Longevity of lobsters is linked to ubiquitous telomerase expression

Wolfram Klapper, Karen Kühne, Kumud K. Singh, Klaus Heidorn, Reza Parwaresch, Guido Krupp*

Institute for Hematopathology, Center for Pathology and Applied Cancer Research, Christian-Albrechts-Universität Kiel, Niemannsweg 11, D-24105 Kiel, Germany

Received 24 August 1998; received in revised form 12 October 1998

Abstract Mammals have high growth rates in embryonic and juvenile phases and no growth in adult and senescent phases. We analyzed telomerase activity in a fundamentally different animal which grows indeterminately. Lobsters (*Homarus americanus*) grow throughout their life and the occurrence of senescence is slow. A modified TRAP assay was developed and the lobster telomeric repeat sequence TTAGG was determined. We detected telomerase activities which were dependent on RNA and protein components, required dGTP, dATP and dTTP, but not dCTP. Telomerase products with a five nucleotide periodicity were generated. High telomerase activities were detected in all lobster organs. We conclude that telomerase activation is a conserved mechanism for maintaining long-term cell proliferation capacity and preventing senescence, not only in cellular models or embryonic life stages but also in adult multicellular organisms.

Key words: Indeterminate growth; Cell proliferation; Senescence; Telomerase assay; Telomeric repeat sequence; *Homarus americanus*

1. Introduction

The distal ends of the chromosomes from most eukaryotic species consist of short tandemly repeated sequences called telomeres [1,2]. DNA replication starts with RNA primers that are subsequently removed. In lagging strand synthesis, the gaps left after removal cannot be filled in at the extreme 5′ end, leading to successive telomere shortening with each cell division, and to a proliferation stop [3,4]. The length of telomeres reflects the predicted number of divisions a cell can undergo [5,6]. Escape from senescence occurs by activation of telomerase, a specialized reverse transcriptase that adds new telomeric repeats to the chromosome ends [1]. In humans, most somatic tissues lack telomerase activity and cell differentiation was shown to result in loss of enzyme activity. Telomerase is expressed in tumor cells, embryonic tissues, lymphocytes and stem cells, in agreement with the expanded proliferative capacity of these cells [7–9].

Humans and other mammals stop growing after reaching the adult stage. During their longest life period, size and weight are kept essentially constant. Lobsters grow continuously throughout their lifespan, only decreasing growth rates with age [10]. Furthermore, and again in contrast to humans, they are able to regenerate whole limbs even at a high age [10]. These characteristics assume a life-long high proliferative capacity also in cells of differentiated tissues and not restricted to the embryonic stage. A common mechanism for cells to retain high proliferative potential is the expression of telomerase and significant telomerase activity is expected in all organs.

We have verified this assumption previously with rainbow trout, another indeterminately growing animal [11]. Extremely high telomerase activity was detected in all organs and we predicted a similar ubiquitous expression in all long-lived metazoa [11]. We assumed that the previously identified repetitive sequence TAGG [12] is the telomeric repeat sequence and we developed an adapted quantitative TRAP assay to determine telomerase activity in lobster tissues.

2. Materials and methods

2.1. Tissue extracts

Five live lobsters (*Homarus americanus*) with a weight range between one and two pounds were obtained from suppliers in Boston (MA, USA). For preparation of extracts the tissue samples (ca. 100 μg) in 500 μl CHAPS lysis buffer were crushed with sterile micropestles and incubated for 30 min on ice [11]. After centrifugation for 30 min at $20\,000 \times g$ the supernatant was removed and flash frozen in liquid nitrogen. The samples were stored at −70°C before analysis. Protein concentrations were determined with the Bradford assay, using reagents supplied by Bio-Rad.

2.2. Telomerase assays

Telomerase activities were determined with the PCR-based TRAP assay essentially as described for human cell extracts [7] with our recent improvement [11,13]. We assumed the recently described repetitive sequence TAGG [12] as the telomeric repeat sequence of lobsters. The reverse primer CX(4-mer)-ext (5′-GTGCCTTCCTTCCTTCCTTCCTTCCTA-3′) was adjusted to this sequence. TS was used as the forward primer; this primer was originally described for the human telomerase assay (TRAP) [7]. Quantitative TRAP assays require the inclusion of an internal amplification standard (ITAS) [14]. The ITAS was prepared as recently described [11,14,15]. As template we used the cloned gene for the RNA component of *Thermus thermophilus* RNase P [16] and primers F-286 (5′-GCTTGCCAATCCGTCGAGCAGAGTTCGGGATGGGCCGCTTGAGGC-3′) and R-376 (5′-GTGCCTTCCTTCCTTCCTTCCTACGCCTCCGGGACGAGGCGTAAG-3′). The primers include the sequences for TS and CX(4-mer)-ext respectively (underlined) and yield a 135 bp product. The samples were analyzed by capillary electrophoresis using the ABI Prism 310 (Perkin Elmer). Integrated values for the telomerase peaks containing seven (one repeat beyond primer dimer size) and up to 13 repeats were added up and divided by the value for ITAS. The average value of all muscle samples of each PCR was defined as 1.0.

TRAP assays were performed with protein amounts between 0.06 and 6 μg per sample. All assays of five lobsters were performed at least in triplicate and standard deviations are indicated. Human telomerase extracts were prepared using the human tumor cell line L428 [13,15], available from DSMZ, Braunschweig, Germany (DSM ACC 197). For ribonuclease inactivation, extracts containing 2 μg protein from different organs were preincubated with 100 ng RNase A (Boehringer Mannheim) for 10 min at 30°C. Heat inactivation was performed with the same amount of extract at 95°C for 5 min. The telomerase reaction (elongation) was done at 30°C for 30 min. After this step the sample was incubated at 95°C for 5 min for inactivation of telomerase. The PCR amplification was carried out with 30 cycles

*Corresponding author. Fax: (49) (431) 597 3426.
E-mail: gkrupp@path.uni-kiel.de

PII: S0014-5793(98)01357-X

(95°C for 30 s, 50°C for 30 s, 72°C for 30 s) on a Mastercycler (Eppendorf).

2.3. Cloning and sequencing of lobster telomerase products

TRAP products were cloned using Topo TA Cloning (Invitrogen). Sequencing was carried out on an ABI Prism 310 (Perkin Elmer) with the Big Dye cloning reagents (Perkin Elmer).

3. Results

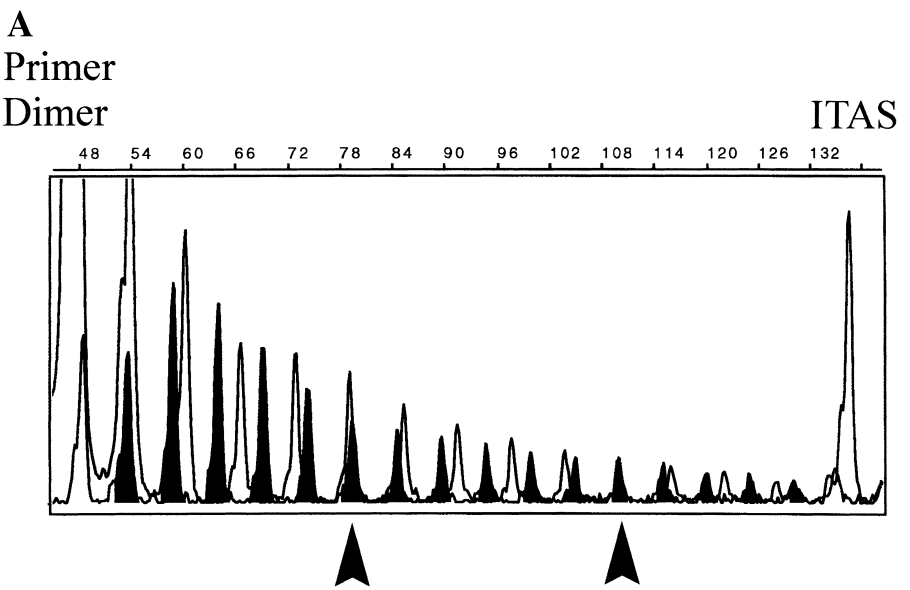
3.1. Characterization of lobster telomerase

With our modified TRAP assay we were able to detect the typical ladder forming activity of a processive telomerase in extracts of lobster tissues. We expected a 4 bp periodicity and the reverse primer CX(4-mer)-ext was constructed accordingly (see Section 2). In Fig. 1A the 6 bp telomerase ladder of the human cell line L428 is shown in white, while the lobster telomerase in hepatopancreas extracts is shown in black. The pictures of the samples are superimposed and the size of the products in base pairs is shown at the top. As indicated by the arrows, the peaks of both types of telomerase products coincide after every five peaks of the human telomerase products. From this ladder pattern we deduced a 5 bp periodicity for lobster telomeric repeats (5×6-mer repeats are equal to 6×5-mer repeats). To determine the sequence of lobster telomerase products, we performed direct cloning of the PCR products. Several clones were sequenced and a sample result is shown in Fig. 1B. The TS primer is elongated by lobster telomerase with several repeats of the sequence TTAGG. At the end of the insert the reverse primer with a different repeat sequence can be seen (bold italics). Although the reverse primer CXL-ext used for the lobster TRAP anneals only with five bases to the assumed telomerase products, it is sufficient to generate enough products to be detected by capillary electrophoresis (Figs. 1A, 2 and 3).

We further tested whether lobster telomerase shows the same features as known telomerases. Tests were performed as described for the characterization of plant [17] and fish [11] telomerases. As shown in Fig. 2, the activity of the enzyme could be abolished by treating the extract with RNase or heat prior to the TRAP assay. Omitting dTTP or the TS primer in the telomerase reaction and adding it for the PCR only also abolished the activity. Similar results were obtained by omission of dATP or dGTP (data not shown). Prior to PCR amplification, the absence of dCTP or the CX(4-mer) primer in the telomerase elongation step did not affect the activity.

3.2. Telomerase expression patterns in lobster tissues

The ITAS allows us to determine telomerase activity in a semiquantitative manner. Hepatopancreas, heart, skin and muscle from five lobsters were analyzed. Each sample was measured at least in triplicate. The average value of all muscle samples in one PCR was defined as 1.0. As shown in Fig. 3, telomerase is expressed in all tissues that were analyzed, with the highest relative activity in extracts of hepatopancreas (5.1 ± 3.3) and heart (2.9 ± 1.8). Moderate levels of telomerase



B 5′-AATCCGTCGAGCAGAGTTAGGTTAGGTTAG

GTTAGGTTAGGTTAGGTTAGGTTAGGTTAGGTT

AGGTTAGGT***TAGGAAGGAAGGAAGGAAGGAAG***

GCAC-3′

Fig. 1. A: Telomerase assay. The telomerase products of human L428 tumor cell (white peaks) and lobster hepatopancreas tissue (black peaks) were analyzed by capillary electrophoresis and the two scans are superimposed. Arrows indicate peaks that coincide. The length of the products is indicated at the top. B: Sequences of the lobster telomerase products were determined by sequencing of cloned PCR products. TS primer (underlined) was elongated by lobster telomerase with several TTAGG repeats. At the 3′ end of the insert the CX(4-mer)-ext reverse primer with a different repeat sequence can be seen (bold italics).

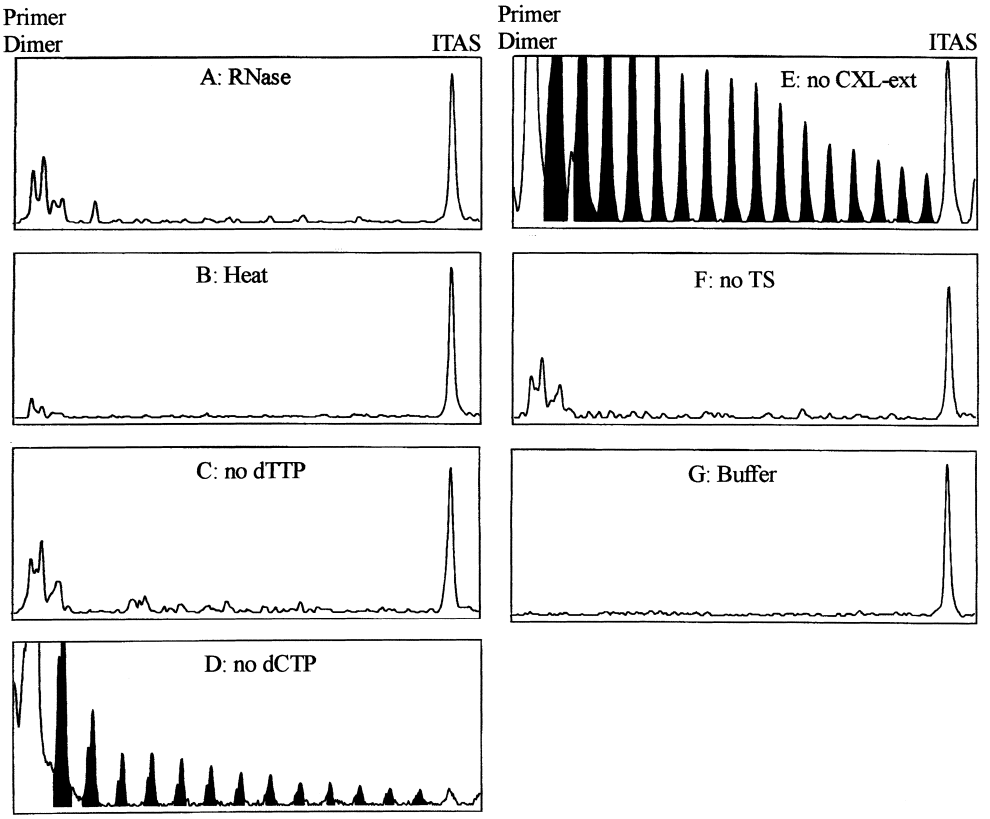


Fig. 2. Characterization of telomerase reaction. Pretreatment of lobster extracts with RNase (A) or heat (B) prior to the assay abolished the telomerase activity. Omitting dTTP (C) or the forward primer and telomerase substrate TS (F) during the telomerase reaction also abolished the signal, although the missing reagents were added for the PCR step. The absence of the reverse primer CX(4-mer)-ext (E) or dCTP (D) during the telomerase reaction did not affect the activity. Panel G shows the negative control with lysis buffer only.

activity were detected in skin (1.6 ± 1.2) and muscle (1.0 ± 0.7). Direct comparison of the lobster telomerase activity with the human tumor cell line L428 is not possible since the different telomeric repeat sequences require different reverse primers. Nevertheless the activity in lobster extracts is rather high because strong activity could still be seen using only 0.06 μg protein. This is comparable to 0.01 μg, the minimum amount needed for the L428 tumor cell line [11,15].

4. Discussion

In normal somatic tissues of adult humans, only cells with high proliferation capacity express telomerase activity, this includes the proliferating descendants of stem cells as well as stimulated lymphocytes [7–9]. The regulation of telomerase expression is less stringent in adult mice, which express significant telomerase activity in liver and low levels in kidney and spleen [18]. The level of activity is tightly linked to proliferation as was shown for cell lines [15,19] and primary cell cultures [20]. The proliferation linkage was also demonstrated for plants. Mitotically active meristematic tissue and cultured cells express telomerase, whereas non-dividing cells from leaves and axillary buds are telomerase negative [17,21].

Our data demonstrate that all investigated fully differentiated lobster tissues retain telomerase expression. The need for this activity can be explained by the continuous growth of all lobster tissues, which must be based on cell proliferation [10]. Telomerase inactivation during development from embryonic to adult eukaryotic organisms was observed in humans [22], but it does not seem to be necessary for the generation of fully differentiated tissues. Interestingly, the telomeric repeat sequence that we characterized in lobsters was found previously in a very distantly related arthropod, the silk worm *Bombyx mori* [23].

Although other mechanisms for telomere maintenance are known like in *Drosophila* [24] or yeasts [25] and postulated for humans [26], we conclude that telomerase activation is a conserved mechanism for maintaining long-term cell proliferation capacity. Therefore similar patterns of telomerase activity are predicted in other species with similar growth features, for example fungi, molluscs, reptiles, amphibia and fish [27–29]. Recently, we have confirmed this assumption for the indeterminately growing rainbow trout. Similar to the data reported here for lobster, high levels of telomerase are expressed in all organs [11]. Further studies with more divergent species will provide more insight into the linkage of ubiquitous telomerase expression and the longevity of multicellular eukaryotes. But clearly, ageing is a multifactorial process and should not be reduced to cellular replicative senescence. Lobsters and similar multicellular organisms may provide abundant sources of telomerase with different repeat patterns, versatile tools to investigate properties of the enzyme.

Acknowledgements: We thank U. Kellner and A. Rott for help with cloning and sequencing. This work was supported by IntelliGene Ltd., Jerusalem, Israel, Eppendorf-Netheler-Hinz GmbH, Hamburg and the Kinder-Krebs-Initiative, Buchholz.

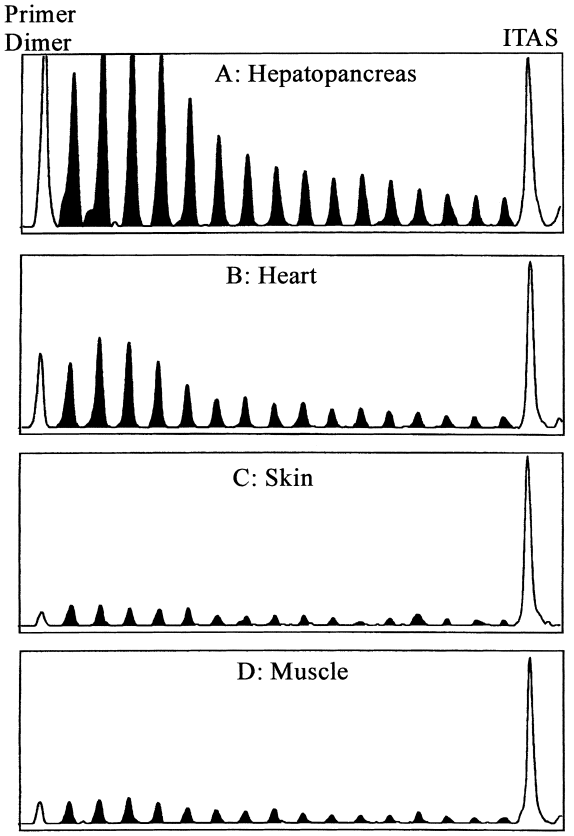


Fig. 3. High telomerase activity was observed in all organs examined, with the highest activity in the hepatopancreas (A) and the heart (B). Lower activity was found in skin (C) and muscle (D). The ITAS was adjusted to the same height for all shown panels. As indicated in Section 2, several other protein amounts were also analyzed (data not shown). Here, one series of examples with the same protein content (2 μg per assay) is shown.

References

[1] Blackburn, E.H. (1991) Nature 350, 569–573.
[2] Lejnine, S., Makarov, V. and Langmore, J.P. (1995) Proc. Natl. Acad. Sci. USA 92, 2393–2397.
[3] Olovnikov, A.M. (1971) Dokl. Akad. Nauk. SSSR 210, 1496–1499.
[4] Watson, J.D. (1972) Nature New Biol. 239, 197–201.
[5] Harley, C.B., Futcher, A.B. and Greider, C.W. (1990) Nature 345, 458–460.
[6] Harley, C.B. (1991) Mutat. Res. 256, 271–282.
[7] Kim, N.W., Piayszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, M.G., Wright, W.E., Weinrich, S.L. and Shay, J.W. (1994) Science 266, 2011–2015.
[8] Hiyama, K., Hirai, Y., Seishi, K., Akiyama, M., Hiyama, E., Piatyszek, M.A., Shay, J.W., Ishioka, S. and Yamakido, M. (1995) J. Immunol. 1995, 3711–3715.
[9] Härle-Bachor, C. and Boukamp, P. (1996) Proc. Natl. Acad. Sci. USA 93, 6476–6481.
[10] Govind, C.K. (1995) in: Biology of the Lobster *Homarus americanus* (Factor, J.R., Ed.), pp. 291–312, Academic Press, San Diego, CA.
[11] Klapper, W., Heidorn, K., Kühne, K., Parwaresch, R. and Krupp, G. (1998) FEBS Lett. 434, 409–412.
[12] Fowler, R.F. and Skinner, D.M. (1985) J. Biol. Chem. 260, 1296–1303.
[13] Krupp, G., Kühne, K., Tamm, S., Klapper, W., Heidorn, K., Rott, A. and Parwaresch, R. (1997) Nucleic Acids Res. 25, 919–921.
[14] Wright, W.E., Shay, J.W. and Piatyszek, M.A. (1995) Nucleic Acids Res. 23, 3794–3796.
[15] Klapper, W., Singh, K.K., Heidorn, K., Parwaresch, R. and Krupp, G. (1998) Biochim. Biophys. Acta (in press).
[16] Hartmann, R.K. and Erdmann, V.A. (1991) Nucleic Acids Res. 19, 5957–5964.
[17] Fitzgerald, M.S., McKnight, T.D. and Shippen, D.E. (1996) Proc. Natl. Acad. Sci. USA 93, 14422–14427.
[18] Prowse, K.R. and Greider, C.W. (1995) Proc. Natl. Acad. Sci. USA 92, 4818–4822.
[19] Holt, S.E., Aisner, D.L., Shay, J.W. and Wright, W.E. (1997) Proc. Natl. Acad. Sci. USA 94, 10687–10692.
[20] Belair, C.D., Yaeger, T.R., Lopez, P.M. and Reznikoff, C.A. (1998) Proc. Natl. Acad. Sci. USA 94, 13677–13682.
[21] Heller, K., Kilian, A., Piatyszek, M.A. and Kleinhofs, A. (1996) Mol. Gen. Genet. 252, 342–345.
[22] Wright, W.E., Piatyszek, M.A., Rainey, W.E., Byrd, W. and Shay, J.W. (1996) Dev. Genet. 18, 173–179.
[23] Okazaki, S., Tsuchida, K., Maekawa, H., Ishikawa, H. and Fujiwara, H. (1993) Mol. Cell. Biol. 13, 1424–1432.
[24] Pardue, M.L., Danilevskaya, O.N., Lowenhaupt, K., Slot, F. and Traverse, K.L. (1996) Trends Genet. 12, 48–52.
[25] Lundblad, V. and Blackburn, E.H. (1993) Cell 73, 347–360.
[26] Bryan, T.M., Engelzou, A., Gupta, J., Bacchetti, S. and Reddel, R.R. (1995) EMBO J. 14, 4240–4248.
[27] Patnaik, B.K. (1994) Gerontology 40, 200–220.
[28] Kara, T.C. (1994) Gerontology 40, 161–173.
[29] Patnaik, B.K., Mahapatro, N. and Jena, B.S. (1994) Gerontology 40, 113–132.