ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 9, pp. 1017-1021. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 9, pp. 1248-1253.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM11-097, July 3, 2011.

Telomerase Activity in the Bats *Hipposideros armiger* and *Rousettus leschenaultia*

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Received March 17, 2011

Abstract—Telomerase activity was examined in two species of bat, *Hipposideros armiger* and *Rousettus leschenaultia*, which have similar body mass and lifespan but differ in use of hibernation. We found that telomerase activity was present in all tissues sampled, but it was greater in metabolically active tissues such as liver, spleen, and kidney. Of special interest is the raised activity found in the heterothermic bat *H. armiger*, and the hibernating bats having raised values for spleen, heart, and kidney. These findings show that maintenance of high levels of telomerase is an essential part of the regulation of cellular activities during hibernation.

DOI: 10.1134/S0006297911090057

Key words: bat, body mass, hibernation, telomerase activity

The predominant function of telomeres is the protection of chromosome extremities from end-to-end fusion and also from degradation [1], thus maintaining chromosomal integrity and cell viability [2, 3]. Studies on telomerase biology have been conducted in several mammalian orders. In Primates, especially in humans, normal somatic cells have no detectable telomerase activity, low activity is detected in spleen, thymus, and digestive tract, with high activity being found in testes and their telomeres shorten with every division [4-6]. Similar features of telomere biology have been found in Ungulates, Carnivores, and Lagomorphs [7-11]. Replicative senescence is believed to have evolved as an adaptive mechanism to protect organisms from uncontrolled cell proliferation and cancer [12], and telomerase is seen to be activated in most human tumors [13].

Replicative senescence is not, however, a universal phenomenon. Laboratory mice can express telomerase in most of their somatic tissues [14]. The difference between human and mouse telomerase regulation is generally explained by their differences in lifespan and body mass [15, 16]. One hypothesis is that the longer-lived organisms experience more cell divisions and thus a greater risk of multistage carcinogenesis. Another hypothesis is that telomerase activity is thought to have coevolved with increases in body mass, thus larger animals, having more cells than smaller ones, could experience a greater susceptibility to cancer [17]. However, within the order Rodentia telomerase activity appears to have coevolved inversely with body mass, independent of lifespan [18-20]. In contrast to rodents, bats have very long lifespans that would be predicted for their body mass, but few studies have addressed the molecular or physiological mechanisms involved in the relationship between telomerase and extreme longevity of bats [21].

Bats account for almost one fourth of all mammalian species with approximately 1100 species of bats worldwide. The order Chiroptera is divided into two suborders, Megachiroptera and Microchiroptera. Body size of bats varies from the 2-g hog-nosed or bumblebee bat (*Craseonycteris thonglongyai*) to the 1.2-kg large flying fox (*Pteropus vampyrus*). Once adjusted for body mass, bats are the longest-lived mammalian order [22-24]. On average, species of Chiroptera live three times longer than non-flying eutherian mammals of comparable size [23].

Many factors have been considered in the comparative analysis of longevity among different bat species both in the Megachiroptera and Microchiroptera including diet, colony size, reproductive rate, body mass, and use of hibernation. Hibernating bats are those that are able to reduce their body temperature in a controlled and regulated manner, thus reducing metabolic rate and reducing energy expenditure during times of energy shortages [25]. Bats that can hibernate are also known as heterotherms as they

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can regulate body temperature both at normothermic values ("normal" body temperatures of about 37°C) and also at lower values. In the Microchiroptera, almost all species, such as *Hipposideros armiger*, live at latitudes with large seasonal temperature fluctuations and are typically heterothermic, using hibernation to survive long winters without food, and daily torpor to reduce energy expenditure while roosting [26]. The Megachiroptera, most of the bats, including the fulvous fruit bat *Rousettus leschenaultia*, live in tropical areas, or migrate to warmer regions during the winter and are typically homeothermic because they can only regulate at normothermic values and do not hibernate. Hibernating species may live on average 6 years longer than species that do not hibernate [24].

To determine whether telomerase activity coevolves with lifespan, body mass, or hibernation, we tested the hypothesis that telomerase activity coevolved with use of hibernation. Thus we examined telomerase activity in two bat species. We chose *H. armiger* and *R. leschenaultia*, which have almost the same lifespan (table) and body mass (table) to study evolution of telomerase regulation in Chiroptera. Values for body mass and lifespan are within the range of those described by Brunet-Rossini and Austad for bats [21]. Then we compared the telomerase activity in some tissues of *H. armiger* while hibernating and also aroused from hibernation to explore whether the telomerase activity changes during hibernation.

MATERIALS AND METHODS

Animal samples. *Rousettus leschenaultia* were collected from a cave in Jinlundong (23°33′19″ N, 108°15′41″ E) of Guangxi province (China) in November 2009, and *H. armiger* were captured from Yulongdong (31°32′199″ N, 116°08′609″ E) in Anhui province (China) in December 2009. Male Kunming (KM) mice (*Mus musculus*) were used for procedural controls and quality assurance, and they were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. Two-to-four animals were used in each species, and these animals were young adults. Exact age was known for laboratory animals and was estimated for wild-caught animals from body measurements and coat color, which changes with age [27]. *Hipposideros armiger*, which had been in hibernating state, were euthanized before arousal, and the liver, spleen, kidney, lung, and heart were stored immediately in liquid nitrogen. Then all the tissues were transported in liquid nitrogen and stored at −80°C until further processing. All other tissues were collected in laboratory and the same five tissues were rapidly frozen in liquid nitrogen. All tools and tubes used in collecting the animals' tissues were RNase-free. Animals were collected and euthanized using the European Union (86/609/ EEC) guidelines.

Preparation of tissue extract. For telomerase extraction approximately 30 mg of tissue was washed twice in ice-cold phosphate-buffered saline (PBS). The sample was pulverized by pestle and mortar. The thawed sample was transferred to a sterile 1.5 ml microcentrifuge tube and resuspended in an appropriate amount of 200 μl CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM benzamidine, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol [13]) and then incubated on ice for 30 min. The extracts were centrifuged at 12,000*g* for 20 min at 4°C, and the supernatants were collected, divided into aliquots, and frozen at −80°C.

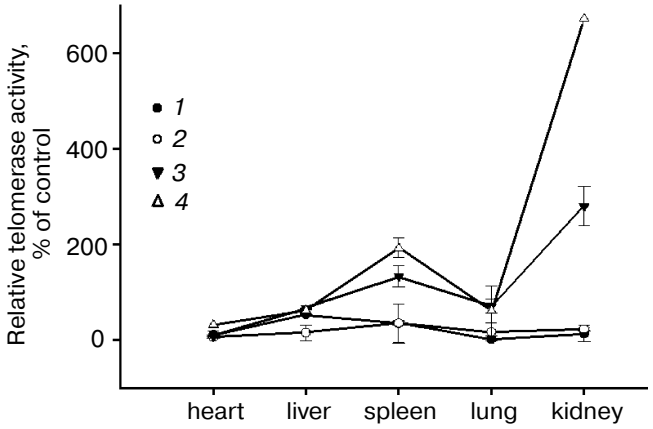
The protein concentration was determined from a standard curve of BSA.

Telomeric repeat amplification protocol. The telomeric repeat amplification protocol (TRAP) assay was performed using TRAPeze® XL Telomerase Detection Kit (Millipore, USA) according to the manufacturer's instructions. First, serial dilutions (1 : 5) of the stock concentration (0.2 pM) of TSR8 (control template, instead of the sample extract) were used to generate a standard curve that permits the calculation of the amount of TS primers with telomeric repeats extended by telomerase in a given extract. Second, the reaction was carried out as follows and run on a thermocycler (ABI, USA): 48 μl of the reagents containing Taq DNA polymerase (Takara, Japan), dH_2O and 5× TRAPEZE® XL Reaction Mix mixed with 2 μl tissue extracts (0.5-1 μg/μl) on ice, the reaction condition was 30°C for 30 min, then 36 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 1 min, and followed by a 72°C for 3 min extension step and then at 55°C for 25 min, and finally the reaction products were incubated at 4°C. The reaction products (20 μl) of the TRAP assay were mixed with 180 μl of buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM $MgCl_2$) and were transferred to a black 96-well plate. The fluorescein and sulforhodamine emission were measured in a Synergy™ 4 microplate reader (BioTek Instruments, USA) using appropriate emission and excitation filters (485 and 620 nm, respectively).

Telomerase expression profiling. To determine the telomerase expression profile in the bats, we assayed telomerase activity in a panel of five tissues (including liver, spleen, kidney, lung, and heart) using the TRAP. In all experiments, testicular tissue extracts of bats and the same five tissue extracts of mice were used as positive controls and specimen quality. Telomerase activity was found to be present in the testes of all mammals studied so far, hence it was used as a positive control [18]. Second, a telomerase-positive cell was used in each reaction set as a reference for quantification. Third, in each reaction, the K2 primer and the TS primer, which are involved in the semi-competitive amplification of the internal control template TSK2, generated a 56-bp sulforhodamine amplification product that served as a control for amplification efficiency in each reaction and was used for quantitative analysis of the TRAPEZE® XL products.

Raw telomerase activity values expressed as percent of the activity in the telomerase-positive cell were used to calculate tissue-specific relative telomerase activity, and the values were averaged for each tissue for each animal sampled. For each animal species, values of the total telomerase activity summed across the five tissues were calculated as an average between two or three animals. The assays were repeated at least two times for each individual animal to ensure reproducibility.

Statistical analysis. All data are expressed as means ± SD. Statistical analysis was performed with the SPSS statistical software package for Windows, v.13.0 (SPSS Inc., USA). To determine differences between groups, where data were sufficient a one-way ANOVA followed by the Student–Newman–Keuls test was used. When comparing differences between hibernating and non-hibernating *H. armiger*, a two-tailed Student's *t*-test was used. A *P*-value less than 0.05 was considered statistically significant.



Relative telomerase activity in *M. musculus* (*1*) and homeothermic *R. leschenaultia* (*2*) and heterothermic *H. armiger* bats (during both normothermia (*3*) and hibernation (*4*)). Data are expressed as mean ± SD

RESULTS

Telomerase activity: general observations. In all three species telomerase activity was seen in the five tissues sampled (table). With the exception of the heart, whose values were similar in the three species, telomerase activity tended to be higher in *H. armiger* than the other species (figure).

When telomerase activities were summed to get the total telomerase activity, it was seen that there were significant differences among the three species, with normothermic *H. armiger* having about fivefold more activity than *R. leschenaultia* and *M. musculus* ($P < 0.005$ for *R. leschenaultia*, $P < 0.05$ for *M. musculus*) and hibernating *H. armiger* having about twice as much as normothermic individuals and about 10 times as much as the two other species (*R. leschenaultia* and *M. musculus*) (table).

Telomerase activity during hibernation and normothermia in the heterothermic bat *H. armiger*. Telomerase activity was expressed differentially between tissues in both hibernating and normothermic bats, with liver and lung expressing less activity than that found in spleen and kidney (figure). Telomerase activity from most of these tissues did not differ between groups, although the activity in the spleens from hibernating *H. armiger* was signifi-

Percentage of relative telomerase activity of different tissues in *M. musculus*, *R. leschenaultia*, and *H. armiger*

Species	Number of animals analyzed	Maximum lifespan, years	Body mass, g	Relative telomerases activity, %					
				heart	liver	spleen	lung	kidney	total****
M. musculus	2	4*	30*	11.0 ± 7.8	53.4 ± 1.7	34.7 ± 40.5	2.0 ± 0.3	13.8 ± 17.0	114.9 ± 33.2
R. leschenaultia	4	12**	70-90**	6.5 ± 1.3	25.7 ± 18.0	42.6 ± 8.1	14.6 ± 13.9	22.9 ± 2.9	97.8 ± 6.2
H. armiger normothermic	3	~10	45-55**	7.7 ± 3.6	61.4 ± 7.6	110.0 ± 43.0	61.3 ± 20.0	280.4 ± 41.3	558.1 ± 2.4
hibernating	3	~10	45-55**	32.2***	61.4 ± 4.8	193.0 ± 20.8	61.3 ± 52.1	672.4***	1072.5

Note: All data are expressed as mean ± SD (n = 2-3).

* Reference [28].

** Reference [24].

*** Only one tissue of *H. armiger* (hibernating) was collected, so the data in kidney and heart tissues were pooled when analyzed later for *H. armiger* (the total data for heart is 15.8 ± 14.4, and the data for kidney is 411.1 ± 228.2).

**** All data were calculated using only individuals with the complete five tissue samples collected.

cantly higher than those from normothermic *H. armiger* ($P < 0.05$). Although the telomerase values from the kidney and heart of hibernating *H. armiger* were higher than normothermic *H. armiger*, insufficient data precluded analysis. Because of the insufficient data in kidney and heart tissues, the data were pooled for *H. armiger* before proceeding to the next analysis.

Telomerase activity in homeothermic *R. leschenaultia* and *M. musculus* and heterothermic *H. armiger*. When percentage telomerase activity was compared in the same tissues across the three species, some differences were found (figure). There were significant differences in telomerase activity of spleens between species (ANOVA $F3,8 = 19.90$, $P < 0.0005$). Telomerase activity was higher in spleens from hibernating *H. armiger* than for normothermic *H. armiger* ($P = 0.05$) and higher in *H. armiger* than for *R. leschenaultia* ($P < 0.05$) and *M. musculus* ($P < 0.05$) (figure). Telomerase activity did not differ in spleens from *R. leschenaultia* and *M. musculus*. Telomerase activity was higher in lungs from *H. armiger* than from that of the other species (ANOVA $F3,8 = 3.134$, $P = 0.087$), although activity did not differ between hibernating and normothermic *H. armiger* (figure). When data for *H. armiger* were pooled, it was found that for kidney tissues telomerase activity was higher in *H. armiger* than the other species, but the data were not significant ($F2,4 = 5.065$, $P = 0.08$). While for heart telomerase activity did not differ between the three species during normothermia, it was higher in hibernating *H. armiger*, although insufficient data precluded statistical analysis. Telomerase activity was significantly higher in liver tissue from *H. armiger* (ANOVA $F3,8 = 6.93$, $P = 0.013$) than for the liver of the other species (figure).

DISCUSSION

Our study showed that telomerase activity was present in the two bat species in all tissues sampled. The expression profile was similar to the laboratory mouse, with telomerase activity in almost all tissues. However, the total relative telomerase activities were significantly different among the three species. The normothermic *H. armiger* had about five times more activity than the other species (*R. leschenaultia* and *M. musculus*) and hibernating *H. armiger* had about 10 times as much as the other species. Our data suggests that the coevolution of the telomerase activity and body mass maybe is not related in bats, because the two species of bats are a similar body mass, which contrasts to previous studies [18, 20]. The higher telomerase activity in *H. armiger* may be because it is a heterothermic species; therefore, hibernation might be the key regulation factor affecting the relationship of the telomerase activity and metabolism in bats. Telomerase activity was greater in metabolically active tissues such as liver and spleen in the three species, and telomerase activity was higher in the lung and kidneys of *H. armiger* than in the other two species. This interesting relationship warrants further investigation using more samples and a wider range of heterothermic and homeothermic species.

To determine whether the telomerase activity was related to hibernation, we studied the telomerase expression profile during the hibernation and arousal state of *H. armiger*. Of special interest is that the telomerase activity differed between hibernating and normothermic individuals, with hibernating bats having significantly raised values for spleen, and higher values for heart and kidney were observed. The telomerase activity in liver and lung did not change between normothermia and hibernation. From these results, we could conclude that during the hibernation the telomerase activity is not only maintained, but in some tissues it is raised, even though body temperature is reduced from about 40 to 6°C, and the bats will maintain low body temperatures for several weeks at a time. The data interestingly suggest that maintenance of high levels of telomerase is an essential part of the regulation of cellular activities during hibernation.

High telomerase activity has been found in short-lived species, such as the laboratory mouse, and this high activity has been linked as a reason for longevity failure in these species [18]. In contrast, the naked mole-rat, a small, exceptionally long-lived rodent, exhibits high telomerase activity [24]. Moreover, in fish high telomerase activity is correlated with tissue regeneration, not longevity [29]. Our data suggests that high tissue telomerase activity is also not related to longevity, but is more likely to be related to effective cellular regeneration and repair in these small, long-lived, metabolically active mammals. Therefore, bats may have different tumor-suppressor mechanisms to suit the different telomerase activity present. Since any increase in lifetime cell divisions should increase the opportunity for tumorigenic somatic mutation, tumor suppressor mechanisms other than telomerase repression may coevolve with the lifespan seen in many bats.

Furthermore, the laboratory species most commonly used in aging and cancer mechanistic studies are distinguished by their lack of success at combating the ravages of aging. For this reason, comparative biology of aging studies that focus on animals that are exceptionally long-lived, such as naked mole-rats and many bats as well as on traditional short-lived laboratory species, may be useful. Understanding the cellular mechanisms employed by long-lived bats with high telomerase activity might provide valuable information for understanding the role of telomerase and cancer development in humans. Future studies aimed at understanding why telomerase activity is so high in bats may shed new light on the mechanisms of proliferative cellular activity and evolution of longevity.

We thank JunPeng Zhang and PanYu Hua from East China Normal University for collecting bat samples and especially thank Prof. ShuYi Zhang for editing the manuscript.

This work was funded by a special grant from East China Normal University to S. Y. Zhang.

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