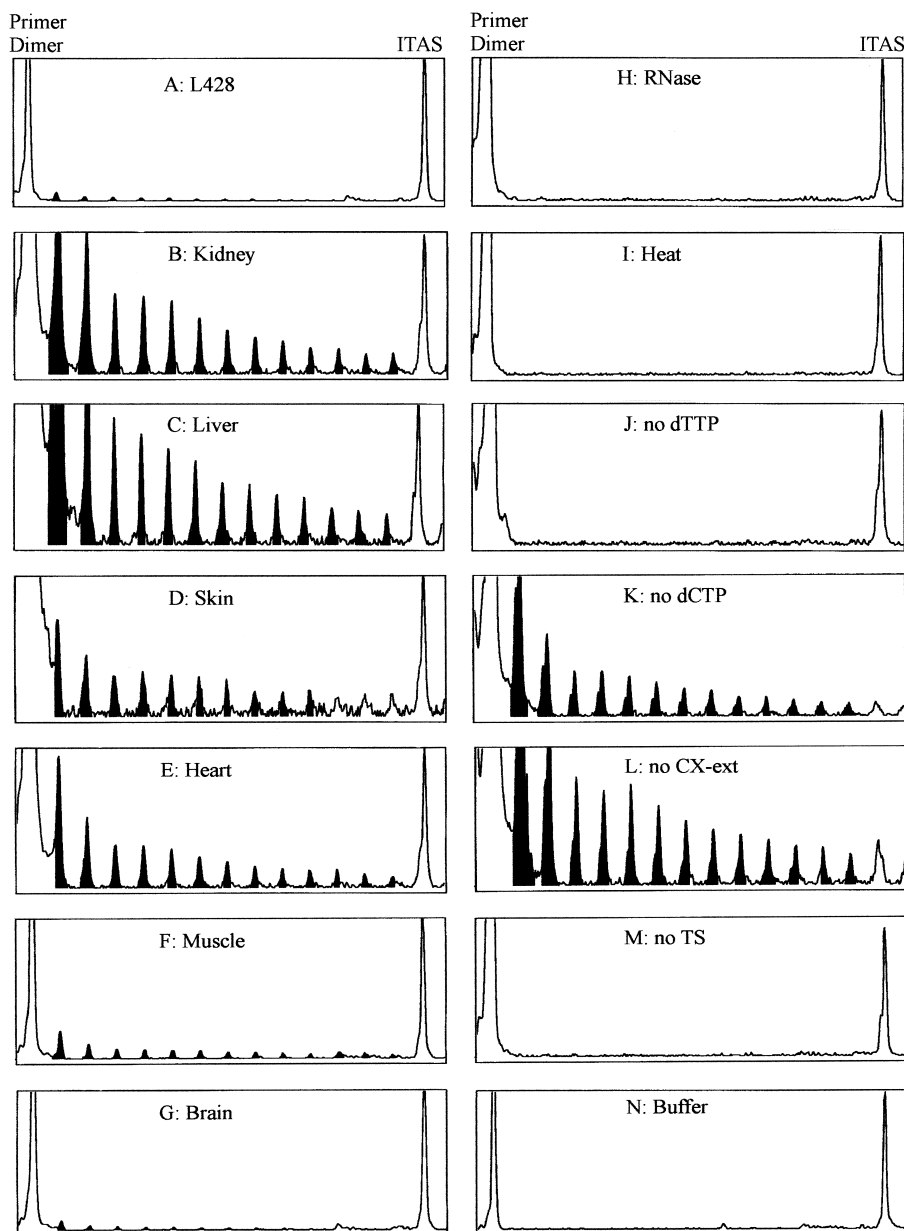


Fig. 1. Telomerase activities were determined with the TRAP assay, including an internal amplification standard [14,15]. Fluorescence-labeled PCR products were analyzed by capillary electrophoresis [14]. Telomerase elongation products are shown in black; positions of primer dimer and ITAS (internal amplification standard) are indicated. For comparison, all examples which are shown here were obtained with the same, very low amount of protein (0.02 μ g) and vertical scales were adjusted to include the ITAS signal (white peak at the right). Extracts from the human tumor cell line L-428 and from organs of medium size trout (see also Fig. 2) were analyzed. Additional data were obtained with a range of 0.04–2 μ g protein (not shown). A: Human tumor cell line L-428; B–G: extracts from trout organs, as indicated. H–M: Various controls; H,I: inactivation of telomerase in extract from trout liver by pretreatment with RNase A (sample H) or by heating at 85°C for 10 min (sample I); J–M: in control experiments with liver extracts, components were omitted in the telomerase elongation step, but added in the subsequent PCR amplification; J: without dTTP; K: without dCTP; L: without reverse primer CX-ext; M: without forward primer TS; N: negative control, without extract (buffer only).

diluted extracts containing the same, very low amount of 0.02 μ g protein; this means the relatively low telomerase activities in the human cell line L-428, trout brain and muscle were barely detectable. Accordingly higher activities were observed with higher protein amounts, up to 2 μ g (not shown). As expected for a ribonucleoprotein, telomerase activity was abolished by heating or by pretreatment with ribonuclease. Similar to previous studies with plant extracts [16], we also obtained the expected results after omitting the following individual components in the telomerase elongation step only.

But the missing compound was supplemented in the subsequent PCR amplification. In agreement with the telomeric repeat sequence TTAGGG, telomerase activity was abolished after omitting dTTP, dATP or dGTP, but unaffected by the absence of dCTP. The omission of the reverse primer CX-ext had no effect, whereas no telomerase elongation products were detected in the absence of forward primer TS (Fig. 1, samples H–M).

For comparison of fish telomerase activities with human tumor cell lines, we show data with the Hodgkin tumor cell

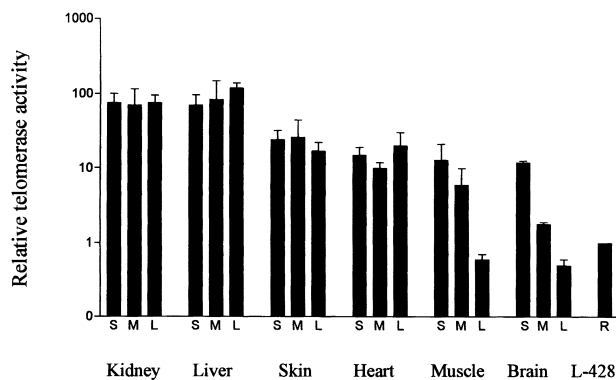


Fig. 2. Relative telomerase activities in extracts from trout organs. For quantitative comparison, the human cell line L-428 [14] was used as reference and its value was defined as 1. Relative values were obtained by comparison with a calibration curve for extracts from L-428 cells (0.02–2 µg). For example, a value of 20 was obtained if telomerase activity with 0.02 µg fish extract was equivalent to 0.4 µg L-428 extract. At least two trout of each size group were analyzed and all assays were performed at least in triplicate; standard deviations are indicated. Fish organs are indicated and three size groups of trout were analyzed: S (weight: 4 ± 2 g; age: 1 month); M (400 ± 50 g; 30 months) and L (2000 ± 200 g; 42 months).

line L-428 [14]. In extracts from other cell lines, like HeLa cells or the lymphoma cell line U-937 (ATCC CRL-1593), we observed variations of telomerase activities, but based on protein amounts these variations were threefold or less (not shown). Compared to the human cell lines, we found similar telomerase levels in fish brain and muscle, even higher values in skin and heart, and up to 100-fold higher activities in trout kidney and liver samples (Figs. 1 and 2). Noteworthy, the already lower telomerase activities in fish brain and muscle show a further decrease in older fish, but they retain a level comparable to fast growing human tumor cell lines (Fig. 2).

4. Discussion

In normal mammalian somatic tissues, the stem or progenitor cells with high proliferation capacity have telomerase competence or the ability to express telomerase. In addition, increasing evidence has linked telomerase expression to cell proliferation rates [17,18]. This model was extended by findings in the plant kingdom. Cells with high proliferation capacity and high mitotic activity reside in meristematic tissue. Telomerase activities were detected in these tissues as well as in cultured cells, whereas non-dividing cells from leaves and axillary buds were telomerase negative [16,19].

We have shown that indeterminate growth of fish and the very slow occurrence of senescence is accompanied by high telomerase activities in all investigated fish tissues. Interestingly, the gradual decrease of telomerase activity in trout brain agrees with an age-dependent decline of proliferative activities, which was previously observed in trout brain in an age range from 1 to 24 months [20]. As an *in vitro* effect, telomerase activity in normal cells is expected to facilitate their immortalization to permanent cell lines. Accordingly, the less stringent telomerase repression in murine cells has been linked to the relative ease in obtaining immortalized murine fibroblasts, as compared to human fibroblasts [21]. In agreement with the observed high telomerase activity in

fish tissues, permanent lymphoid cell lines have been readily obtained from peripheral blood cells of channel catfish (*Ictalurus punctatus*), in contrast to corresponding mammalian cell lines [22].

Our data demonstrate that all investigated tissues in rainbow trout retain telomerase competence and extend the current telomerase hypothesis from unicellular organisms and cell lines to a multicellular organism. At least for vertebrates, our data suggest that continuous telomerase expression is the primary route for maintaining long-term cell proliferation capacity. Quite different possibilities are also known, like the distinct primary route for maintaining the unique telomere structures in *Drosophila* [23] and as a secondary option, a different route was characterized in yeast [24] and suggested for humans [25,26]. It seems possible that prevention of telomere erosion can prevent senescence not only at the cellular level but also in adult animals. But we have no direct proof that slow senescence in fish is caused by switching off the mitotic clock as a consequence of the high telomerase activity.

We propose that ubiquitous somatic telomerase activity will be found in all species with indeterminate growth. Since telomerase activity is linked to proliferation activity [17,18], it will be restricted to indeterminately growing tissues, as previously shown for meristematic tissues in plants [16,19]. Expected additions to this list are metazoan organisms, evolutionarily as diverse as the long-lived and indeterminately growing micelles of fungi and numerous animals, including various species of molluscs, reptiles and amphibia [27,28]. Trout tissues provide a conveniently available, rich source of telomerase which will stimulate further studies of vertebrate telomerase, its composition, expression and regulation.

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