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Telomere length regulation during postnatal development and ageing in *Mus spretus*

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ABSTRACT

Telomere shortening has been causally implicated in replicative senescence in humans. To examine the relationship between telomere length and ageing in mice, we have utilized *Mus spretus* as a model species because it has telomere lengths of approximately the same length as humans. Telomere length and telomerase were analyzed from liver, kidney, spleen, brain and testis from >180 *M.spretus* male and female mice of different ages. Although telomere lengths for each tissue were heterogeneous, significant changes in telomere lengths were found in spleen and brain, but not in liver, testis or kidney. Telomerase activity was abundant in liver and testis, but weak to non-detectable in spleen, kidney and brain. Gender differences in mean terminal restriction fragment length were discovered in tissues from *M.spretus* and from *M.spretus* × C57BL/6 F1 mice, in which a *M.spretus*-sized telomeric smear could be measured. The comparison of the rank order of tissue telomere lengths within individual *M.spretus* showed that certain tissues tended to be longer than the others, and this ranking also extended to tissues of the *M.spretus* × C57BL/6 F1 mice. These data suggest that telomere lengths within individual tissues are regulated independently and are genetically controlled.

INTRODUCTION

Telomeres, the natural ends of linear eukaryotic chromosomes, consist of evolutionarily conserved, repetitive DNA and its associated specific DNA binding proteins. In most vertebrates, telomeric DNA consists of a short G-rich sequence, 5′-TTAGGG-3′, repeated hundreds to thousands of times at the ends of all chromosomes (1). Telomeres function to protect the loss of vital DNA sequences through nuclease degradation or illicit recombination (reviewed in 2). They have been shown to bind to the nuclear matrix (3) and may participate in chromosome pairing during meiosis (4). Telomeres have also been implicated in the control of cell immortalization (5,6) and cellular senescence (7,8). Cellular senescence was first described by Hayflick and Moorehead (9) as the point at which a cessation of cell growth occurs, concomitant with a lack of response to normal growth stimuli, in a population of human fibroblasts in culture. The number of possible cell doublings decreased as a function of donor age, presumably reflecting the replicative history of the cells *in vivo*. This limited cell division capacity has since been observed in numerous other somatic cell types, and has led to the idea of a cellular mitotic clock, which measures cell divisions rather than chronological age. Evidence has accumulated which suggests that telomeres play a role in the clocking mechanism.

In humans, telomeres are known to shorten during replicative ageing in various types of somatic cells at rates ranging from 40 to 200 bp/division depending on the cell type (5,10–12). This shortening of telomeres was predicted based upon the end-replication problem of eukaryotic linear chromosomes (13,14). DNA polymerases require an RNA primer to initiate replication and synthesize DNA unidirectionally in the 5′ to 3′ direction. The removal of the RNA after each round of DNA replication results in a loss of sequence at the 5′ end of the daughter strands. The telomere hypothesis of cellular senescence (15) proposes that when a critically short length of telomeric sequence remains on one or a few chromosomes in normal somatic cells, the cells cease to divide to prevent self-destruction. Without a mechanism to overcome this under replication and maintain a complete set of chromosomes, cellular and ultimately organismal viability would be impaired. In germline, stem cells and most immortal and tumor cells, a unique DNA polymerase, telomerase, acts to compensate for incomplete replication of chromosomes, thus permitting continued cell division (reviewed in 16). Telomerase, a ribonuclear protein complex, synthesizes telomeric DNA *de novo* onto the 3′ end of the parental G-rich strand using its integral RNA as a telomeric sequence template (17–19). The newly synthesized telomeric DNA provides a template to allow the conventional DNA polymerase complex to synthesize a complete daughter strand.

The biochemical properties of telomerase and regulation of telomere length between human and mice differ. Partially purified mouse telomerase extracts adds predominately only one repeat onto a telomeric primer *in vitro* under conditions where the human enzyme adds hundreds of repeats (20). The telomerase RNA components between human and mice share only 65% sequence identity and differ in their template regions (19,21). A recently identified, non-catalytic telomerase-associated protein shows 75% amino acid identity between humans and mice in the ORF (22). The terminal restriction fragment (TRF) lengths, comprising both telomeric and non-telomeric sequences, also differ between human and mouse DNA. Tissues from humans have mean TRF lengths that range from 5 to 15 kb (10,23), whereas tissue TRFs from most inbred strains of *Mus musculus* are >50 kb (24,25). Telomeric sequence binding proteins

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identified in mouse and human cells share an overall homology of 67% indicating perhaps a rapid evolution of this gene as well (26). In humans, most normal somatic cells do not have detectable telomerase activity (5,27) and telomeric DNA is lost during replicative cell division in culture (10) and during ageing *in vivo* (11,28). However, in mice, many normal somatic cells do express detectable telomerase activity (29,30), but the effect of this activity on telomere length during ageing was largely unknown.

To examine the relationship between ageing and telomere length in mice further, a suitable mouse model is necessary. Because of the long TRF lengths of *M.musculus*, detection of any changes in TRF length with age is difficult by conventional gel separation techniques. In contrast, *Mus spretus* and *Mus caroli*, both wild-derived species, have been shown to have significantly shorter mean TRF lengths (5–25 kb) than *M.musculus* strains (25). In a previous study, we examined seven additional wild-derived mouse species, all of which had shorter telomere lengths than *M.musculus* strains (29). We chose to follow telomere length regulation in *M.spretus* because of its readiness to interbreed with laboratory strains of *M.musculus*, thus providing further opportunities for genetic studies of telomere length.

Previously, we reported differences in telomere length between tissues within a few individual *M.spretus* (29). Due to the limited number of mice sampled in the study, it was not possible to detect changes in tissue telomere length with respect to ageing in *M.spretus*. In this study, we have examined telomere lengths and telomerase activity in five tissues in a large population of randomly bred male and female *M.spretus* at different ages. Statistically significant TRF length changes with age were found in brain (0–4 months) and spleen (0–30 months), but not in liver, testis and kidney (0–30 months). A significant difference between male and female tissue TRF lengths in *M.spretus* was also discovered. In addition, a *M.spretus*-like telomeric smear was detected in F1 mice from *M.spretus* × C57BL/6 F1 crosses, which displayed similar tissue and gender TRF length differences. Abundant telomerase activity was present in liver and testis, while weak or no activity was detected in spleen, kidney and brain. These results are discussed in relation to the role that telomere length and telomerase may play in replicative ageing in mice.

MATERIALS AND METHODS

Mice

Ten male and 10 female *M.spretus* were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained as breeding pairs. The colony was expanded by using a random breeding system among the offspring over a 2.5 year period in a licensed research animal facility. Mice were maintained in VCL™ ventilated caging units (Lab Products, Maywood, NJ) in a clean room environment and were sustained on a rodent breeder diet (Harlan Teklad #8760) and acidified water, pH 3.0, + 0.4 μg/ml Vitamin K. Several female C57BL/6 mice were also purchased from the Jackson Laboratory, crossed to male *M.spretus*, and maintained as described above. The ageing study was conducted according to an IACUC (Institutional Animal Care and Use Committee) approved protocol. Mice were euthanized with ether at the different time points and samples of kidney, spleen, liver, brain, testes and serum were collected and frozen on dry ice. Some of the *M.spretus* brain samples were dissected, separating out the cerebellum, hippocampus and the subventricular zone of the basal ganglia and frozen on dry ice.

Isolation and restriction enzyme digestion of genomic DNA

Genomic DNA was prepared from mouse tissue samples according to a standard protocol (29). Briefly, frozen tissues were rapidly homogenized (PowerGen 35, 5 × 95 mm generator probe; Fisher Scientific) in DNA extraction buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA, pH 8, 0.5% SDS, 0.1 mg/ml Proteinase K). The homogenate was then treated as described previously. DNA was dissolved in 1× TE and heated at 50–55°C for 1–5 h. A fluorimeter was utilized to quantitate the isolated genomic DNA. The integrity of the undigested DNA was analyzed by gel electrophoresis. Degraded, uncut DNA appeared as a smear extending from limit of mobility to the bottom of the gel, while intact DNA appeared as a band at limit of mobility. An aliquot (3–5 μg) of DNA was digested with *Hin*FI/*Rsa*I (2 U each/μg DNA) and DNase-free RNase at 37°C for 16 h. The digests were monitored for completeness by gel electrophoresis. Incomplete digests appeared as a band or smear at the top of the gel, while the complete digests appeared as a smear at the bottom of the gel.

TRF analysis

Aliquots (1–2 μg) of digested DNA were separated on a 0.5% agarose gel (20 × 25 cm) in 1× TBE for a total of 800–900 V h. The gels were dried for 20–25 min at 60°C, denatured (0.5 M NaOH, 1.5 M NaCl) for 8 min and neutralized (0.5 M Tris pH 7, 1.5 M NaCl) for 4 min. Gels were prehybridized in 30 ml of a standard hybridization solution (5× Denhardt's solution, 5× SSC, 10 mM Na_2HPO_4, 1 mM $Na_4P_2O_7$) at 37°C for 1–4 h. An aliquot (0.25 μg) of a single-stranded telomeric oligonucleotide, $(TTAGGG)_3$, was end-labeled with 50 μCi of [γ-^{32}P]ATP and 10 U of T4 polynucleotide kinase and added to the prehybridization buffer. The gels were incubated at 37°C for 16 h, and washed with 0.5× SSC at 37°C (3 × 10 min). To determine the TRF length, a Phosphorimager (Molecular Dynamics) was used to quantitate the position and strength of the radioactive signal in each of the lanes, as described (7,29).

Tissue extracts and telomerase assays

Mus spretus tissues were harvested and frozen as described above. Samples were kept cold and homogenized with a motorized disposable pestle in CHAPS lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenlymethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) using 20 μl of lysis buffer/4–10 mg of tissue and placed on ice for 30 min. Samples were microcentrifuged at 12 000 *g* for 30 min at 4°C. A Coomassie assay (Pierce) was used to quantitate the amount of protein in each extract and TRAP assays were performed using 2 μg of protein as described previously (27). Samples with detectable signal above the background in the negative control lanes were normalized to an internal control PCR product and related to a known amount of DNA standard. The quantitation was expressed as total product generated.

RESULTS

Telomere length changes in *M.spretus* during ageing

In this study, we have analyzed a population of *M.spretus* for telomere length in multiple tissues throughout postnatal development and ageing. Tissues from 109 female and 73 male

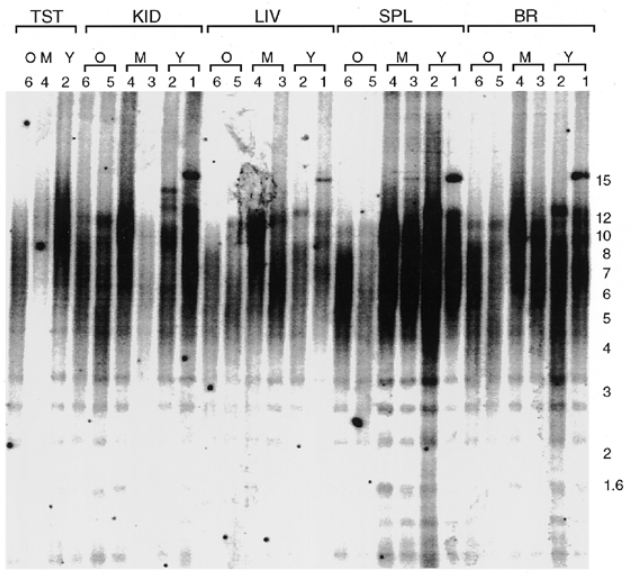

Figure 1. Telomere lengths in tissues of young, middle-aged and older *M.spretus*. Genomic DNA was prepared from testes (TST), kidney (KID), liver (LIV), spleen (SPL) and brain (BR) of *M.spretus* at different times throughout postnatal development and ageing, and analyzed for telomere length as described in Materials and Methods. A representative gel containing tissues from male (#2, 4, 6) and female (#1, 3, 5) *M.spretus* of 1–2 months (Y, young), 12 months (M, middle-aged) and 22–24 months (O, old) is shown. The position of molecular weight markers are indicated in kb on the right side of the figure.

M.spretus were collected at different times over a 2.5 year period, and genomic DNA was isolated from kidney, liver, spleen, testes and brain. The mean TRF length of each sample was determined using a phosphorimager as described in the Materials and Methods (7,29). Figure 1 shows a representative tissue gel of three male and three female *M.spretus* of different ages. As expected, the signal was distributed over a wide size range (4–15 kb) representing both strongly hybridizing smears and weakly hybridizing, distinct bands of DNA. The smears were determined to be telomeric by sensitivity to Bal31 exonuclease digestion, while most distinct bands represented internal tracts of TTAGGG (data not shown). The mean TRF lengths for each tissue were varied among individuals. For example, the liver DNAs in Figure 1 show a difference in mean TRF length between each pair of same-aged mice, as well as among the different ages.

The number of samples analyzed, line equation parameters and the *P* value for each tissue are given in Table 1. Liver and kidney TRFs showed no significant change in length with age in the population as a whole (Fig. 2), or when the populations were divided into young (0–11 months) and old (≥12 months) groups (data not shown). The remaining three tissues (spleen, testes and brain) each showed changes in telomere length with age. Spleen TRF lengths (Fig. 3A) decreased significantly with age in the total population ($P = 4 \times 10^{-7}$) at ~50 bp/month (Table 1), and when the populations were divided into young (0–10 months) and old (≥11 months) groups ($P = 6.5 \times 10^{-5}$). Testes telomeres showed a non-significant decrease ($P = 0.1$) in length at a rate of 37 bp/month (Fig. 3B, Table 1). However, when the samples were divided into young (0–11 months) and old (≥12 months) age groups for statistical analysis, a highly significant difference in TRF length was revealed ($P = 0.00056$). This was somewhat surprising given the degree of significance in the testis samples as a whole. When each of the two age groups were analyzed separately, the regression line for the 0–11 month group showed a statistically significant increase in TRF length (m = 0.147, $P = 0.0045$) whereas the regression line of the older age group (≥12 months) showed a non-significant increase (m = 0.027, $P = 0.62$). In addition, the mean TRF of the older group was ~1 kb shorter than the younger age group. The results of these analyses suggest that some unusual telomere dynamics may be occurring in the testes at ~12 months of age. Telomere lengths in *M.spretus* brain showed a much clearer biphasic distribution with age (Fig. 4A), with the division occurring between 4 and 5 months. Student's *t*-test analysis of the 0–4 and ≥5 month age groups showed a highly significant difference between the two groups ($P = 1.3 \times 10^{-14}$). Regression analysis of the separate groups also revealed a 10-fold difference in the rate of telomere loss (–289 versus –28 bp/month). The loss rate was significantly different from 0 in the 0–4 month group ($P = 0.02$), but showed no difference in the ≥5 month group ($P = 0.17$). A change in the range of mean TRF lengths was also apparent. In the younger group, the mean TRFs showed a tighter r correlation and ranged from 5 to 7 kb, while in the older group the mean TRF lengths were more heterogeneous and ranged from 6 to 10 kb.

To investigate the biphasic TRF length changes in *M.spretus* brain, we examined telomeric DNA from several regions of the brain known to show continued cell division in the adult mouse (31,32). We reasoned that perhaps cell turnover in specific parts of the brain may contribute to the observed changes in TRF length (although it is equally possible that other factors such as selective cell death, telomerase activity levels or changes in telomere binding proteins could play a role). Telomeric DNA was analyzed from the cerebellum, hippocampus and subventricular zone of the basal ganglia from a total of nine female *M.spretus*, three each from 4, 5 and 6 months postpartum (Fig. 4B). No significant differences in TRF length were found between any of the three brain regions within individual mice or between mice of the different ages. However, when the TRF lengths of the separate regions were compared with whole brain TRFs, a significant difference was observed, but only at the 4 month time point ($P = 0.0025$). The total brain average TRF length for 4 months was 2.5 kb shorter than the average TRF length of the separate regions at the same age.

Comparison of telomere lengths within individual *M.spretus*

As different tissues may differ in their turnover rate *in vivo*, we compared telomere lengths of different tissues within individual mice. Fifty mice (26 male and 24 female), ranging in age from 2 to 26 months postpartum, were used for the analysis; only mice with TRF length data for all tissues were included, and the data were not separated initially by age of the mouse. We first calculated the average TRF length for each tissue and compared those values (Table 2). On average, testis and brain TRFs were the longest and spleen TRFs were the shortest. However, because the heterogeneity in TRF lengths between mice of the same (as well as different) ages made absolute length comparisons difficult, we also rank ordered the tissues within each mouse from longest (1) to shortest (4 or 5), calculated the average rank for each tissue for males and females, and performed pairwise *t*-test analyses on the ranked data (Table 2). Although an exact order of tissues could not be definitively determined, significant differences between tissue TRFs within individual mice in the population were found. In males, testis and brain TRFs were statistically different from

each other and all other tissues examined. No differences were found between liver, kidney and spleen. In females, brain TRFs were also significantly different from kidney and spleen, and spleen was different from liver. When we examined tissue TRF order with respect to age by dividing male and female mice into two roughly equal-sized age groups, similar results were obtained (data not shown). These data suggest that although there were variations in order among individual mice, telomere length maintenance within the different tissues and relative to other tissues may be genetically pre-programmed. If the relative lengths within each tissue are controlled genetically, controlling factors may be the presence or absence of telomerase activity in each tissue and/or the degree of cell turnover.

Table 1. Regression analysis of *M.spretus* tissue TRF length with age

Tissue		N	Regression analysis TRF length (kb) versus age (months)			
			P value	Slope (m)	Pearson r	Y-intercept
All	Kidney	107	0.45	–0.009	0.073	7.06
	Liver	130	0.66	–0.005	0.038	7.21
	Spleen	145	*4.00E-07*	–0.050	0.405	7.36
	Brain (0–4 months)	34	*0.02*	–0.289	0.394	7.38
	Brain (≥5 months)	88	0.17	–0.028	0.146	7.85
Females	Kidney	57	0.41	–0.011	0.111	7.36
	Liver	71	0.73	0.004	0.042	7.40
	Spleen	90	*1.60E-04*	–0.042	0.386	7.47
	Brain (0–4 months)	31	*0.02*	–0.342	0.431	6.88
	Brain (≥5 months)	46	0.12	–0.031	0.231	8.20
Males	Kidney	50	0.61	0.010	0.073	6.53
	Liver	59	0.54	–0.011	0.082	6.94
	Spleen	55	*0.02*	–0.046	0.315	7.01
	Brain (≥5 months)	42	0.48	–0.029	0.111	7.53
	Testis	64	0.10	–0.037	0.205	8.61

Values in italics are statistically significant at the 0.05 level. Slope is kb/month, Y-intercept in kb.

Table 2. Intra-mouse tissue TRF length comparison

Tissues		Average TRF	Average rank[a]	*t*-test on ranked data			
				Brain	Liver	Kidney	Spleen
Females (24)	Brain	7.67 ± 1.11	1.92 ± 1.21	X	X	X	X
	Liver	7.53 ± 0.99	2.38 ± 0.88	0.1347	X	X	X
	Kidney	7.36 ± 1.03	2.71 ± 1.12	*0.0233*	0.2571	X	X
	Spleen	7.06 ± 0.79	3.00 ± 1.02	*0.0016*	*0.0275*	0.3513	X
Males (26)	Testis	7.83 ± 1.09	1.69 ± 1.19	*0.0196*	*5.39E-07*	*5.34E-06*	*2.04E-08*
	Brain	7.03 ± 1.14	2.54 ± 1.33	X	*0.0044*	*0.0200*	*0.0005*
	Liver	6.67 ± 0.68	3.58 ± 1.17	X	X	0.5619	0.4668
	Kidney	6.55 ± 0.65	3.38 ± 1.20	X	X	X	0.1909
	Spleen	6.47 ± 0.76	3.81 ± 1.10	X	X	X	X

[a]1 = Longest TRF. Values for pairwise two-tailed *t*-tests on *M.spretus* tissue rankings are shown. Values in italics are at least significant at the 0.05 level.

Table 3. Tissue telomerase activity in *M.spretus*

Tissue		Telomerase positive			Telomerase negative
		Number	Percent	Age (months)	Age (months)
Kidney		1/8	12.5	15	2,3,6,12,14,23,25
Spleen		2/8	25	6,21	2.5,2.5,6,12,13,14
Testis		6/8	75	1,2,12,12,22,23	0.13,0.13
Liver		6/8	75	1,2,4,12,12,24	0,24
Brain	Total	0/10	0	0	1,3,4.5,6,6,10,14,15,21,24
	Cerebellum	0/9	0	0	4,4,4,5,5,5,6,6,6
	Hippocampus	0/9	0	0	4,4,4,5,5,5,6,6,6
	SVZ Basal ganglia	0/9	0	0	4,4,4,5,5,5,6,6,6

Total number, percent and ages of telomerase positive tissue samples are shown. Ages of telomerase negative samples are also given.

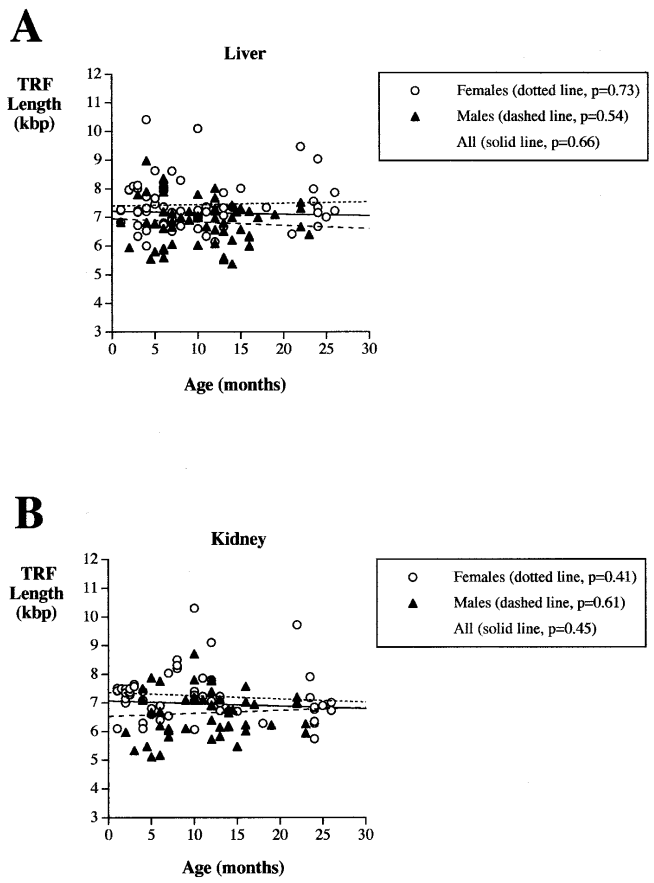

Figure 2. TRF lengths remain stable in *M.spretus* liver and kidney with age. Genomic DNA from liver (**A**) or kidney (**B**) was isolated at different ages and analyzed for TRF length. The mean TRF length (kb) for each sample was plotted against age (months), and regression lines for males (filled triangles, dashed line), females (open circles, dotted line) or the total population (solid line) are shown.

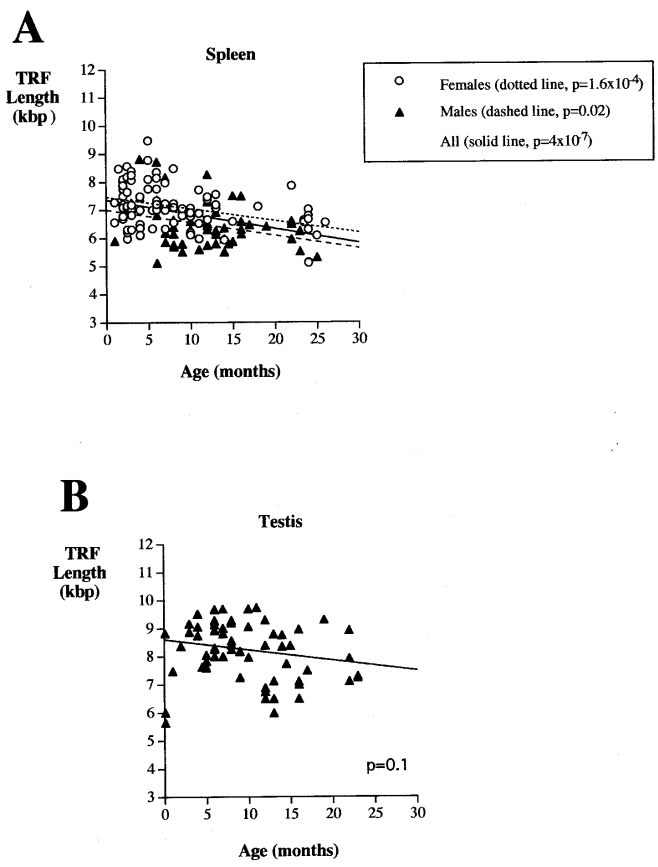

Figure 3. Telomere lengths change with age in spleen and testis. Genomic DNA from spleen (**A**) or testis (**B**) was isolated at different ages and analyzed for TRF length. The mean TRF length (kb) for each sample was plotted against age (months), and regression lines for males (filled triangles, dashed line), females (open circles, dotted line) or the total population (solid line) are shown.

Telomerase activity in *M.spretus* with age

We had previously detected telomerase activity in the liver and testes of a single *M.spretus* male, while no activity was detected in kidney or brain (29). However, weak telomerase activity has been detected in the kidney and spleen of BALB/c (29) and FVB/N (30) mice, and variability in telomerase activity between individual mice had also been observed (29,30). To examine the expression of telomerase activity in *M.spretus* tissues with age, we randomly sampled tissues from different male and female mice of various ages and analyzed a total of 69 samples for telomerase activity using the TRAP assay (Table 3).

The presence or absence of telomerase activity in different tissues was somewhat variable, but trends in activity could be observed. Weak activity was detected in one out of eight kidney samples, and two out of eight spleen samples. These results suggest that telomerase activity in total kidney and spleen is weak to non-detectable at all ages, and supports previous findings from single *M.spretus* and *M.musculus* (29,30). Stronger activity was detected in six out of eight samples of both testis and liver. The two testis samples which tested negative for telomerase were from two 4 day postpartum mice, which agrees with our previous finding of undetectable telomerase activity in the immature BALB/c testis (29). Telomerase activity was detected in all other ages of *M.spretus* testis. The two liver samples which tested negative were from a 21 day fetus and 24 month postpartum mouse. However, telomerase was also detected in a 1 month old and another 24 month old *M.spretus*. These findings suggest that telomerase activity is expressed in the liver of mice at all ages, although individual variation among *M.spretus* samples can occur. No telomerase activity was detected in total *M.spretus* brain samples at any age or in the separated cerebellum, hippocampus or SVZ of the basal ganglia between 4 and 6 months. This result was somewhat surprising, given the difference in telomere length between total brain and the individual regions tested at 4 months of age, as well as the difference in total brain TRF lengths at 4 and 5 months. Further time point studies are required to rule out the possibility that a transient activation of telomerase may occur between 4 and 5 months postpartum.

Telomere lengths reflect gender differences

TRF lengths were plotted versus age for each tissue, and linear regression analyses were performed for males, females and the total population (Figs 2–4, Table 1). When male and female TRF lengths were compared for each of the tissues, significant gender differences were observed (Table 4). The regression line for females in both liver and kidney was ~+0.5 to +1.0 kb offset from

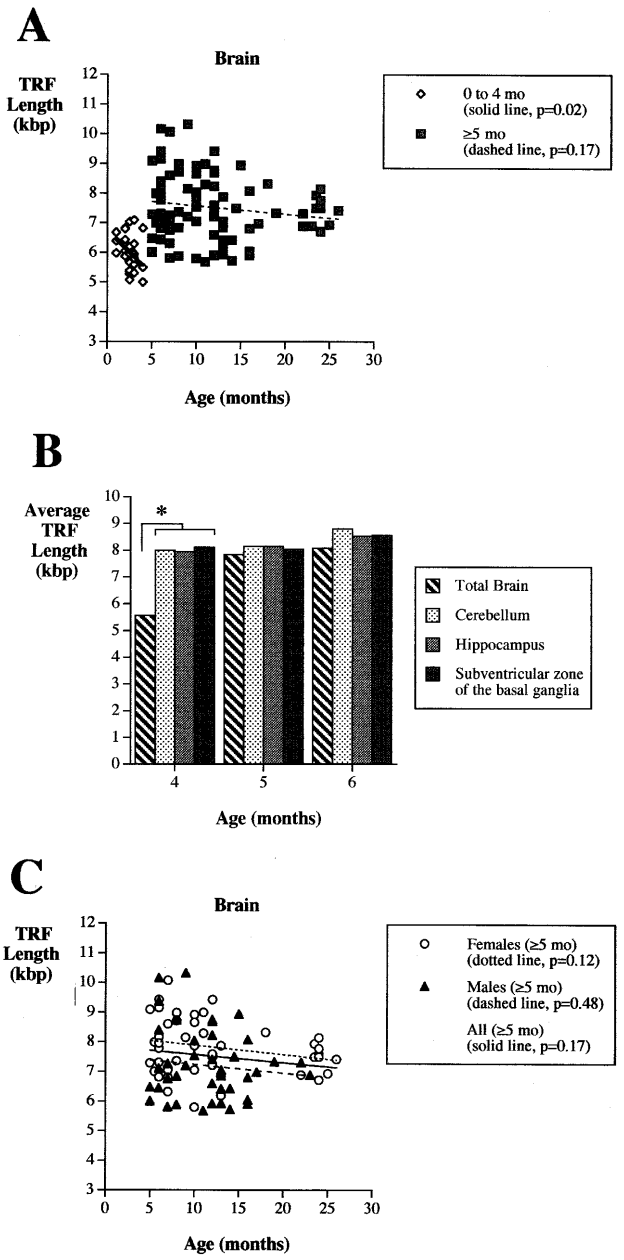

Figure 4. Telomere dynamics with age in *M.spretus* brain. Genomic DNA from total brain (A and C) or brain regions (B) was isolated at different ages and analyzed for TRF length. (**A**) The total population was divided into two age groups, the mean TRF length (kb) for each sample was plotted against age (months), and regression analysis was performed. Filled diamonds, 0–4 months, solid line; open squares, ≥5 months, dashed line. (**B**) Graph contrasting average TRF lengths for total brain (hatched), cerebellum (dotted), hippocampus (gray) and subventricular zone of the basal ganglia (black) in female *M.spretus* at 4, 5 or 6 months. (**C**) Graph showing regression lines for males (filled triangles, dashed line), females (open circles, dotted line) or the total population (solid line) for mice ≥5 months postpartum.

the male regression line. In the spleen, male and female TRF lengths each showed significant decreases (P = 0.02 and 0.00016) at approximately the same rate (46 and 42 bp/month). Again, a significant gender difference (P = 0.00001) was observed between the regression lines of the two populations (Table 4), with female TRFs being ~0.5 kb longer. Gender differences were also present in brains of *M.spretus* ≥5 months old. When males and females in the ≥5 month group were compared for telomere lengths (Fig. 4C), female TRFs again showed a significantly longer mean TRF length (+0.5 kb, P = 0.0075). A limited number of male mice prevented the analysis of gender effects in the 0–4 month group.

Two other lines of evidence support that telomere length is influenced by gender. First, pairwise analyses of the ranked TRF length data yielded differences in significance between tissues with respect to gender. Second, in a limited analysis of brain and spleen DNAs from four male and four female *M.spretus* × C57BL/6 F1 mice, preliminary data showed two peaks of hybridization signal in the F1 samples. Peak 1 (>30 kb) appears to correspond to the C57BL/6 parent while peak 2 (14–20 kb) may derive from the *M.spretus* parent (data not shown). A statistically significant difference in TRF peak 2 between genders in both tissues was observed (Table 4). In addition, the average spleen TRF lengths for both male and female F1 mice (15.21 and 18.94, respectively) were shorter than those for brain (16.07 and 19.87). Both these results are consistent with the data from *M.spretus* tissue TRF length analysis described above, and suggest that peak 2 in the F1 mice derives from the paternal *M.spretus* chromosomes.

Table 4. Significant gender differences in tissue TRF length

Tissue			Student *t*-test[a] Males versus females (*P* value)
Kidney			*0.0002*
Liver			*0.00002*
Spleen	*M.spretus*		*0.00001*
	spr × BL/6		*0.02*
Brain	*M.spretus*	all ages	0.87
		≥5 months	*0.0075*
	spr × BL/6		*0.014*

[a]Two-tailed *t*-test. Values in italics are statistically significant. spr × BL/6, F1 mouse from *M.spretus* × C57BL/6 cross.

DISCUSSION

Differences in telomere length and telomerase activity between and within mouse and human cells and tissues have previously been reported (20,29,30,33). The TRF lengths of mouse species can differ quite dramatically, from >50 kb in *M.musculus* laboratory strains (24,25) to the 5–15 kb in *M.spretus* and other wild-derived mice (25,29). However, the TRF lengths in human tissues are considerably shorter (5–15 kb) and less variable than the hypervariable *M.musculus*. In addition, unlike most human somatic cells, telomerase is expressed in many mouse somatic tissues (29,30). Furthermore, telomere binding proteins, and telomerase activity, RNA and one associated protein component show different sequences and properties between mouse and human (20,22,26,34). Given these distinctions, it is not unlikely that telomere length regulation in mouse may involve some altered or additional pathways from telomere length regulation in humans. In this study, we have utilized the wild-derived mouse species, *M.spretus*, to examine the relationship between cell ageing and telomere length regulation in the mouse.

TRF lengths in the same tissue types between many individual mice of different ages in a population were compared. In this analysis, we determined whether telomere length regulation in mice followed the telomere hypothesis of cell senescence proposed

for human cells (15). The telomere hypothesis proposed that telomeres will shorten during replicative cell division due to the inability of normal DNA polymerases to completely replicate the ends of linear chromosomes in those cells that lack sufficient telomerase activity. Once a critical telomere length is reached, the shortened telomere(s) would signal the cell to stop dividing, perhaps using DNA damage pathways. In cells that have sufficient telomerase activity, telomere length would be maintained from one generation to the next, and allow for continued cell division. Some of our results from mice support the telomere hypothesis of cell ageing, while other results may support alternative or additional explanations for maintaining telomere length.

Mus spretus tissues differed in TRF lengths and telomerase activity with age. None of the TRF lengths in any tissue or at any age were <5 kb, which is in agreement with the TRF length found in human cells at senescence (8). Liver and kidney samples showed no statistical difference in TRF length with age. Although data regarding accurate cell turnover is difficult to obtain, some reports suggest that both of these tissues show little cell division in normal tissues in adult mice (35,36), so no changes in TRF length might be expected. However, a difference in telomerase activity between the two tissues is apparent: telomerase was detected in 75% of liver samples but only 12.5% of kidney samples from all ages. To account for this discrepancy, the telomere hypothesis would propose that telomerase activity is present in these non-dividing, adult liver cells to maintain telomere lengths in the event of liver damage. This activity could allow the necessary cell divisions to promote regeneration of damaged tissue to occur. Contrary to the liver and kidney data, the spleen showed distinctive TRF length changes with age. A portion of the spleen contains different types of proliferating cells that continue to divide throughout the lifespan (37). The low level of telomerase activity detected in 25% of spleen samples probably derived from the small population of self-renewing progenitor cells as previous work (38) has shown. For our purposes in this study, whole spleens were homogenized into extracts without separation of the progenitor population. The decrease in whole spleen TRF length with age probably reflects the non-self-renewing cell proliferation occurring in the spleen, in agreement with the telomere hypothesis.

No significant change in testis TRF length with age was observed in the population as a whole. However, in separate age group analysis, testis TRFs show a statistically significant increase with age up to 12 months. Interestingly, an increase in the TRF length of mature human sperm with age has also been detected (7). This increase in mouse TRF lengths correlates with the presence of telomerase activity in the testis. Between 4 and 8 weeks postpartum, the testis is ~80–90% spermatogonial cells and the first mature spermatozoa are produced (39). Telomerase activity had previously been detected by the conventional telomerase assay in *M.musculus* between 4 and 6 weeks postpartum (29), suggesting that telomerase is present at some stage of spermatogonial development. Likewise, we detected telomerase activity by TRAP assay in the testis of all *M.spretus* 4 weeks or older, but not in two 4 day old mice. Testis telomerase expression is therefore consistent with the ability to maintain or increase telomere length in the dividing germ cells.

The testis, spleen and kidney appear to support the telomere hypothesis in mice and the liver data could also speculatively support the hypothesis. Telomere length dynamics in brain, however, do not support the telomere hypothesis of ageing. Brain TRFs show a biphasic regulation of length, which appears unrelated to cell turnover or telomerase. TRF lengths in brain decrease at a rate 10-fold faster from 0 to 4 months than in mice ≥5 months, though the decrease in the older group is non-significant. The significant TRF decrease in the younger group does not seem to arise from rapid cell proliferation because in the >2 week postpartum brain, cell division seems limited to a small population of stem cells within the hippocampus, cerebellum and subventricular zone of the basal ganglia (31,32,40). In the younger age group, the range of mean TRF lengths is quite homogeneous, while in the older group it is very heterogeneous. Brain telomere lengths also increase substantially on average at 5 months and are maintained at the new length thereafter. However, telomerase activity seems not to be involved because telomerase is not detected in total brain samples at any age. In addition, no activity was detected in any of the individual regions thought to have continued cell proliferation, although it is possible that the isolated stem cells may contain telomerase activity which may be masked by the bulk of the tissue. The significant decrease in TRF length in the brain, followed by a significant increase at 5 months may therefore involve alternate mechanisms of telomere-length modulation.

The finding of gender differences in tissue TRF lengths in all four tissues examined is one line of evidence which points to a genetic component of mouse telomere length regulation in tissues. Male *M.spretus* had TRF lengths that were on average 0.5–1 kb shorter TRFs than females in every tissue examined. Male *M.spretus* × C57BL/6 F1 mice also had shorter peak 2 TRFs than the female F1 mice, although in these mice the difference was ~3.7 kb rather than 0.5–1 kb. Although we have not observed a consistent weight difference between adult male and female *M.spretus*, a growth rate or metabolic distinction between genders may be a possible explanation for the TRF length difference. Gender TRF length differences in at least one other species has been found. Rhesus monkey males displayed lymphocyte TRF lengths that were, on average, 2.5 kb longer than those of female monkeys (unpublished data). However, in human lymphocytes, no significant difference between males and females was detected (11). This finding of a gender effect on telomere length in mice could be another difference between mice and humans or it could simply be that not enough human tissues have been examined to detect a difference. Further telomere regulation and metabolism studies in humans and mice may provide a possible explanation.

Significant differences between tissue TRF lengths within individual mice also support a genetic component of telomere length regulation. When tissue TRFs within individual *M.spretus* were compared, specific tissues tended to have longer or shorter TRF lengths compared to other tissues. The finding of TRF length differences between tissues suggests that TRF lengths of each tissue are regulated independently. This tendency is also observed in the preliminary data from *M.spretus* × C57BL/6 F1 mice since both the gender and size difference were maintained. To follow this phenomena further, a significant number of F1 mice as well as subsequent generations and backcrossed animals should be examined. One other line of evidence which suggests a genetic component is the fact that the American colonies of *M.spretus* have shorter TRF lengths than their European counterparts (41). The telomeres of European *M.spretus* are similar in size to *M.musculus* strains. The American colonies presumably derived from a small population of the European *M.spretus* in which

perhaps one or more changes occurred which affected telomere length regulation (41).

Although most tissue data in this study support the telomere hypothesis of cell ageing, the results from the brain data suggest that telomere lengths may decrease due to factors other than the end-replication problem during cell division, and that alternate pathways in addition to those utilizing telomerase may be involved in maintaining and regulating telomere length in the mouse. Both environmental as well as genetic factors could be involved. Recently, hyperoxic conditions have been shown to rapidly shorten telomeres in cultured cells without cell turnover (42). The mechanism for this rapid decrease is thought to be due to free radical-induced single strand breaks in the telomeric DNA. Endogenous factors, including those involved in telomere structure, DNA repair and cell cycle regulation, also very likely play a role in regulating telomere length in the mouse. For example, differential regulation of the mammalian telomere-repeat binding factor (TRF1, 26) could be one mechanism involved in setting the telomere length in distinct tissues by regulating the availability of telomeric sequences to modifying factors. Likewise, perhaps, DNA repair enzymes, such as DNA polymerase β which has a high activity level in the brain (43), or other DNA replication factors play a direct or indirect regulatory role in the telomere dynamics of the mouse brain. Telomere length regulation during ageing in *M.spretus* tissues may be a complex dynamic process involving not only the coordinated regulation of telomerase activity and cell cycle controls, but also additional, perhaps tissue-specific, factors. Further studies should yield important clues to understanding telomere length regulation in mouse.

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