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Real-time PCR Assay for Measurement of Mouse Telomeres

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Measurement of telomeres by polymerase chain reaction (PCR) amplification has been problematic due to the formation of dimers by the primers designed to hybridize to the telomere repeats. Recently, a set of primers that overcome this problem has been created and used to develop an assay to measure human telomeres by real-time quantitative PCR. We modified this assay to measure mouse telomeres. Results showed that the primers do indeed amplify mammalian telomere repeats without forming dimers. Results obtained from the real-time quantitative PCR assay of mouse DNA were similar to terminal restriction fragment analysis by pulsed-field gel electrophoresis followed by Southern hybridization. The assay performed with mouse DNA in a similar manner as it performs with human DNA. Preliminary linkage mapping suggests a gene influencing telomere length on the X chromosome. This assay will aid in the study of telomere function and importance in diseases associated with aging and cancer formation.

Abbreviations: ATLR, average telomere length ratio; PCR, polymerase chain reaction; PFGE, pulsed field gel electrophoresis; TRF, terminal restriction fragment

Telomeres are oligonucleotide repeats found at the end of eukaryotic chromosomes. In mammals they consist of the repeat sequence TTAGGG. They serve to cap the ends of chromosomes and to protect these ends from degradation and to prevent chromosomal end to end fusions and genomic instability.[24,28] Telomere length has been implicated in cell senescence, as a mitotic clock for aging, and as a factor in tumorigenesis.[2,21,34]

Telomere lengths are known to vary between mammalian species, between animals of a particular species, and between the cells in the various tissues and organs of an individual animal. Genotype, cell type, and cell replicative history all are known to influence telomere length.[5,29,32] Human telomeres are relatively short, with terminal restriction fragment lengths of 10 to 15 kb,[17] whereas in the laboratory mouse, *Mus musculus*, telomeres are relatively long (>20 kb). Interestingly a wild-derived mouse species, *M. spretus*, displays shorter telomeres, closer in length to the telomeres found in human cells.[8,19,24,25,32,41] Yet the underlying genetic mechanisms that control telomere length in mammals have not been well characterized.[40]

Average telomere length usually is measured by performing mean terminal restriction fragment (TRF) analysis by Southern blotting. This method is time-consuming and requires a relatively large amount of DNA for analysis. Quantitative fluorescence in situ hybridization may also be used to determine average telomere length and to measure the telomere length of individual chromosomes. However, this method is technically challenging, expensive, and time-consuming. Therefore, a specific set of primers and a quantitative PCR method was developed to measure relative telomere lengths in humans. Specially designed primers were required because of the propensity of primers directed toward repetitive DNA sequences to form primer dimer-derived products. Here we adapted this method for use in the mouse. This assay provided a simple, inexpensive, and rapid way to quantify telomere lengths.[6] We then used the quantitative PCR assay to score genotypes of an interspecific backcross DNA panel to perform a preliminary linkage analysis to map genes involved in telomere length regulation in the mouse.

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Materials and Methods

DNA sources. DNA used for assay development in this study was extracted from mouse tissues obtained through a tissue sharing program operated by the Comparative Medicine Program at Texas A&M University. These mice were housed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, and were maintained on animal use protocols approved by the University Laboratory Animal Care and Use Committee at Texas A&M University. Bovine and hamster DNA were used to evaluate the amplification of the telomere primers and as negative controls for gradient PCR experiments. Bovine DNA was extracted from the JEW38 cell line and hamster DNA was extracted from the A23 cell line; both of these cell lines are maintained in the Womack laboratory for experimental use. Phenol extraction with ethanol precipitation[31] was used to extract DNA from the tissue samples and cell lines. Mouse DNA used for the telomere length comparisons and mapping was purchased from The Jackson Laboratory (Bar Harbor, ME).

Standard PCR. All primers were evaluated using standard PCR techniques[26] and gel electrophoresis with 2% agarose containing ethidium bromide. A graduated thermal cycler was used to optimize annealing temperatures. Each reaction contained 1 µl 10× PCR buffer with 15 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.2 mM each dNTP, 0.5 U Amplitaq Gold (Applied Biosystems), 250 nM each of the forward and reverse primers, 50 ng genomic DNA, and enough double-distilled H_2O to yield a 10-µl reaction. Thermal cycler reaction conditions were set at 94 °C for

10 min followed by 35 cycles of 94 °C for 30 s, 56 °C (telomere reactions) or 52 °C (36B4 control; see following section) annealing for 30 s, and extension at 72 °C for 30 s, with final extension for 5 min at 72 °C.

Real-time polymerase chain assay. Average telomere length was measured from total genomic mouse DNA by using a real-time quantitative PCR method previously described.[6] The premise of this assay is to measure an average telomere length ratio by quantifying telomeric DNA with specially designed primer sequences and divide that amount by the quantity of a single-copy gene. Here we followed the same protocol as Cawthon[6] and chose the acidic ribosomal phosphoprotein PO (36B4) gene, which is well-conserved and has been used for gene-dosage studies. Modified primer sequences provided by Richard Cawthon (Eccles Institute of Human Genetics, University of Utah) were used for the telomeric portion of the assay. Forward and reverse telomeric primers were 5′ CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT 3′ and 5′ GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3′ respectively. Primers for the mouse 36B4 gene were generated by Primer Express software (Applied Biosystems, Foster City, CA). Forward and reverse primers for the 36B4 portion of the assay were 5′ ACT GGT CTA GGA CCC GAG AAG 3′ and 5′ TCA ATG GTG CCT CTG GAG ATT 3′, respectively. Each reaction for the telomere portion of the assay included 12.5 µl Syber Green PCR Master Mix (Applied Biosystems), 300 nM each of the forward and reverse primers, 20 ng genomic DNA, and enough double-distilled H_2O to yield a 25-µl reaction. Two 20-ng samples of each DNA were placed in adjacent wells of a 96-well plate. An automated thermocycler (Prism 7000 Sequence Detection System, Applied Biosystems) was used with reaction conditions set at 95 °C for 10 min followed by 30 cycles of data collection at 95 °C for 15 s and a 56 °C anneal–extend step for 1 min. Master mix concentrations for the 36B4 portion contained 12.5 µl Syber Green PCR Master Mix (Applied Biosystems), 300 nM forward primer, 500 nM reverse primer, and enough double-distilled H_2O to yield a 25-µl reaction. The Prism 7000 reaction conditions were set at 95 °C for 10 min followed by 35 cycles of data collection at 95 °C for 15 s, with 52 °C annealing for 20 s, followed by extension at 72 °C for 30 s. The 36B4 portion of the assay was performed on two 20-ng samples of DNA from each animal, placed in adjacent wells. To serve as a reference for standard curve calculation, an individual sample of mouse DNA was serially diluted over a 24-fold range for the telomere PCR, from 3.75 to 90 ng per well, and over a 16-fold range, 3.75 to 60 ng per well, for the 36B4 portion of the assay.

Real-time PCR results were exported to an Excel (Microsoft, Redmond, WA) spreadsheet for analysis. Standard curves were generated using the user's manual for the Applied Biosystems Prism 7700 Sequence Detection System[38] according to the relative standard curve protocol. Input amounts were calculated for each sample by using the same protocol. The relative input amount of the telomere PCR then was divided by the relative input amount of the 36B4 PCR of the same sample. Real-time PCR was performed a minimum of 3 times for each sample, and the ratio of telomere:36B4 was calculated. The average of these ratios was reported as the average telomere length ratio (ATLR).

Pulsed field gel electrophoresis. Pulsed field gel electrophoresis (PFGE) was performed on 2 to 5 µg genomic DNA from C57BL/6J and SPRET/Ei mice according to a standard protocol.[12] Briefly, DNA was digested overnight with *Tru*1I (Fermentas, Hannover, MD) and then separated with a PFGE apparatus (CHEF Mapper, Bio-Rad, Hercules, CA) at conditions for separating 4- to 60-kb DNA in 0.5% agarose gels. Sizing standards included 8- to 48-kb standards (Bio-Rad), Low-Range PFG marker, and 1-kb DNA ladder (Invitrogen, Carlsbad, CA). After PFGE, DNA was transferred to a nylon membrane by the downward capillary transfer method of Southern blotting.[3] After DNA transfer, the membrane was hybridized overnight with ^{32}P-labeled oligonucleotide probe ($[TTAGGG]_8$) as previously described.[4] After hybridization, the membrane was washed twice in 2× SSC, 0.1% SDS for 10 min each wash followed by 2 washes in 0.2× SSC, 0.1% SDS for 10 min each wash. Blots then were visualized using a phosphor screen and a phosphoimager (Storm Phosphoimager, Molecular Dynamics, Sunnyvale, CA).

Genetic mapping. DNA was obtained from the Backcross DNA Panel Mapping Resource BSS panel (The Jackson Laboratory). The BSS panel contains DNA samples from 94 mice from a series of backcrosses between (C57BL/6JEi ×SPRET/Ei) F_1 females by SPRET/Ei males.[37] This panel has been typed for numerous markers and allows linkage analysis to be performed according to segregation of a locus influencing telomere length. The C57BL/6J strain is known to have relatively long telomeres, whereas the SPRET/Ei strain has shorter telomeres. The 94 DNA samples from the BSS panel were analyzed using the real-time PCR assay, and the ATLRs for each sample were calculated. By using the calculated ATLR, each sample was scored as either S (ATLR < 1) for the Spretus parental genotype or B (ATLR ≥ 1) for the C57BL/6J parental genotype. The genotypes of the 94 samples were submitted to The Jackson Laboratory, and a linkage analysis was performed using the database of previously typed markers.

Statistical methods. Statistical calculations were performed with SPSS for Windows (SPSS, Chicago, IL) software program. The sample means of the 2 groups of different mouse species were compared using an independent samples *t* test with equal variances not assumed and were considered to be statistically different at $P \leq 0.05$. A correlation coefficient was calculated between the ATLR and the mean TRF data, and the equation for the linear regression line that best fit the data was added. Correlation coefficients were calculated for each of the standard curves as they were generated in the Excel spreadsheet.

Results

Standard PCR. Repetitive elements of DNA have been historically difficult to amplify with PCR techniques. This difficulty is due largely to the predisposition of primers designed for these elements to form primer dimer-derived products and not true amplification products of the desired sequence. Special primers were designed to overcome this limitation for human DNA,[6] and theoretically these should work for the DNA of any mammalian species. However, there are varying amounts of interstitial telomeric repeats located throughout the genomes of various species.[5,41] To address these concerns, mouse (C57BL/6J and SPRET/Ei), bovine (JEW38 cell line), and hamster (A23 cell line) DNA were used to evaluate the use of these primers in species other than humans. Primers were evaluated with no genomic template present and with DNA from *E. coli* to address the primer-dimer product issue. Mammalian DNA of all species tested produced the expected pattern of results, with the majority of products in the 79-bp range. No PCR products were noted when the genomic DNA template was omitted or when *E. coli* DNA was substituted in the reaction (Figure 1).

Real-time PCR assay. Two interfertile mouse species, *M. mus-*

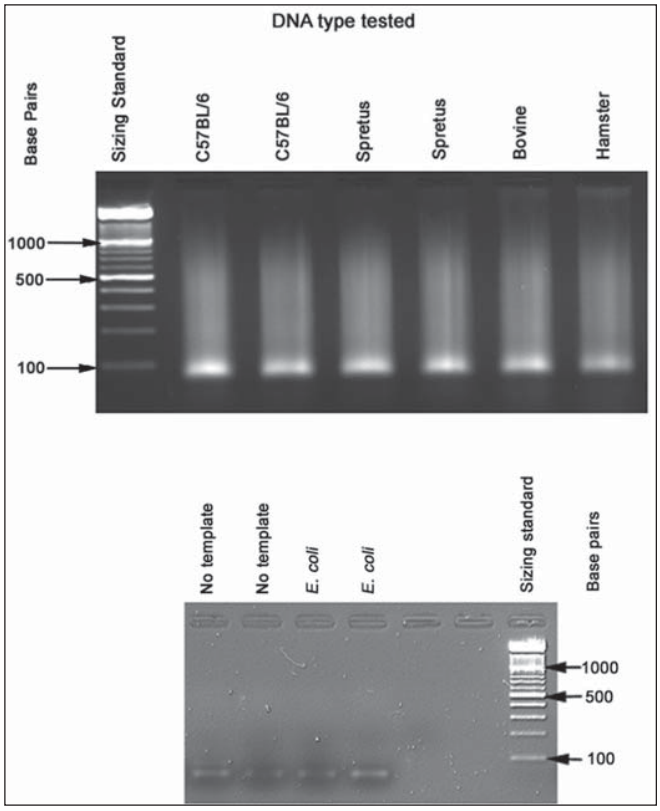

Figure 1. Agarose gel electrophoresis of telomeric PCR products. Various samples of DNA are shown. The majority of products migrate to the expected 79-bp region when mammalian genomic DNA is present. No PCR products are seen when genomic DNA is omitted or when bacterial DNA is included.

culus and *M. spretus*, were used to evaluate the real-time PCR method. These 2 species express differing telomere length phenotypes. *M. musculus* animals have long telomeres with repeats of >20 kb, and *M. spretus* mice have short telomeres (similar to those in humans) with 5- to 10-kb repeats. Samples of DNA from 5 mice of each species were analyzed by real-time PCR assay. Correlation coefficients for standard curves were similar to those previously reported (Figure 2). Sample ATLRs were calculated and fell within expected ranges for each mouse species (Table 1). The average standard deviation for the ATLRs, 7.1%, was similar to the standard deviation reported by Cawthon.[6] The mean ATLRs for the 2 groups were compared and found to be statistically different (Figure 3).

PFGE. To verify the results from the real-time assay, terminal restriction fragments of DNA from both mouse species were analyzed by PFGE followed by Southern blotting. Mean TRF sizes were observed to be in the expected ranges for both species (Figure 4). Correlation between the mean TRF and ATLRs was similar to results previously reported (Figure 5).

Genetic mapping. Our analysis revealed 52 B genotypes and 42 S genotypes across the BSS panel. The best fit to the backcross panel database was on the distal X chromosome, although 21 misfit scores prohibited assignment to a specific locus. Our best interpretation of these data is a locus with a major effect on telomere length on the distal X chromosome with modifying effect from an undefined locus elsewhere in the genome.

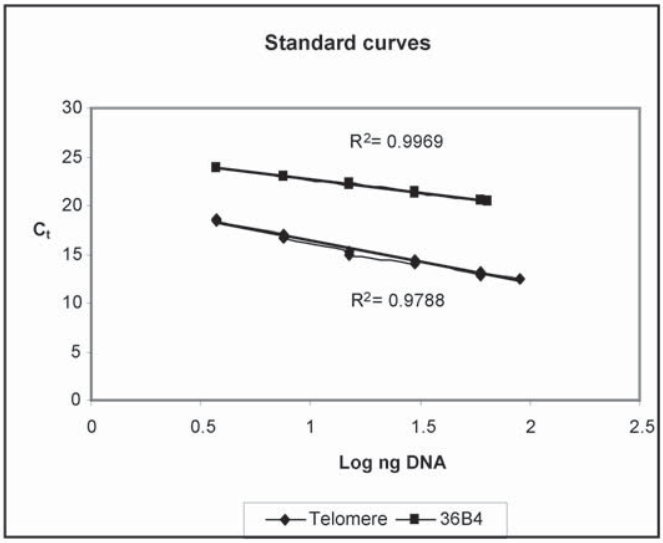

Figure 2. Standard curves used to calculate relative DNA concentrations for the real-time PCR. C_t is the fractional number of PCR cycles at which enough fluorescent product has been accumulated in order to cross a set threshold of magnitude. The correlation coefficients depicted were similar to those previously reported for this assay. Diamonds, telomere; squares, 36B4 control.

Table 1. Average telomere length ratio (ATLR) and standard deviation calculated for 5 samples from each mouse species

Species	Mouse no.	ATLR	Standard deviation
C57BL/6J	P36035	1.424224	0.060836
C57BL/6J	P36036	1.563648	0.181006
C57BL/6J	P36037	2.014800	0.135288
C57BL/6J	P36038	2.197379	0.065451
C57BL/6J	P36039	2.536917	0.057753
M. spretus	P33911	0.403874	0.069840
M. spretus	P35415	0.657047	0.089712
M. spretus	P35616	0.344427	0.052409
M. spretus	P35618	0.588413	0.088878
M. spretus	P33930	0.538101	0.068919

Average standard deviation was similar to that previously reported for this assay.

Discussion

Telomeres tend to lose base sequences each time a cell divides because of the end-replication problem, which is due to the inability of the DNA replication machinery to synthesize the very end of the 3′ strand.[24,27] This problem can be overcome by expression of the enzyme telomerase, which can synthesize de novo telomere repeats. Telomere length is maintained by the enzyme telomerase, which consists of a RNA component and a reverse transcriptase denoted as Tert.[14] The mouse, even with relatively long telomeres, often expresses telomerase in adult somatic tissues.[7,8,32] Human cells by contrast, even though they have relatively short telomeres, do not express telomerase in most somatic cells. In addition to telomerase, telomeres are associated with several proteins that regulate their function. These include the telomere binding proteins TRF1 and TRF2, TANK1 and TANK 2, and TIN2. The ends of mammalian chromosomes in conjunction with these associated proteins are capped by large lasso-shaped structures, referred to as t loops.[5,16] These loops are thought to protect the end of the telomere from degradation; they may also be involved in the interaction with telomerase.

Although these proteins and their functions in relationship to

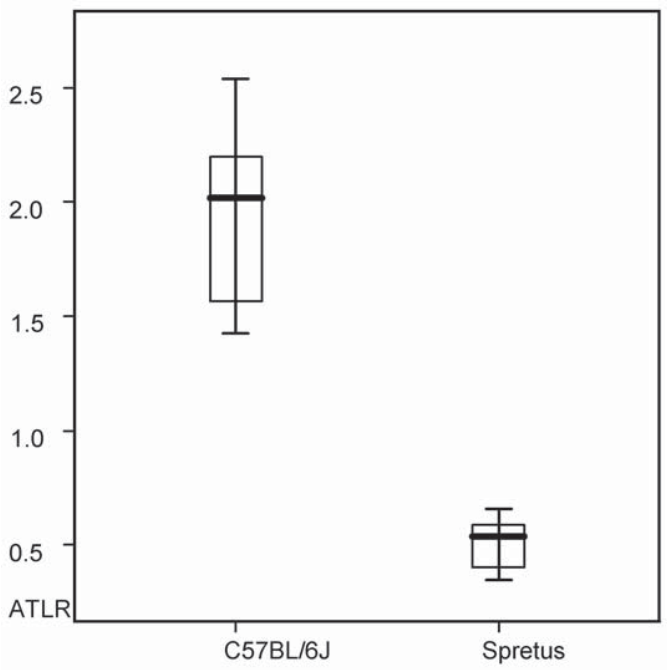

Figure 3. Comparison of the average telomere length ratios (mean ± standard error) between 2 mouse species. The C57BL6/J group, which has longer telomeres, varies over a wider range, as expected. The means of the 2 groups were compared using an independent samples *t* test with equal variances not assumed and were found to be significantly different ($P = 0.002$).

telomere function have been studied widely, the mechanisms underlying the genetic regulation of overall telomere length are not well characterized. Many studies have been done using plants and lower organisms.[13,33,35,36] These studies in lower eukaryotes have shown that telomerase is only part of the picture. Telomeres are maintained within species-specific lengths through interactions between the telomerase enzyme and the telomere-associated proteins.[5,15,24,30]

Little is known about the genetic and molecular bases for the differences in telomere biology in mammals.[24] Few studies have been reported regarding the genetic regulation of mammalian telomere length.[40] Telomere length is known to vary between species and even between individuals of a particular species. Even within an individual, telomere length varies considerably between the different organs.[8,15,23,41] However, this variation appears to occur within a preset range. The genes controlling this range appear to be independent of the genes coding for the proteins known to be associated with telomeres.[40] Zhu and others estimated that 2 to 3 unlinked loci exert influence on telomere length between *M. musculus* and *M. spretus*.[40] The major influence was mapped to a locus on distal chromosome 2, and another possible locus was on the X chromosome. However, neither the exact location of this locus nor candidate genes for this influence were found.

Ding and others[9] subsequently located on distal chromosome 2 the gene *Rtel* which is thought to be responsible for the major differences in telomere phenotypes between *M. musculus* and *M. spretus*. This gene is the mouse homolog of a human novel helicase-like gene and encodes a helicase-like protein.[9] This gene was shown to be necessary for development, as null mutants died before embryonic day 11.5. Even though *Rtel* was shown to modulate telomere length significantly, it does not appear to account

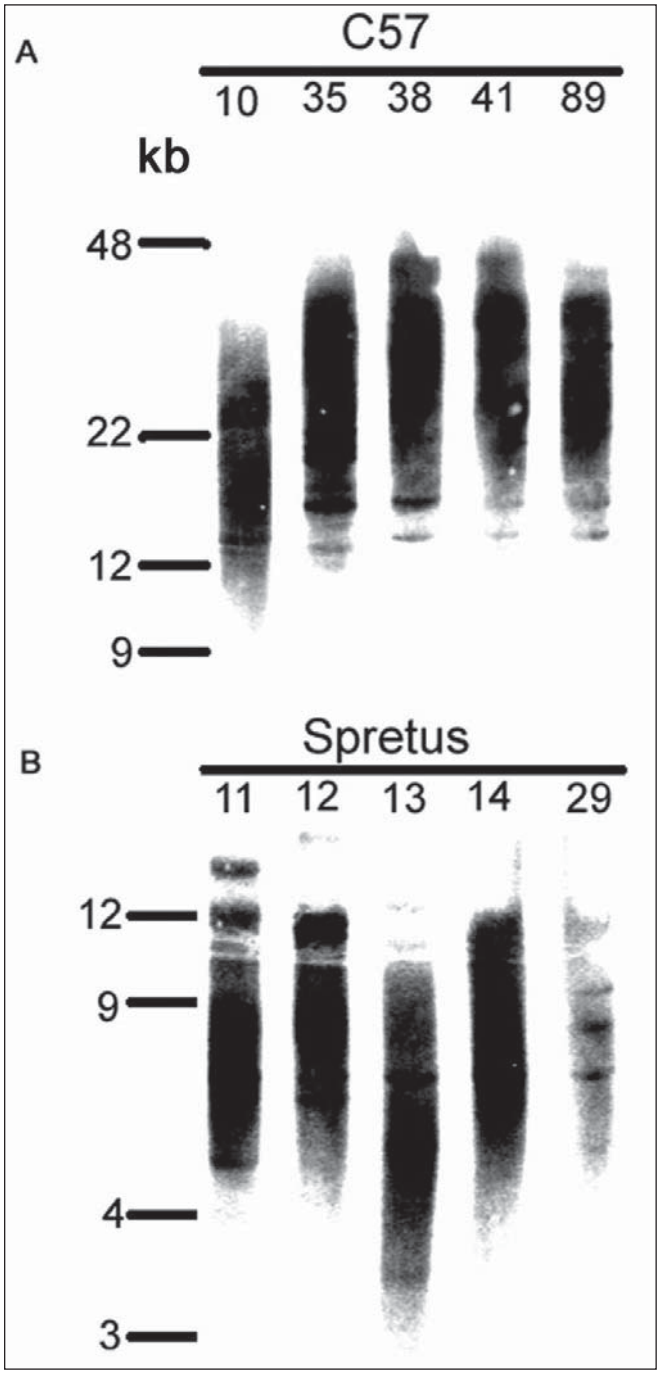

Figure 4. Pulsed field gel electrophoresis and hybridization of 10 individual mouse DNA samples (animal identification is given at the top of each lane), demonstrating the difference between telomere lengths of the C57BL/6J and *M. spretus* mice.

for the total difference between the 2 species.[9] More studies are needed to clarify the function of this gene and how it regulates telomere length in mice.

As cells divide, telomeres shorten each time due to the end-replication problem mentioned previously. This shortening of the telomeres limits the proliferative capacity of cells in culture as they reach a certain number of divisions, called the Hayflick limit.[18,21] As 1 or more telomeres in a cell reach a critical length,

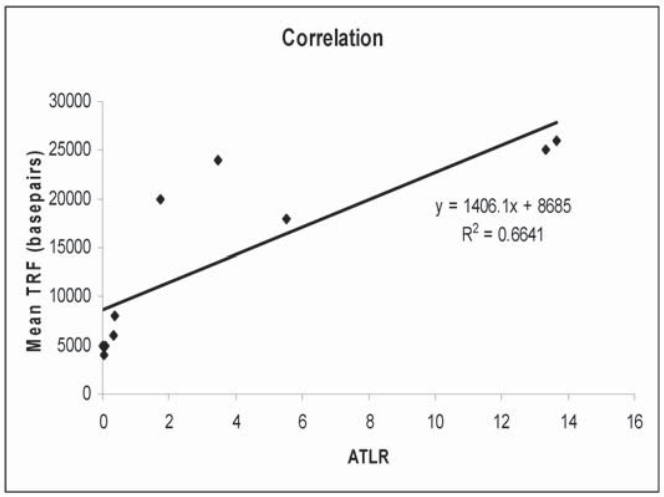

Figure 5. Correlation of average telomere length ratios (ATLR) and mean terminal restriction fragment (TRF) lengths determined by pulsed field gel electrophoresis and Southern blot analysis. The linear regression line that best fit the data ($P = 0.004$) is included.

those chromosomes affected become unstable and prone to breaks and end-to-end fusions. Under normal circumstances, cells stop dividing before genomic instability occurs and enter a state of senescence. This state of senescence is thought to be a contributing factor to the aging process.[20,21] The amount of nucleotide repeats lost indicates the number of times a cell has undergone division. This correlation led to the hypothesis that telomeres function as a mitotic clock in vivo.[5,20]

Other than senescence, death and genomic instability are 2 possible outcomes to telomere dysfunction. The cell's attempt to repair genomic instability leads to a predisposition for neoplastic transformation.[39] Therefore, senescence and cell death (apoptosis) may be thought of as tumor suppressive elements due to their prevention of the survival of cells that have developed genomic instability.[5] However, mutations that cause defects in the genes that are required for senescence accumulate throughout life.[5,10,11] The next line of defense, cell death, requires a functional p53 pathway. Mutational defects in the genes required for a functional p53 pathway also accumulate over time.[5,22] Defects in the senescence and cell death pathways allow cells with genomic instability to survive. The cells in turn attempt to repair the genomic instability by stabilizing their telomeres. This response puts them at great risk for tumor formation. One method for stabilizing telomeres is increased expression of telomerase. Frequently telomerase is activated in tumor cells, suggesting that it may help ensure their survival.[5] Telomerase deficiency favors the development of carcinomas, epithelial tumors, which are common age-associated cancers in humans.[1]

The study of telomeres has value with regard to both human and animal health. Telomere biology and function have importance for understanding normal cellular biology as well as aging and cancer. The wide variance of telomere length and the biologic controls of that length are central to the understanding of their biology. Measurement of telomere length has, in the past, required time-consuming or expensive, technically challenging procedures. The repetitive DNA sequences of telomeres have been one of the limiting factors with regard to PCR analysis. The primers designed for the human telomere assay proved to amplify other mammalian DNAs effectively. This success is to be expected because all mammalian telomeres are composed of the same repetitive sequence. With the initial step of designing a new set of primers followed by optimizing the real-time PCR reaction, we adapted the human assay for use in mice.

Results were in line with the expected values for each mouse species. The ATLRs of *M. musculus* animals were significantly larger ($P < 0.05$) than those of *M. spretus* mice. These values correlated well with the TRF analysis of each species: the PCR measures of the longer telomere set varied to a greater degree than the short set. These results are similar to those from other methods of telomere quantification, which also vary more as the telomere length increases. The assay appears to work for mice as it does in humans.[6]

Initial mapping results point to a locus on the distal X chromosome that influences telomere length. Unfortunately a tightly linked marker could not be resolved. The DNA used was extracted from whole mouse tissues. Because telomeres are known to vary widely from one tissue to another,[5,41] this method of DNA preparation may have obscured the results somewhat. Single tissue-type DNA would most likely improve the power of the assay and offer better resolution.

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