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Developmental and tissue-specific regulation of mouse telomerase and telomere length

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ABSTRACT **Telomere shortening and telomerase activation in human somatic cells have been implicated in cell immortalization and cellular senescence. To further study the role of telomerase in immortalization, we assayed telomere length and telomerase activity in primary mouse fibroblasts, in spontaneously immortalized cell clones, and in mouse tissues. In the primary cell cultures, telomere length decreased with increased cell doublings and telomerase activity was not detected. In contrast, in spontaneously immortalized clones, telomeres were maintained at a stable length and telomerase activity was present. To determine if telomere shortening occurs *in vivo*, we assayed for telomerase and telomere length in tissues from mice of different ages. Telomere length was similar among different tissues within a newborn mouse, whereas telomere length differed between tissues in an adult mouse. These findings suggest that there is tissue-specific regulation of mouse telomerase during development and aging *in vivo*. In contrast to human tissues, most mouse tissues had active telomerase. The presence of telomerase in these tissues may reflect the ease of immortalization of primary mouse cells relative to human cells in culture.**

Accurate chromosome transmission during cell division is essential for cell survival. When chromosome integrity is lost, cell death or unregulated growth may occur. Telomeres stabilize natural chromosome ends and inhibit aberrant fusions and rearrangements that occur on broken chromosomes. Telomere sequences consist of short G-rich repeats that are highly conserved in eukaryotes, although the exact repeat sequence is species-specific (reviewed in ref. 1). Telomere repeats are synthesized *de novo* onto chromosome ends by the enzyme telomerase (2, 3). This addition compensates for the inability of conventional DNA polymerases to completely replicate DNA ends (refs. 4 and 5; reviewed in ref. 1). Telomerase is a ribonucleoprotein enzyme; the essential RNA component provides the template for telomere repeat synthesis. Telomerase activity has been isolated from the ciliates *Tetrahymena*, *Euplotes*, and *Oxytricha* as well as from immortalized human and mouse cells lines (2, 6–9).

Telomere length regulation in mammalian cells is complex. Human sperm telomeres are longer than those in somatic cells (10). Somatic cell telomeres shorten with each cell division *in vitro* and telomere length correlates with the life-span of fibroblasts in culture. Cells with longer telomeres undergo more divisions than those with short telomeres (11–13). Telomere shortening is also observed *in vivo*. In a variety of tissues telomeres are shorter in older individuals than in younger individuals (13–16).

Telomere length also differs between normal somatic cells and cancer cells (14, 17, 18). The average telomere length in colorectal carcinoma tissue and ovarian carcinoma is shorter than that in the corresponding normal tissue from the same individual (14, 18). To determine what occurs when telomeres from cancer cells become very short, telomere length and telomerase activity were assayed in primary human cells transfected with simian virus 40 large tumor (SV40 T) antigen (19). The expression of SV40 T antigen in primary human cells allows growth for an extended number of cell doublings, and the culture enters "crisis" where most of the cells die; however, at a low frequency, some cells will become immortalized (20). During the extended life-span, telomeres shortened and telomerase activity was not detected. After crisis, the cells that survived had stabilized telomere length and telomerase activity was detected (19). This suggests that telomerase activity in the immortal cells allows maintenance of telomere length. Since telomeres are required for chromosomes stability, those cells that express or reactivate telomerase may be selected for at crisis. Thus telomerase activity may be required for the growth of immortalized human cells (19).

To develop a mouse model to study telomere length and telomerase we initially characterized mouse telomerase activity (9). Detection of changes in telomere length is difficult in most inbred mouse strains because they have telomere restriction fragment lengths of up to several hundred kilobase pairs (21, 22). We therefore studied telomere length regulation *in vivo* and *in vitro* in *Mus spretus*. We chose *M. spretus* because its has telomere restriction fragment lengths similar to those of humans (22) and are thus easily resolved in agarose gels. In contrast to primary human cells, mouse cells will spontaneously immortalize in culture (23). Thus telomere length and telomerase activity during immortalization can be assayed without introduction of exogenous agents. In one mouse primary culture where telomeres shortened, telomerase activity was not detectable. After an apparent crisis, and telomere length stabilization, telomerase activity was detected. Although two primary fibroblast cultures lacked detectable telomerase, many mouse tissues had high levels of telomerase activity. The presence of telomerase activity in normal mouse cells may play a role in the ease of immortalization of these cells in culture.

MATERIALS AND METHODS

Mice. BALB/c, W/W^V, and breeding pairs of *M. spretus* were obtained from The Jackson Laboratory. Familial samples of *M. spretus* were obtained from Verne Chapman (Roswell Park Cancer Institute, Buffalo, NY).

Primary Fibroblasts. Skin explants from newborn mice (1–2 mm^2 pieces) were excised and placed dermis side down in a 25-cm^2 T flask in 0.5 ml of Dulbecco's modified Eagle's medium and 10% of fetal bovine serum (plus penicillin and streptomycin). The explants were allowed to adhere for 15 min; then the flask was inverted and 4.5 ml of medium was added to the opposite face of the flask. The cells were grown inverted overnight, the flask was then inverted, and the cultures were

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Abbreviations: PD, population doubling; MPD, mean PDs; TRF, terminal restriction fragment.

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refed every third day. When fibroblasts had migrated to cover the flask (typically at 10–14 days), the explant was removed and the cells were trypsinized and counted. The initial population doubling (PD) level was calculated by assuming an initial outgrowth of 200 cells per explant (24). At each passage, fibroblasts were plated at 5×10^5 cells per 10-cm dish in 8 ml of medium, and the remaining cells were frozen in medium plus 10% dimethyl sulfoxide. The cells were passaged every 3–4 days and the cell number and PD were determined for each passage.

DNA Isolation. Genomic DNA was prepared from cultured cells by standard protocols. Briefly, cells were washed in phosphate buffered saline (PBS), resuspended in TE at 5×10^7 cells per ml, and added to 10 vol of a buffer containing 10 mM Tris, 100 mM EDTA (pH 8), 100 M NaCl, 0.5% SDS, and 100 μg of proteinase K per ml. The mixture was incubated at least 3 hr at 50°C and then cooled to 37°C and incubated with 100 μg of DNase-free RNase per ml for 1 hr. The samples were extracted with phenol and precipitated with 0.2 M NaCl and ethanol, and the DNA was dissolved in TE. For tissue samples, the tissues were placed in 10 ml of the above buffer per g of tissue and Dounce homogenized to disrupt the tissue. The extract was then placed at 50°C and treated as above.

Gel Hybridization and Terminal Restriction Fragment (TRF) Length Analysis. Total genomic DNA was digested for 16 hr with *Hin*fI at 37°C. The DNA was extracted with phenol, precipitated with ethanol, and dissolved in TE, and equal aliquots (2-μg samples) were separated in 0.5% agarose gels. The gels were dried for 1.5 hr at 60°C as described (25), denatured for 30 min in 0.5 M NaOH/1.5 M NaCl, and neutralized for 30 min in 0.5 M Tris, pH 7/1.5 M NaCl. After rinsing briefly in water, the gels were hybridized for 16 hr in 0.5 M Na_2HPO_4/1 mM EDTA/7% SDS/1% bovine serum albumin at 37°C. The gels were washed with 2 liters of 0.5× SSC and 0.1% SDS at 37°C before autoradiography. To determine TRF length, a Fuji BAS2000 PhosphorImager was used to quantitate the radioactive signal in each of the lanes. Each lane was graphically divided into quadrants and the amount of radioactivity in each was determined. The molecular weight range of each quadrant was calculated by plotting the position of the radioactive markers versus the logarithm of their molecular weights. The mean length of the telomere restriction fragments was then calculated as described (13).

Extracts and Telomerase Assays. S-100 extracts from FM3A and cultured fibroblast cells were prepared as described (9, 19). To prepare mouse tissue S-100 extracts, freshly dissected tissues were rinsed in cold PBS, minced, and weighed. The minced tissue was placed in 0.75 vol of cold "hypo" buffer per g of tissue for 10 min on ice and disrupted by Dounce homogenization. The homogenate was then treated as described (9, 19). All S-100 extracts were fractionated on DEAE-agarose columns and eluted with 0.2 M NaCl (9). For some extracts, the 0.2 M DEAE fractions were then passed over a phenyl Sepharose column. Conventional telomerase activity was assayed as described (9). The PCR-based assay (TRAP assay) was performed as described (26).

RESULTS

Telomeres Shorten During Growth of Mouse Fibroblasts in Culture. Telomeres in primary human cells shorten with age *in vitro* and *in vivo* (11, 13, 14, 16). To determine whether telomere shortening occurs in mice, we assayed telomere length in primary mouse fibroblast cultures. Primary *M. spretus* fibroblasts were obtained from skin explants taken from newborn mice using conditions similar to those for culturing human skin fibroblasts (see *Materials and Methods*). We began with three independent skin explants from three newborn mice, and all three sets led to actively growing cultures. The fibroblast cultures were passaged every 3–4 days and the growth kinetics (Fig. 1*B*) and morphology of the cells were monitored. Genomic DNA was isolated at regular intervals, digested with *Hin*fI, and separated on a 0.5% agarose gel. To avoid loss of high molecular weight DNA during Southern transfer, the gel was dried and hybridized directly with the telomeric d(TTAGGG)$_3$ probe (Fig. 1*A*) (25). This probe

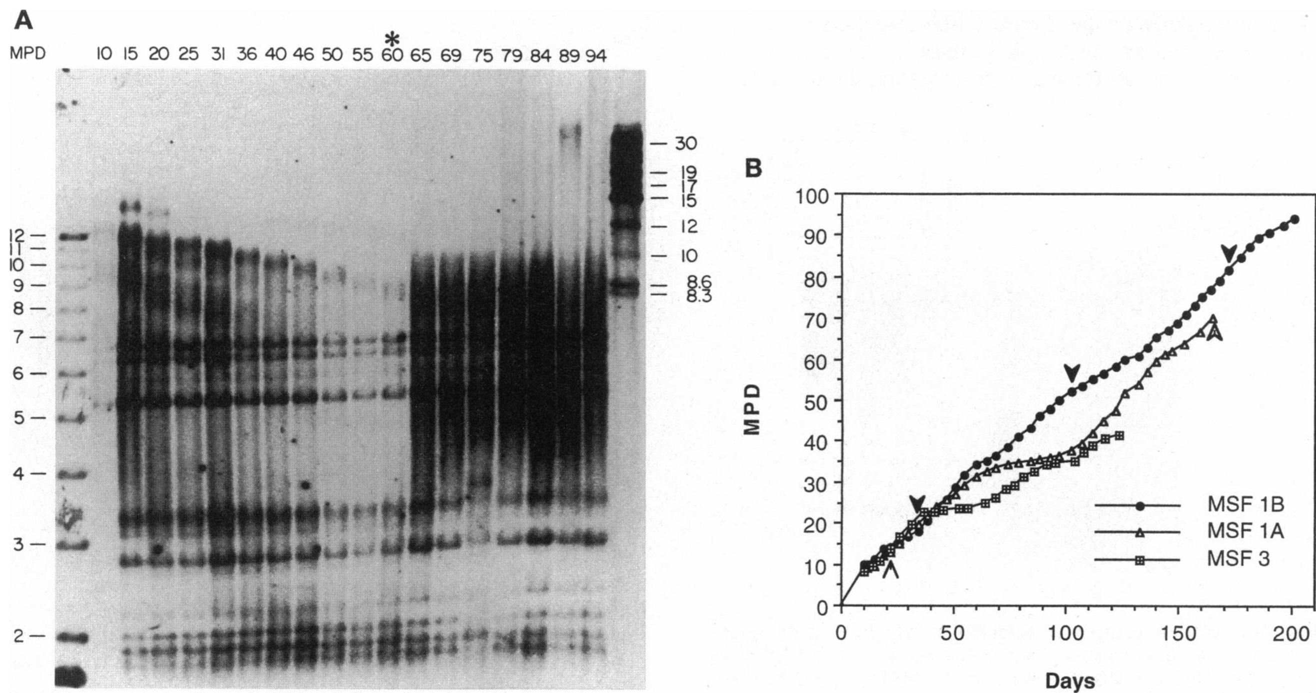

FIG. 1. (*A*) Telomere shortening in cultured *M. spretus* fibroblasts. Telomere length was determined at different PD levels from *M. spretus* fibroblast culture MSF1B. The asterisk marks the PD level where cell morphology changes were first evident. (*B*) Graph of the growth rate of three different mouse fibroblast cultures. The estimated number of mean PDs (MDP) are plotted versus the number of days in culture. The arrowheads mark the points at which telomerase was assayed.

hybridizes to restriction fragments containing internal sequence repeats in the mouse genome as well as to the telomeric fragments (27). The telomeric bands were identified by their sensitivity to BAL-31 exonuclease digestion (data not shown). We refer to the telomeric bands as TRFs because they comprise telomere adjacent DNA sequence in addition to the telomeric d(TTAGGG) repeats.

The TRF length decreased by 75 ± 9 bp per cell generation *in vitro* up to 60 doublings in MSF1B (Fig. 1*A*). After PD 60 the TRF length stabilized and remained constant as the population continued growing. Similar results were obtained with the MSF1A cell culture (data not shown), although the growth kinetics and time of immortalization and subsequent TRF length stabilization differed between the two cultures. We were not able to measure telomere length over time in the third culture (MSF3) due to contamination in the culture. The shortening and subsequent stabilization of telomere length in MSF cells were similar to that seen pre- and postcrisis during immortalization of human cells (19, 28, 29). In contrast to human cells, the precise point of immortalization was difficult to determine in the mouse cell cultures. In culture 1A the growth rate of the culture slowed between PD 30 and PD 35; however, in culture 1B there was little change in growth rate. The cells in culture 1B initially had a uniform fibroblast morphology; however, at PD 60 a morphological change was apparent in some cells and by PD 65 the majority of the cells were enlarged and non-uniform in size (data not shown).

Telomerase Activity Detected after Immortalization. We assayed cell extracts from MSF1A and MSF1B for telomerase activity at different PD levels using the conventional telomerase assay (data not shown) and the PCR-based "TRAP" assay (26) (Fig. 2). Telomerase was not detected early (PD 18) in MSF1B when telomere lengths were shortening; at PD 52 some telomerase was detected, and activity was detected at the latest time point, PD 80. Activity was also detected at late points in MSF1A and at one early point in MSF3 (see below). Although the conventional assay using mouse telomerase generates one predominant product (9), the TRAP assay results in a ladder of products with a 6-base periodicity. The ladder is the result of greater processivity of the mouse telomerase under the PCR conditions as well as the length increase of the repeated sequences due to out-of-frame primer annealing during PCR (L. Mantell, K.R.P., N. Kim, K. Buchkovich, and C.W.G. unpublished data). The lack of a distinct crisis in the mouse cultures makes it difficult to pinpoint the exact point of immortalization. However, the presence of telomerase in the late stage but not early MSF1B culture, together with the telomere length data (Fig. 1), suggest that telomerase-positive cells were selected for in the growth of the culture.

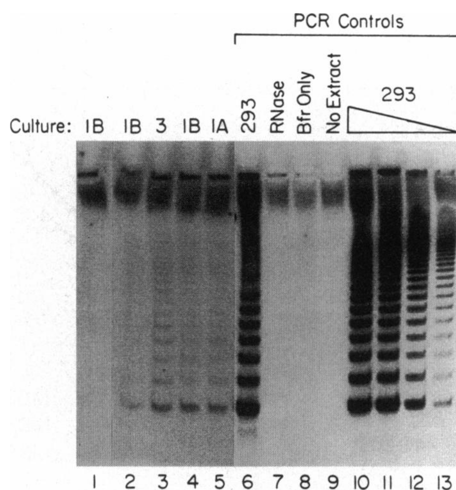

FIG. 2. Telomerase activity detected at late PDs in *M. spretus* fibroblasts. Telomerase activity was assayed using the TRAP assay. Lane 1, MSF1B cells at PD 18 (early); lane 2, MSF1B at PD 52 (mid); lane 5, MSF1B cells at PD 80 (late); lane 3, MSF3 cells (early) (PD 20); lane 4, MSF1A cells at PD 70 (late). Lanes 6–13, controls for the PCR. Lane 6, 293 cell extract (positive control); lane 7, RNase-treated 293 cell extract; lanes 8 and 9, no extract controls; lanes 10–13, 5-fold serial dilutions of the 293 cell extract.

Developmental and Tissue-Specific Telomere Length Regulation. To determine whether telomere shortening occurs in mouse tissues *in vivo*, we examined telomere length in *M. spretus* tissues at different ages (Fig. 3). As was found in humans, TRF length in a given tissue differed between individuals of the same age. For example, the two newborns examined in Fig. 3 are littermates, yet the mean size of the tissue TRFs in Ms1 are longer by several kilobase pairs as compared to the mean TRF lengths in Ms2. We attribute the length differences to the outbred genetic background in these wild-derived mice. We have analyzed liver TRF length in 12 different species of *Mus musculus* and 9 different wild-derived species. The TRFs from all of the *musculus* strains were at limit mobility (>30 kb), while the TRFs of all wild-derived species were resolvable (<25 kb) (data not shown). This suggests that the *musculus* species may have unusually long telomeres among mice. Due to the degree of genetic variation in the population, a large number of *M. spretus* at different ages will be needed to obtain statistically significant data on telomere length changes during aging.

Comparing the relative lengths of TRFs between tissues within individual mice, we found interesting differences. Telomere lengths within the tissues analyzed of any individual newborn mouse were similar, while those within any individual adult mouse (2 months or older) differed between tissues (Fig. 3). The most striking difference lies in the length of the major telomeric band in the testis sample relative to the kidney and brain samples of the same adult mouse. The mean TRF length of the adult testis was quantitated using a PhosphoImager; it was consistently 2–3 kbp longer relative to the kidney and brain TRFs. In addition to longer testis telomeres, telomere length in liver was also 1 kbp longer than the kidney and brain telomeres. This difference in

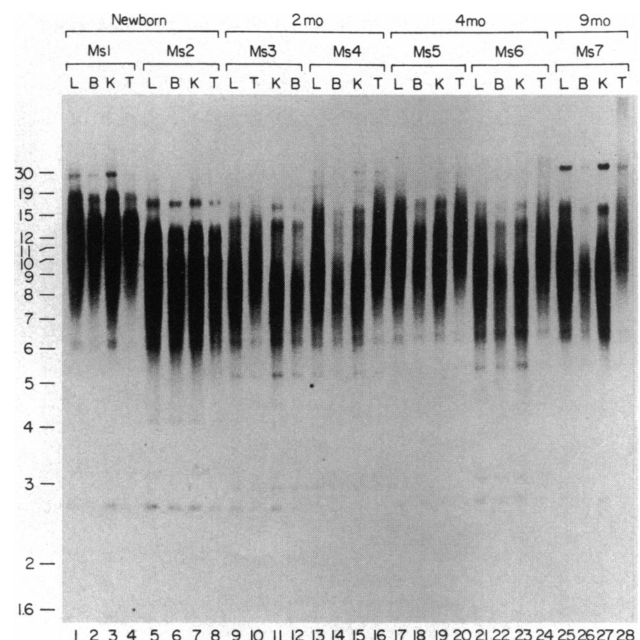

FIG. 3. Tissue telomere lengths change with age. Genomic DNA was isolated from individual male *M. spretus* (Ms) tissues and analyzed as in Fig. 1. Ms1–Ms6 are offspring of Ms7. Ms1 and Ms2, newborn littermates; Ms3 and Ms4, 2-month littermates; Ms5 and Ms6, 4-month littermates; Ms7, 9-month parent. L, liver; B, brain; K, kidney; T, testis.

tissue TRF length within an individual mouse was apparent by 2 months of age and was manifested in all adults up to 9 months, the oldest mouse examined.

Telomerase Activity in Mouse Tissues. The difference in telomere length between tissues of the same adult mouse can be interpreted in several ways. The telomeres in newborn mouse tissues could shorten during postnatal development only in specific tissues while shortening might not occur in other tissues. Alternatively, telomeres in some tissues could become longer during development. Finally, a combination of both processes could be occurring. To determine whether telomerase is active in specific mouse tissues, we assayed extracts from adult mouse brain, liver, kidney, spleen, and testis. No activity was detected in brain. However, readily detectable telomerase activity was observed in extracts from testis and liver, and weaker activity was also detected in kidney and spleen extracts (Fig. 4 and data not shown). The presence or absence of telomerase in these tissue extracts was confirmed using the TRAP assay (Fig. 4*B*) (26). Under the conditions of this PCR-based assay, quantitative differences between tissues cannot be determined, although the qualitative differences such as the presence or absence of a signal are meaningful (N. Kim, K. Buchkovich, L. Mantell, and C.W.G., unpublished data). Telomerase was detected in testes, liver, and kidney from C57BL/6 and BALB/c mice and in the spleen from BALB/c mice (data not shown). Initially all of the telomerase assays were done in these strains of *M. musculus*; however, to confirm these results in a second species, we also assayed tissues from *M. spretus*. Activity was present in both testes and in liver from *M. spretus* (Fig. 4*B*). The presence of detectable telomerase activity in these two tissues correlates with the longer telomere lengths observed in testes and liver in adult mice and suggests an active mechanism for maintaining long telomeres in these tissues.

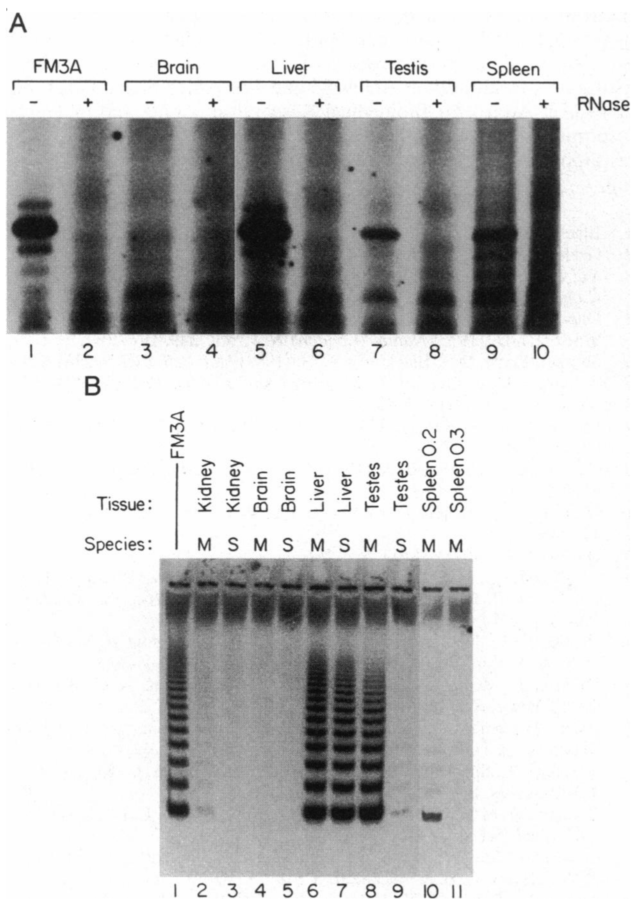

FIG. 4. Telomerase activity in mouse tissues. (*A*) The brain and spleen S-100 extracts were prepared from a pool of four BALB/c mice (7 months); the liver and testis S-100 extracts were obtained from individual BALB/c mice (4 months and 3 months, respectively). All S-100 extracts were fractionated over DEAE-agarose columns. The spleen and testis DEAE fractions were subjected to further fractionation over phenyl Sepharose. An aliquot (40 μl) of each sample was treated with (+) or without (−) RNase and then assayed for telomerase activity using the conventional telomerase assay. Lanes 1 and 2, FM3A DEAE; lanes 3 and 4, brain; lanes 5 and 6, liver; lanes 7 and 8, testis; lanes 9 and 10, spleen. (*B*) Telomerase activity assayed by the TRAP assay. M and S refer to extracts made from *M. musculus* (M) (pooled tissues from 6 animals) or tissue from a single *M. spretus* (S). Lane 1, FM3A DEAE; lanes 2 and 3, kidney; lanes 4 and 5, brain; lanes 6 and 7, liver; lanes 8 and 9, testes; lanes 10 and 11, spleen DEAE fraction (0.2 and 0.3 M NaCl eluates).

To explore the temporal regulation of telomere length changes in testis and liver, telomerase activity was assayed in these tissues at different developmental stages in *M. musculus*. Activity was detected in liver at the earliest point tested (4 weeks) and was present at all subsequent ages tested (data not shown). However, in the testis, telomerase activity was not detected at 4 weeks but was detected at 6 weeks of age and older (Fig. 5). Although a direct comparison cannot be made because the telomere length and telomerase activity were from two different mouse species, it was interesting that the developmental stage at which telomere lengthening was observed is similar to the stage where telomerase was first detected. The first mature sperm are produced about 5–6 weeks after birth, and after 6 weeks of age spermatocytes constitute a significant proportion of the mass of the testis. The longer TRF length and detectable telomerase activity may reflect this increased mass of spermatocytes in the testis after 6 weeks.

To determine whether testis telomerase activity was due to developing sperm or to another cell type within the testis, we utilized a mutant strain of *M. musculus* (W/W^V) that lack primary spermatogonia but otherwise have a structurally normal testis (30). The W/W^V mutation causes sterility in homozygotes by reducing the proliferation of the primordial germ cells and their migration into the genital ridge during development. Heterozygotes have normal germ cell proliferation and migration. Mutant (homozygotes) and normal (heterozygotes) mouse testes were assayed for telomerase activity. No activity was detected in 8-week-old mutant W/W^V testis, whereas in siblings that contain normal testes, telomerase

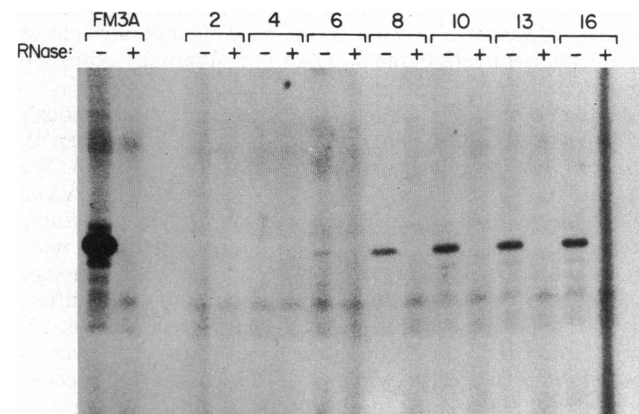

FIG. 5. Testis telomerase activity is developmentally regulated. Testis telomerase activity in mice of different ages. Testis S-100 extracts were prepared from pools of four BALB/c mice from each of the indicated ages and were fractionated over DEAE-agarose and phenyl Sepharose columns. An aliquot (40 μl) of each sample was treated with (+) or without (−) RNase and then assayed for telomerase activity. Lanes 1 and 2, FM3A; lanes 3–16, testis at 2 weeks (lanes 3 and 4), 4 weeks (lanes 5 and 6), 6 weeks (lanes 7 and 8), 8 weeks (lanes 9 and 10), 10 weeks (lanes 11 and 12), 13 weeks (lanes 13 and 14), and 16 weeks (lanes 15 and 16).

activity was detected (data not shown). This suggests that the telomerase activity originates from the progenitor sperm cells and not another cell type within the testis.

DISCUSSION

Telomerase in Mouse Tissues. Studies on human telomere length and telomerase activity have suggested that telomerase activity may be restricted to germ-line cells and cancer tissue. We have used the mouse as a model to study the regulation of telomerase in mammalian development. We were surprised to find that many somatic mouse tissues, unlike human tissues, have significant telomerase activity. The telomerase activity detected in mouse tissues may be due to telomerase expression in all cells or expression in a limited subset of cells, such as stem cells. Our current assays are not able to distinguish telomerase-positive and -negative cells at the individual cell level.

Our data suggest that tissue specific developmental regulation of telomere length may occur in mice. Telomere lengths between tissues in any individual newborn mouse were similar while those in any individual adult mouse differed in size. Long telomere lengths and telomerase activity were detected in the testis about 5–6 weeks after birth. At this time, the testis increases dramatically in size due to the production of the first spermatocytes. Thus the shorter relative TRF length and lack of detectable telomerase activity in immature mouse testis may reflect the relatively low mass of germ cells. The lack of telomerase activity in mature W/W^V mutant mice that lack germ cells indicates that the testis telomerase activity is present in the germ line and not the somatic cells of the testis.

Telomerase in Primary and Spontaneously Immortalized Mouse Fibroblasts. Two cultures of *M. spretus* fibroblasts showed initial telomere shortening and undetectable telomerase followed by telomere length stabilization and detectable telomerase activity. In the MSF1A culture, the detection of telomerase correlated with an improvement in the growth rate of the culture. This growth rate improvement is often referred to as the point of immortalization of fibroblast cultures (23). In the MSF1B culture there was no change in the growth rate; however, a morphological change in the cells was noted between PD 60 and PD 65. In a third culture, MSF3, telomerase was detected at early passage. The population of cells that first grow out of the skin explant may determine whether telomerase is present or absent at early passage levels. The detection of telomerase late in culture growth could result from an activation of telomerase in a subpopulation of cells or the selection for cells in the population that initially expressed telomerase.

M. spretus cells, like many rodent cells, will spontaneously immortalize in culture, although at a lower frequency than *M. musculus* cells (A. Balmain, personal communication). We established two fibroblast cultures from *M. spretus* that lacked detectable telomerase and one culture that had detectable telomerase. Primary mouse embryo fibroblasts from *M. musculus* have detectable telomerase activity at very early passage (K. Buchkovich and C.W.G., unpublished data). The difference between these cells and the *M. spretus* fibroblasts described here may be due to the tissue origin (fetal versus newborn or mixed origin versus skin fibroblasts) or a species-specific difference between *musculus* and *spretus*.

Telomerase Expression in Human Versus Mouse Tissues. Although telomere shortening and subsequent stabilization in primary *M. spretus* fibroblasts is similar to that found in human cells, the presence of telomerase activity in many mouse tissues is very different (26). No detectable telomerase activity was found using the sensitive TRAP assay in >50 samples of human tissue including human liver samples (ref. 26; J. Shay, personal communication). One explanation for this difference could be that mouse cells lack a telomerase regulatory pathway that is present in human cells. Primary human cells rarely, if ever, spontaneously immortalize, while mouse cells can spontaneously immortalize. If, for example, human cells have two separate pathways that regulate telomerase and mouse cells have only one of these pathways, it will take fewer "hits" to inactivate the one mouse regulatory pathway versus the two for human.

The regulation of proliferation is thought to play a role in the protection against carcinogenesis. The requirement for multiple hits for cancer development is reflected in the increased cancer incidence with increased age. Long-lived species therefore may have more control mechanisms to limit cellular proliferation than short-lived species. The inhibition of telomerase activity may be one mechanism used by humans, a long-lived species, to limit replicative potential. Further studies of mouse telomerase should provide a model to explore the role of telomere length and telomerase in cellular senescence and immortalization.

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