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Influences of inbreeding and genetics on telomere length in mice

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Abstract. We measured telomere lengths of blood leukocytes in several inbred and outbred mammalian species, using a telomere-specific fluorescent probe and flow cytometry. Humans, non-human primates, and three outbred populations of *Peromyscus* mice (*Peromyscus leucopus, Peromyscus maniculatus,* and *Peromyscus polionotus*) have short telomeres. Two common strains of laboratory mice, C57BL/6J and DBA/2J, have telomeres several times longer than most other mammals surveyed. Moreover, the two inbred laboratory mouse strains display significantly different telomere lengths, suggesting the existence of strain-specific genetic determinants. To further examine the effects of inbreeding, we studied three *Peromyscus leucopus* inbred lines (GS109, GS16A1, and GS16B), all derived from the outbred *P. leucopus* stock. Telomeres of all three inbred lines are significantly lengthened relative to outbred *P. leucopus,* and the three lines display strain-specific significantly different telomere lengths, much like the C57BL/6J and DBA/2J strains of *M. musculus.* To further characterize the genetic inheritance of telomere length, we carried out several crosses to obtain hybrid F_1 mice between parental strains displaying the phenotype of long and short telomeres. In all F_1 mice assayed, peripheral blood leukocyte telomere length was intermediate to that of the parents. Additionally, we generated F_2 mice from a cross of the (*P. leucopus* outbred × GS16B)F_1. Based on the distribution of telomere length in the F_2 population, we determined that more than five loci contribute to telomere length regulation in *Peromyscus.* We concluded that inbreeding, through unknown mechanisms, results in the elongation of telomeres, and that telomere length for a given species and/or sub-strain is genetically determined by multiple segregating loci.

Telomeres, the oligonucleotide repetitive sequences that comprise the ends of eukaryotic chromosomes, are evolutionarily conserved structures that, in mammals, play roles in the replicative senescence of cells in vitro and in cancer progression in vivo (Blackburn 2000; Campisi et al. 2001; Sherr and DePinho 2000). In the absence of telomerase, telomeres shorten with cell replication. Thus, it is thought that the stabilizing effects of telomeres on chromosomes are lost in dividing cells, at least in vitro, as they erode to critically short lengths (Allsopp et al. 1992; Hackett et al. 2001; Harley et al. 1990). End-to-end chromosome fusions and genomic instability are among the genetic consequences. The relationship, if any, between telomere dynamics and organismal aging and longevity is largely unknown. Cells of inbred laboratory mice, in which studies of aging and cancer are typically carried out, have anomalously extended telomeres that are several times longer than those of human cells (Hemann and Greider 2000; Kipling and Cooke 1990; Starling et al. 1990). Laboratory mice deficient in mTR, the RNA component of telomerase, a native enzyme that extends and thus stabilizes telomeres in the face of replicative stress, show few physiological consequences for several generations, but eventually fail to reproduce owing to decreased fertility, as well as increased embryonic lethality due to a neural tube closure defect (Herrera et al. 1999a). Late-generation, mTR-deficient mice show defects in highly proliferative tissues (Blasco et al. 1997; Lee et al. 1998). Moreover, telomerase knockout mice generated on a genetic background characterized by strain-specific shorter telomeres show deleterious effects in earlier generations (Herrera et al. 1999b). The physiological effects of telomerase deficiency in humans are manifested in dyskeratosis congenita, a congenital disease caused by mutation of the RNA component of telomerase (Mitchell et al. 1999; Vulliamy et al. 2001). Since human telomeres are shorter than those of most mice, rapidly proliferating tissues are affected early in life in people suffering from the disease, and death usually results in early adulthood from bone marrow failure.

However, recent findings also suggest that telomere dynamics are more complex than originally thought and that a cell's mitotic history and telomerase expression status alone are not sufficient to describe a cell's probability of senescence or neoplastic transformation (Blackburn 2000). The regulation of the t-loop structure of the telomeric end is regulated by telomeric binding proteins, such as TRF2 in humans. The t-loop structure appears to play an important role in protecting the 3′ overhang from nuclease degradation, and also in limiting the access of telomerase to the telomeric end (Blackburn 2000, 2001; Campisi et al. 2001; de Lange 2001).

For mice, even reproductive strategies such as inbreeding, outbreeding, or domestication are thought to influence telomere length (Bickle et al. 1998; Hemann and Greider 2000). In this report, we further examined the effect of breeding strategy in another species, the white-footed mouse, *Peromyscus leucopus*, and two other congeneric species. We conclude that telomere length (a) is increased by inbreeding and (b) is genetically determined by multiple segregating loci that establish a base telomere length upon which inbreeding builds its epistatic effect.

Materials and methods

Animals. Blood from all *Peromyscus* species was obtained from animals housed at the Peromyscus Stock Center, Columbia, S.C. Blood from non-inbred *Mus musculus castaneus* and *Mus spretus* mice housed at the Roswell Park Cancer Institute, Buffalo, N.Y., was provided by Rosemary Elliott. Blood from Rhesus monkeys (Texas Primate Center, Alice, Tex.) housed at the University of Kentucky was provided by Don M. Gash. All other mice were housed under barrier conditions in the University of Kentucky Division of Lab Animal Resources with food and water available ad libitum. DBA/2J, C57BL/6J, CAST/EiJ, SPRET/EIJ, and (C57BL/6J × DBA/2J)F_1 mice were obtained from The Jackson Laboratory, Bar Harbor, Me. Black Swiss, Swiss Webster, and ICR outbred mice were obtained from Taconic, Germantown, N.Y.

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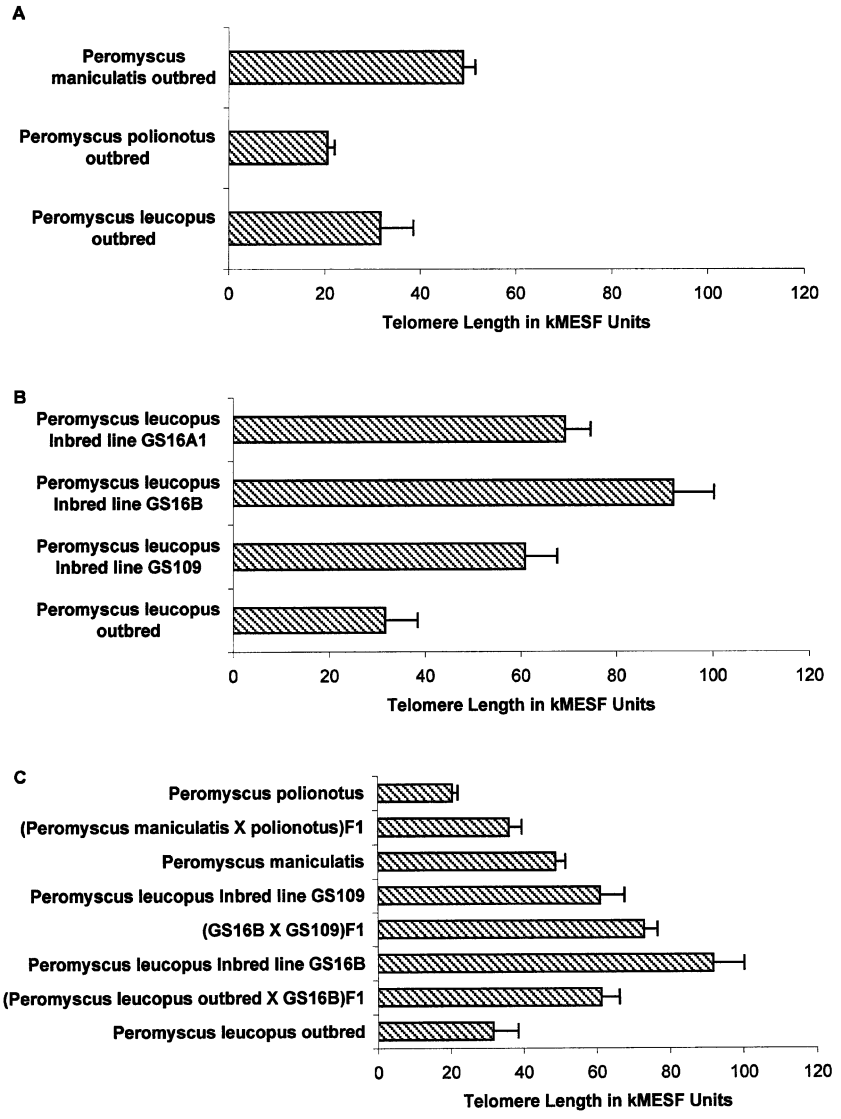

Fig. 1. Leukocyte telomere length in outbred and inbred *Peromyscus* animals. Peripheral blood was collected from mice, treated, and analyzed as described in Materials and methods. **A.** Outbred *Peromyscus leucopus,* Outbred *Peromyscus maniculatus,* and Outbred *Peromyscus polionotus,* ages 5–9 months, n = 21, 5, and 5 respectively. **B.** Inbred lines GS16A1, GS16B, GS109, and outbred *Peromyscus leucopus,* ages 4–16 months, n = 11, 14, 13, and 21, respectively. **C.** Various F_1 crosses and respective parental strains: Outbred *Peromyscus maniculatus,* Outbred *Peromyscus polionotus,* (*Peromyscus maniculatus* × *polionotus*)F_1, Inbred line GS16B, Inbred line GS109, (GS16B × GS109)F_1, Outbred *Peromyscus leucopus,* and (Outbred *Peromyscus leucopus* × GS16B)F_1, ages 4–11 months. n = 5, 5, 5, 14, 13, 4, 21, and 8 respectively.

Telomere length analysis. All blood samples were subjected to ammonium chloride red cell lysis and were washed twice in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Leukocyte telomere length was then determined by a *F*low cytometry based *F*luorescence *I*n *S*itu *H*ybridization (Flow FISH) procedure as described (Rufer et al. 1998). Briefly, cell samples were divided in half by using 5×10^5 cells per tube, and were incubated in hybridization mixture with or without (unstained) a fluorescein-labeled peptide nucleic acid (PNA) probe (5′-Flu-CCCTAACCCTAACCCTAA-3′) (PE Biosystems, Framingham, Mass., or Boston Probes, Bedford, Mass.). Samples were analyzed by fluorescence-activated cell sorting (FACS) on a Becton Dickinson FACScan flow cytometer with laser emitting light at an excitation wavelength of 488 nm. Telomere fluorescence was determined by subtracting the mean channel number of the unstained histogram peak from the mean of the PNA stained histogram peak. To control for daily variations in laser intensity and variations in the sensitivity of the photomultiplier tube, beads with known amounts of fluoroscein isothiocyanate (FITC) label (*M*olecules of *E*quivalent *S*oluble *F*luorochromes, MESF) (Flow Cytometry Standards Corp., Fishers, IN) were run on the flow cytometer before and after biological samples in each experiment. A standard curve was generated from the beads in each experiment, and the slope of the line was used to convert channel number to kMESF units. Flow FISH FACScan data were analyzed by using Becton Dickinson Cell Quest Version 3.3 software.

Data analysis. Data were analyzed by one-way ANOVA, followed by a Tukey test using SigmaStat Version 2.03. Means ±1 standard deviation are depicted.

Results

Figure 1A shows telomere lengths of blood leukocytes from three outbred *Peromyscus* species maintained at the Peromyscus Stock Center at the University of South Carolina. They span a 2.5-fold range with *P. polionotus* having short telomeres roughly the same length as found in humans, *P. maniculatus* having the longest telomeres, and *P. leucopus* is intermediate (each species is significantly different from the other two, $p < 0.001$). Interestingly, and without current explanation, the two species showing the most interspecific difference, *P. maniculatus* and *P. polionotus,* are sister species and interfertile, whereas *P. leucopus* diverged much earlier from either of the sister species. Figure 1B compares telomere lengths of leukocytes from three inbred strains of *P. leucopus* with the outbred parental stock. The GS16B and GS16A1 lines were derived from common ancestors that had been inbred for seven generations from outbred *P. leucopus* stock (Smith et al. 1990). The sub-strains, 16B and 16A1, were then individually derived beginning at the 8th generation, and maintained through ~25 additional generations of inbreeding. Two points are notable. First, telomeres of both inbred lines are significantly lengthened ($p < 0.001$) relative to outbred *P. leucopus.* Telomeres of the GS16B inbred strain are three times longer than those of the outbred stock. Second, the two GS16 lines display strain-specific and statistically significant ($p < 0.001$) differences in telomere lengths.

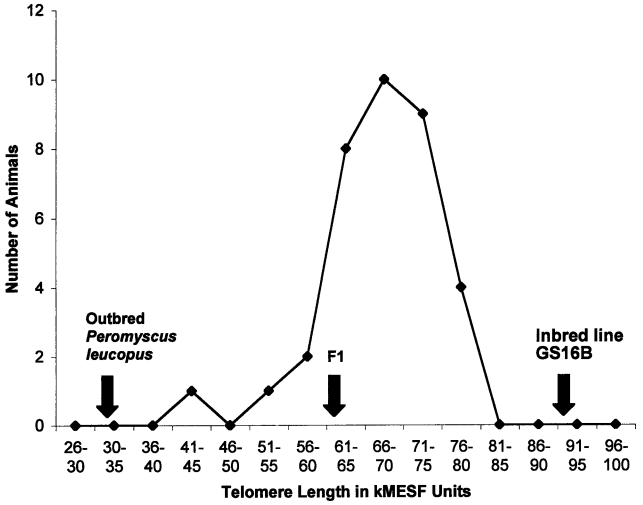

Fig. 2. Distribution of leukocyte telomere length in (*Peromyscus leucopus* × GS16B)F_2 mice. Blood samples were obtained from (Outbred *Peromyscus leucopus* × GS16B)F_2 mice, ages 3–4 months, n = 35, treated and analyzed as described in Materials and methods.

The third inbred strain, GS109, was separately derived from outbred *P. leucopus* stock, and individual mice from inbred generations 24–27 were assayed (Fig. 1B). The GS109 line was characterized by telomere lengths significantly longer than those of outbred animals ($p < 0.001$), but significantly shorter than either of the other two GS16 inbred strains (GS109 vs. GS16B, $p < 0.001$; GS109 vs. GS16A1, $p < 0.05$), suggesting that strain-specific telomere length is genetically determined by multiple, independently segregating loci.

To determine the pattern of inheritance of genetic determinants of telomere length, we carried out several intercrosses and backcrosses (Fig. 1C). *P. polionotus* × *P. maniculatus* F_1 hybrids were characterized by telomere lengths intermediate between the parental species. F_1 hybrids of a cross between the shortest-telomere GS inbred strain (GS109) and the longest-telomere (GS16B) strain also had intermediate length telomeres. Similarly, a backcross hybrid between the longest inbred strain (GS16B) and the *P. leucopus* outbred stock had intermediate length telomeres. Collectively, these data are consistent with a pattern of inheritance in which offspring of short- and long-telomere parents receive one chromosome from each parent, and each chromosome maintains its parental length telomeres. Alternatively, both telomeres of each chromosome pair might assume a length intermediate to the parental strains.

Figure 2 shows the telomere length distribution in a group of 35 (*P. leucopus* outbred × GS16B)F_2 animals. The phenotypes of the parents and the F_1 animals described above are shown for comparison. The mean telomere length of the F_2 mice was 68 ± 7.5 kMESF units, not significantly different from the mean of the F_1 animals (61 ± 5.2). Based on the predicted ratio of parental phenotype to total F_2 animals sampled, the data are consistent with more than five segregating loci contributing to telomere length in this cross. The effect of five loci predicts 1 in 16 parental phenotypes being present among F_2 offspring, and in the population we tested it was <1 in 35 (none had parental phenotypes).

To put the telomere data obtained with *Peromyscus* into perspective, we assayed other mammalian species including other mice from the *Mus* genus, Rhesus monkeys, and humans (Fig. 3). Outbred stock of *Mus spretus* and *Mus musculus castaneus* showed roughly the same length telomeres as humans, monkeys, and outbred *Peromyscus leucopus,* thus underscoring the lack of correlation between natural lifespan and telomere length. However, inbred strains of both *Mus musculus castaneus* and *Mus spretus,* like *Peromyscus leucopus,* both showed longer telomeres than outbred stock, again demonstrating an effect of inbreeding on telomere length. The lack of a statistically significant difference between inbred and outbred *Mus spretus* stocks may possibly be attributed to a limited gene pool in the outbred stock. Two common strains of laboratory mice, C57BL/6J and DBA/2J, have telomeres several times longer than most of the other mammals surveyed (Fig. 3). Moreover, the two inbred laboratory *Mus* strains display significantly different ($p < 0.001$) telomere lengths, further supporting the existence of strain-specific genetic determinants. As observed with the *Peromyscus* hybrid crosses, (C57BL/6 × DBA/2)F_1 animals were characterized by intermediate-length telomeres.

Discussion

The data presented herein support and expand on the overall notion that inbreeding is accompanied by increased telomere lengths. It is possible, but unlikely, that this effect is limited to the blood leukocytes studied here. Rather, our results are in agreement with those of others in which mouse liver, spleen, and kidney cells were analyzed by using terminal restriction fragment (TRF) analysis on Southern blots, all of which showed extended telomeres in inbred strains of laboratory mice (Bickle et al. 1998; Hemann and Greider 2000; Kipling and Cooke 1990; Starling et al. 1990).

In addition to the effect of inbreeding on telomere length, we conclude that specific genetic determinants regulate telomere length, at least in mice. The best evidence for this is the differential and strain-specific telomere lengths of the inbred *M. musculus* and *P. leucopus* lines. The *Peromyscus* lines GS16A1 and GS16B were separately derived after sharing common ancestors through seven generations of inbreeding. By chance, after seven generations, ~1.5% of the loci remained heterozygous in the ancestral line. The fact that the GS16A1 and GS16B strains are characterized by different telomere lengths suggests that alleles affecting telomere length were differentially segregated from the few remaining heterozygous loci at the bifurcation of the strain genealogies, and prior to the attainment of complete homozygosity in the ancestral line.

Similarly, the separately derived GS109 line, with its unique telomere length, is consistent with the independent segregation of multiple loci affecting the trait. The existence of inbred strains with distinguishable and heritable telomere lengths makes it feasible to map and identify regulatory loci through standard genetic approaches. Such an approach was used to identify a locus regulating telomere lengths in an inter-species mouse cross [*M. musculus* (BALB/c) × *M. spretus*]F_1, by reciprocal backcrossing (Zhu et al. 1998). A locus within a 5-cM interval of mouse Chr 2 was found to regulate differential telomere lengths in spleen cells of these mouse species. This region did not contain any known genes associated with the telomere regulatory apparatus.

The pattern of telomere length inheritance observed in the study by Zhu et al. (1998) differs from what we found in the intra-species *P. leucopus* crosses. Zhu et al. found a bimodal distribution of telomere lengths in BALB/c × *M. spretus* F_1 hybrids by both terminal restriction fragment (TRF) analysis and quantitative fluorescence in situ hybridization (Q-FISH). One peak corresponded in size to the long BALB/c telomeres, while the other was intermediate in size to those of the parent strains. The intermediate-sized peak was polymorphic in the F_1 × *M. spretus* backcross and was consequently used in their mapping strategy. In contrast, we found that hybrids of every *P. leucopus* cross and the one *M. musculus* cross we tested invariably showed intermediate telomere lengths. Clearly, there are many polymorphic loci in the strains used in the two studies. Therefore, such a result may not be surprising, since telomere length regulation is likely contributed to by multiple loci.

Aside from the humans and monkeys, all animals in the present study were young adults. It should be mentioned in this regard that

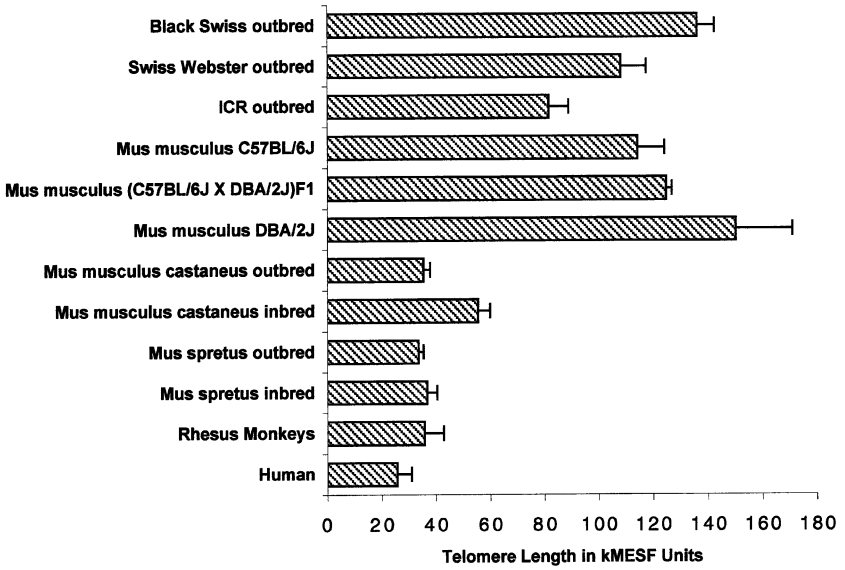

Fig. 3. Comparison of leukocyte telomere length in mammalian species. Peripheral blood was collected from the following subjects: human volunteers ages 26–71 years, n = 5; Rhesus monkeys ages 8–25 years, n = 7; C57BL/6J, DBA/2J, CAST/EiJ, SPRET/EiJ, and (C57BL/6J × DBA/2J)F_1 inbred mice, age 2 months, n = 32, 35, 9, 6, and 6, respectively; Non-inbred *Mus musculus castaneus* and *Mus spretus,* n = 6 and 6 respectively; Outbred Black Swiss, Swiss Webster, and ICR mice, age 4 months, n = 6, 6, and 6 respectively. Blood samples were treated and analyzed as described in Materials and methods.

Peromyscus species housed under laboratory conditions have life spans two- to threefold as long (5–7 years) as most inbred laboratory mice of the *Mus* genus (Sacher and Hart 1978; Smith et al. 1990). *M. musculus* strains were all females, and *P. leucopus* strains were both females and males. We have determined that there is not a sex difference in leukocyte telomere lengths in *M. musculus* laboratory strains that we have studied (Manning et al. unpublished observations). Human subjects were of both genders and of a wide age range. Previous studies have shown that telomere lengths of human leukocytes shorten in a biphasic pattern with age, in which most of the loss occurs in the first year of life followed by a 30-fold slower rate of decline from childhood through old age (Rufer et al. 1999). The fact that we found no relationship between age and telomere length in a sampling of five adult human subjects is not surprising in light of the study by Rufer et al. (1999) in which more than 500 individuals were analyzed to uncover a significant correlation between telomere length and adult age.

Our results corroborate and extend findings of a recent study in which telomere lengths of several of the same mouse strains, including *P. leucopus,* were compared (Hemann and Greider 2000). Our results agree with their conclusion that mice per se do not have long telomeres. Rather, both studies show that recently wild-derived stocks have short telomeres, similar in length to outbred species such as humans and monkeys. Similarly, our findings agree with theirs showing no relationship between natural life span and telomere length. However, our data demonstrating a positive correlation between inbreeding and telomere length in *P. leucopus, Mus musculus castaneus,* and *Mus spretus* are in disagreement with their conclusion regarding the long telomeres of standard inbred laboratory strains of *Mus musculus.* Hemann and Greider concluded that since the outbred *Mus* strains ICR, Black Swiss, and Swiss Webster had long telomeres, similar in length to inbred *Mus* laboratory strains, that inbreeding did not lead to long telomeres. Our data in *P. leucopus* strains show that multiple genes affect telomere length and that segregation of these genes in the derivation of stocks results in strain-specific phenotypes that can vary by as much as 2.5-fold. The outbred *Mus* strains may thus have genetically determined long telomeres that possibly could be further lengthened with inbreeding. A second consideration is that so-called outbred commercial mouse stocks are typically based on a restricted gene pool. Matings are carried out not so much to adhere to a true random breeding strategy where the greatest genetic diversity is sought, but simply to avoid brother-sister matings.

Whereas it is tempting to consider an epigenetic mechanism as contributing to a relationship between inbreeding and telomere length, it is clear that genetic variation for factors affecting telomere length plays a major role. Thus, *P. leucopus* hybrids between the long-telomere inbred and the short-telomere randomized stocks contain telomeres intermediate in length between the two. Telomere length distribution in the F_2 generation suggests the involvement of more than five loci. The curiosity is that alleles associated with long telomeres are focused in the inbred stock, suggesting some selective advantages for them in the inbreeding process. Even for *Mus,* which is one of the easiest species to inbred, it is generally found that their inbred strains are physiologically compromised. Such is manifest as a reduction in a number of fitness characteristics including body size, fecundity, and life span (Green and Witham 1991), as well as those revealed in more demanding experimental manipulations that include in vitro embryo culture (Whitten and Biggers 1968), production of transgenic mice (Brinster et al. 1985), and cryopreservation of spermatozoa (Sztein et al. 2000). Perhaps it is that genes affecting telomere length do so indirectly and that their primary function is associated with some other physiological process (es) required for successful inbreeding.

At the other end of the mouse spectrum are the sister species, *P. maniculatus* and *P. polionotus,* which are poised at the threshold of reproductive isolation. Their reciprocal hybrids exhibit growth deregulation manifest as over- or under-sized offspring, depending on the direction of the cross (Dawson 1965). Recently the phenomenon was found to be associated with epigenetic mechanisms causing disruptions of normal genomic imprinting (Vrana et al. 1998). However, in our studies, nothing unusual was noted in the telomere lengths (*P. maniculatus* × *P. polionotus*)F_1 hybrids. As with all inter-specific and inter-strain hybrids we tested, their telomere lengths were midway between the telomere lengths of the parental species.

In conclusion, our results sound a note of caution in relating telomere lengths to parameters associated with aging, longevity, and carcinogenesis in inbred animals. It should be recognized that the unusually long telomeres of the laboratory mouse, possibly as a result of the homozygosity that makes them so valuable for other genetic studies, may confound their use as a model system here. Of course, valuable data on the effects of telomere length and telomerase expression on tumorigenesis and aging have been, and will continue to be, obtained with mice. As a prime example, a study of aged mice doubly null for telomerase and p53 revealed the emergence of epithelial tumors not normally found in mice, but common in humans (Artandi et al. 2000). This result provides an

important clue in the cause of this class of tumors and, in this case, the long telomeres of the mouse, and their experimental shortening due to a lack of telomerase, were central to revealing potential mechanisms not otherwise evident. Given the exceptionally long telomeres of laboratory mice and their relatively short life spans, it is difficult to invoke gradual telomere shortening as a mitotic clock critical to the aging and senescence of cell populations in vivo, and thus organismal longevity in inbred animals. However, a recently proposed model of telomere regulation takes into account a growing body of evidence that the length of telomeres is but one important telomere parameter relevant to cellular senescence (Blackburn 2000, 2001). Other important parameters include the status of telomerase expression and the association of other critical DNA-binding proteins that in concert determine a 'capped' or an 'uncapped' higher order state of telomere structure that reversibly confers chromosomal stability and instability, respectively.

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