Telomere Shortening Accompanies Increased Cell Cycle Activity during Serial Transplantation of Hematopoietic Stem Cells

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Abstract

Reactivation of telomerase and maintenance of telomere length can lead to the prevention of replicative senescence in some human somatic cells grown in vitro. To investigate whether telomere shortening might also play a role in the limitation of hematopoietic stem cell (HSC) division capacity in vivo, we analyzed telomere length during serial transplantation of murine HSCs. Southern blot analysis of telomere length in donor bone marrow cells revealed extensive shortening (~7 kb) after just two rounds of HSC transplantation. The number of cycling HSCs increased after transplantation and remained elevated for at least 4 mo, while the frequency of HSCs in the bone marrow was completely regenerated by 2 mo after transplantation. Direct analysis of telomeres in HSCs by fluorescent in situ hybridization during serial transplantation also revealed a reduction in telomere size. Together, these data show that telomeres shorten during division of HSCs in vivo, and are consistent with the hypothesis that telomere shortening may limit the replicative capacity of HSCs.

Key words: hematopoietic stem cell • telomere • cell cycle • transplantation • mouse

Introduction

The cellular components of many, if not all, tissues of higher eukaryotes are derived and maintained by stem cells, which are capable of both self-renewal and committed differentiation into one or more lineage subsets. Hematopoietic stem cells (HSCs),[1] which can give rise to all cells of the hematopoietic lineage, can be identified and purified on the basis of cell surface phenotype (1, 2). Murine long-term HSCs reside in the bone marrow at a rare frequency of ~1 in 10,000, where they slowly and continuously turn over to supply cells for the hematopoietic system throughout the life span of the animal (3–5).

Early work by Hayflick and Moorhead showed that cultured human diploid fibroblasts have a finite replicative capacity and eventually undergo senescence (6). In subsequent studies, many other somatic cell types in humans and other organisms were found to have a finite replicative capacity (for a review, see reference 7). Although replicative senescence has not been as well characterized in mice as in humans, serial bone marrow transplantation experiments in mice by Harrison and Astle provide strong evidence that HSCs also have a limited capacity to divide in vivo (8).

Telomeres are the genetic elements capping the ends of chromosomes, and serve to guard against degradation and spurious end to end fusions (9, 10). In vertebrates, the sequence of telomeric DNA is (TTAGGG)n (11). Telomeres can be relatively long in inbred mice, *mus musculus*, ranging from 20 to 100 kb or more (12–15), compared with 3–12 kb in humans (16–18). Telomerase, a ribonucleoprotein complex, functions to complete the replication of telomeres during S phase by synthesis of single-stranded telomeric DNA onto the 3′ overhang at the telomeric terminus (19). Telomerase activity is present in human germ line cells and tumor cells (20, 21), but is absent or present at very low levels in most human somatic cells (21, 22). Consequently, telomeres gradually shorten during division of the latter cells, but not the former (17, 22, 23). Hypotheses have been proposed which predict that the shortening of telomeres during replicative aging of human somatic cells may cause cell senescence (24, 25). Recently, it has been shown that overexpression of terminal reverse transcriptase, the catalytic component of telomerase (26), in human somatic cells grown in vitro allows regeneration of telomere length and immortalization of these cells (27).

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[1]*Abbreviations used in this paper:* FIGE, field inversion gel electrophoresis; FISH, fluorescent in situ hybridization; HSC, hematopoietic stem cell; PNA, peptide nucleic acid; TRF, terminal restriction fragment.

Telomeres also shorten in human hematopoietic cells during replicative aging in vitro (28), and in vivo (18, 29, 30), and accelerated telomere shortening has been reported in the bone marrow of transplant recipients (31). These observations imply that telomeres also shorten during division of HSCs, although this has not been thoroughly established. HSCs also express high levels of telomerase compared with other hematopoietic cells (32). Thus, the relevance of telomere shortening to the finite replicative capacity of HSCs in vivo is not yet clear. To investigate this issue, we have now analyzed the effect of serial transplantation of HSCs in mice on telomere length and cell cycle status of HSCs.

Materials and Methods

Mice. For all transplantation experiments, the C57Bl/Ka-Thy1.1 (Ly5.1) strain (BA strain) was used as HSC donors and the congenic C57Bl/Ka-Thy1.2 (Ly5.2) strain was used as recipients. The initial donor mice and all the recipient mice were 2–3 mo old. All mice were bred and maintained on acidified water (pH 2.5).

Antibodies. The antibodies used in the immunofluorescence staining included E13 (anti–Sca-1, Ly6A/E), C311.1 (anti–c-kit), 2B8 (anti–c-kit), 19XE5 (anti-Thy1.1), Al1-4A2 (Ly5.2), and A20.1(anti-Ly5.1). Lineage marker antibodies included M1/70 (anti–Mac-1), GK1.5 (anti-CD4), KT31.1 (anti-CD3), 53-7.3 (anti-CD5), 53-6.7 (anti-CD8), Ter119 (anti–erythrocyte-specific antigen), 6B2 (anti-B220), and 8C5 (anti–Gr-1).

Bone Marrow Isolation and Immunostaining. Bone marrow cells were isolated and stained with fluorophor-conjugated antibodies as described previously (4). In brief, marrow was aspirated from the tibia and femur bones using staining media (HBSS plus 3% fetal bovine serum, pH 7.2). For HSC analysis or isolation, cells were resuspended in an antibody cocktail (CD3, CD4, CD5, CD8, Ly5.2, Ter119, B220, Mac-1, and Gr-1, all PE-conjugated, Sca-1^{TR}, c-kit $[2B8]^{APC}$, and Thy1.1^{FITC}) and incubated on ice for 30–40 min. Cells were then washed once and resuspended in staining media containing 0.5 μg/ml propidium iodide. Cells were maintained on ice at all times.

Flow Cytometric Analysis and Sorting of HSCs. For isolation of HSC by FACS®, bone marrow cells were first stained with c-kit $(C311.1)^{bio}$ and then enriched by positive selection using MACS (Miltenyi Biotec) streptavidin-conjugated magnetic beads, according to the manufacturer's instructions. The enriched cells were stained with the antibody cocktail described above. HSCs are defined as the c-kit^+Sca-1^+Thy$1.1^{lo}$$Lin^-$ population and can be purified to >95% by double sorting. All analyses and cell sorting were performed on a dual-laser FACS Vantage™ machine (Becton Dickinson).

Cell Cycle Analysis of HSCs. Bone marrow cells were simultaneously stained with antibodies for HSC detection and with Hoechst 34332 dye followed by FACS® analyses of the percentage of HSCs in the S/G2/M phase of the cell cycle as described previously (5).

DNA Isolation and Terminal Restriction Fragment Length Analysis by Field Inversion Gel Electrophoresis. Bone marrow cells were harvested at the time of the initial transplantation and 4 mo or more following each subsequent round of transplantation. Cells were resuspended in lysis buffer (10 mM Tris, 100 mM EDTA, 0.5% SDS, pH 8.0) containing 0.2 mg/ml proteinase K and incubated overnight at 50°C. Cell lysates were extracted one to two times with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. The DNA was ethanol precipitated, and the pellet was washed once with ethanol and resuspended in Tris-EDTA buffer. To obtain DNA that runs clean during field inversion gel electrophoresis (FIGE), each sample was further extracted with DNAzol (GIBCO BRL) according to the manufacturer's instructions and once more resuspended in TE buffer. DNA integrity was assessed by ethidium bromide staining following agarose gel electrophoresis and by Southern blot analysis of the uncut DNA using a telomeric probe as described (23, 29). For each sample, DNA was digested to completion with the restriction enzymes HinfI, RsaI, HhaI, and HpaII. The digested DNA samples (1–0.5 μg) were then resolved in 0.75% agarose gels using FIGE (pulse conditions: 0.8 V forward; 0.4 V reverse for 12 h). The gels were then dried down and terminal restriction fragments (TRFs) were detected by hybridization to a ^{32}P-end labeled telomeric oligomer as described previously (23, 29).

Fluorescent In Situ Hybridization. 1,000 HSCs were double sorted into staining media and cytospun (500 rpm for 5 min) onto glass slides. The slides were air dried and the cells were fixed to the slides by immersion in a 4% paraformaldehyde/PBS solution for 5 min at room temperature. The slides were then air dried overnight before performing fluorescent in situ hybridization (FISH). Telomeres were stained with an FITC-conjugated peptide nucleic acid (PNA) telomeric oligomer, (CCCTAA)3 (Applied Biosystems) using the FISH protocol developed by Lansdorp and coworkers (33). Images were collected and quantitative analysis of the fluorescent signal intensity was performed using a ZEISS confocal microscope. Control slides of unstained cells were used to correct for background fluorescence.

Results

Telomeres Are <50 kb in Size for Hematopoietic Cells from Some mus musculus Strains. FIGE allows high resolution of DNA fragments in the size range of 5–200 kb (34). Since the previously reported size of TRFs in *mus musculus* is within this range (12–15), we used FIGE to perform Southern blot analysis of TRF length for DNA samples from mouse hematopoietic cells. A typical Southern blot analysis showing the range in TRF sizes for bone marrow cells and splenocytes from four different mouse strains is shown in Fig. 1. For all strains, the TRFs are predominantly in the size range of 10–50 kb. The TRF size range for the C57Bl/Ka-Thy1.1 (BA) strain, the donor strain used for serial HSC transplantation, is ~10–40 kb, somewhat less than the TRF size range for early passage mouse embryonic fibroblasts established from the same strain (~30–50 kb; Fig. 1).

An Increase in Frequency of Cycling HSCs Accompanies Restoration of the Donor HSC Pool after Transplantation. Long-term HSCs divide slowly and continuously (8% per day) in the bone marrow of adult BA mice (5), with 5% of the HSC pool in the S/G2/M phase of the cell cycle at any given time (4, 5). After transplantation into irradiated hosts, virtually all bone marrow and splenic HSCs initially enter the cell cycle. However, little is known regarding the long-term cycling activity of the transplanted HSC pool during hematopoietic reconstitution in recipient mice. Also, the

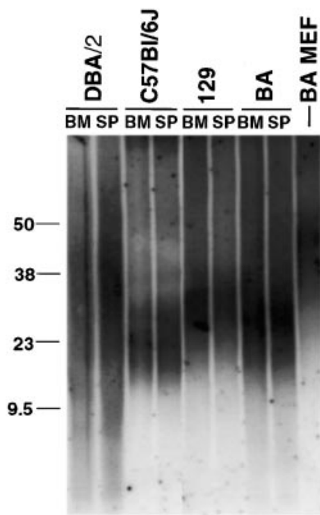

Figure 1. FIGE analysis of TRF length for hematopoietic cells from different *mus musculus* strains. DNA was isolated from bone marrow cells (BM) and splenocytes (SP) from young adult mice, as well as murine embryonic fibroblasts (MEF) established from the BA strain, and digested with the restriction enzymes as described (see Materials and Methods). 1 μg of each DNA sample was resolved in a 0.75% agarose gel by FIGE (pulse conditions, 0.8 V forward and 0.4 V reverse for 12 h). The gel was dried and the DNA hybridized to a ^{32}P-end labeled telomeric oligomer as described (see Materials and Methods). Sizes of molecular weight standards (in kb) are shown on the left.

kinetics and extent of restoration of the donor HSC pool, as assessed directly by surface phenotype analysis, during serial transplantation have not been well characterized. Therefore, bone marrow cells from BA mice and HSC transplant recipient mice were stained simultaneously for HSC detection and with Hoechst dye to determine the frequency of HSCs and the fraction of HSCs in the S/G2/M phase of the cell cycle (Fig. 2 A). 4 wk after transplant, a significant increase in the number of HSCs in the S/G2/M phase of the cell cycle was observed for recipient mice reconstituted with bone marrow containing either 30 HSCs (P = 0.0005) or 3,000 HSCs (P = 0.003; Fig. 2 B). Remarkably, even 4 mo after the transplant, the frequency of HSCs in the S/G2/M phase of the cell cycle was still significantly elevated for both recipients of bone marrow containing 30 HSCs (P = 0.02) or 3,000 HSCs (P = 0.05; Fig. 2 B). We also observed, for both primary and secondary recipients reconstituted with 100–200 donor HSCs, a restoration of the donor HSC pool to levels normally found in young adult BA mice (~0.02%; Table I) by 2 mo after transplant. However, there was a marginal decrease in the total number of donor HSCs after each round of transplantation reflected in a drop in frequency of donor cells in the recipient mice after each round of transplantation (Table I). The reason for this slight drop in donor cell frequency is not clear. We believe that it may be accounted for in part by the increased cell cycle activity of the transplanted HSCs, since our data suggest that engrafted HSCs do not quickly return to the low levels of turnover observed for untransplanted HSCs (Fig. 2), and we have established previously that in various conditions, S/G2/M phase HSCs engraft after transplantation less well than G0/G1 HSCs (see Discussion, and references 35, 36). It is also possible that a small proportion of the transplanted HSCs may either die, for reasons other than poor engraftment, or perhaps exhaust their replicative capacity during hematopoietic reconstitution in primary recipients and, to a greater extent, in secondary recipients.

Size of TRFs from Donor Hematopoietic Cells Decreases during Serial Transplantation of HSCs. It is well established that telomere length shortens in hematopoietic cells during aging in humans and during expansion in vitro (18, 28, 29). To assess whether telomere shortening also accompanies

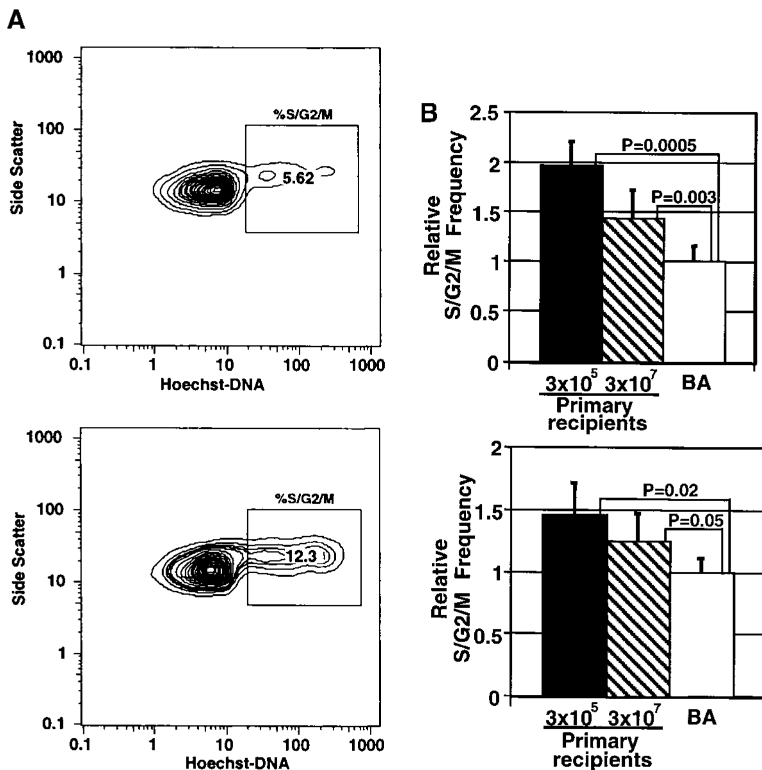

Figure 2. Analysis of cell cycle status of HSCs after transplantation. (A) Detection of HSCs in the S/G2/M phase of the cell cycle by FACS® analysis. Bone marrow was isolated from adult BA mice and primary recipients (1 mo after transplant) and immunostained for HSC detection with Hoechst dye (see Materials and Methods). Shown are sample side scatter (y-axis) and Hoechst fluorescence (x-axis) density plots of HSCs (Sca1$^+$c-kit$^+$Lin$^-$Thy1.1^{lo} cells) from a typical adult BA mouse (top) and a primary recipient reconstituted with 3 × 10^5 bone marrow cells (~30 HSCs) (bottom). Boxes indicate the subpopulation of HSCs in the S/G2/M phase of the cell cycle, showing the percentage of S/G2/M phase HSCs. (B) Analysis of the frequency of cycling HSCs. The average relative frequency of HSCs in S/G2/M for adult BA mice and primary recipients reconstituted with ~3 × 10^5 or ~3 × 10^7 bone marrow cells (30 and 3,000 HSCs, respectively) is shown at 4 wk after transplant (top) and 4 mo after transplant (bottom). Error bars (standard deviation) and P value (Student's t test) are shown.

Table I. *Quantitation of HSCs during Serial Transplantation*

	Total WBCs*	Donor cells‡	HSCs§	Total HSCs
		%	%	
BA	9.1×10^7	100	0.019 ± 0.001	1.7×10^4
Primary‖	8.5×10^7	92 ± 4	0.021 ± 0.002	1.6×10^4
Secondary‖	8.4×107	81 ± 5	0.020 ± 0.002	1.4×10^4

*The total WBCs from the two tibia and femur bones.
‡Analysis was done 8 wk after transplantation. For all sets of mice, $n = 6$.
§Taken as the percentage of the Ly5.1$^+$ marrow population.
‖Both primary and secondary recipients were reconstituted with either 100–200 HSCs isolated by FACS® or bone marrow aliquots containing 100–200 HSCs. For the secondary transplant, primary recipients at 4 mo or more after transplant were used as donors.

the increased replicative demand on HSCs after transplantation, we serially transplanted either whole bone marrow cell populations containing 100-200 HSCs or 100-200 pure HSCs isolated by FACS®. Subsequently, FIGE was used to analyze the TRF length of bone marrow cells from young adult BA mice, primary recipients, and secondary recipients. To ensure that all donor cells in the recipient mice were derived from the donor HSC population, we performed the TRF length analysis at 4 mo or more after transplantation since only the donor HSC population is capable of maintaining donor-derived hematopoietic cell levels for this duration (4). All transplant recipient mice used for telomere length analysis had a donor hematopoietic cell frequency of 80% or greater (data not shown).

As shown in Fig. 3 A, a substantial reduction in TRF length after each round of transplantation is observed. Notably, the range in TRF length for donor-derived bone marrow cells from secondary recipients was just ~7–23 kb, comparable to the TRF size range for human germ line or fetal cells (16, 23, 30). The mean TRF length was also calculated and averaged for all experiments (Fig. 3 B). We observed a significant decrease in mean TRF length after each round of transplantation (Fig. 3 B). After just two rounds of HSC transplantation, the telomere length had decreased by ~7 kb. Similar results were observed after FIGE analysis of TRF length for donor-derived splenocytes (data not shown). The hematopoietic profile of the donor marrow cells, as assessed by analysis of the frequency of various hematopoietic lineages, was not altered during serial transplantation (data not shown).

To assess whether the extent of reduction in telomere length was dependent on the initial dose of transplanted HSCs, we compared the mean TRF length for primary recipients reconstituted with either 30 or 3,000 HSCs. At 4 mo after transplant, mice ($n = 7$) reconstituted with 30 HSCs had significantly shorter telomeres (21.7 kb) than mice ($n = 6$) reconstituted with 3,000 HSCs (22.7 kb; $P = 0.04$).

Fluorescent Telomeric Signal Intensity Decreases in HSCs during Serial Transplantation. We used FISH rather than FIGE to assess telomere length directly in HSCs during serial transplantation because of the scarcity of these cells. 1,000 HSCs were isolated by FACS® from two adult BA mice and secondary recipients, cytospun onto glass slides, and stained by hybridization to an FITC-conjugated PNA telomeric oligomer (33). There is a general decrease in telomeric signal intensity for individual HSC nuclei from secondary recipients compared with HSC nuclei from adult BA mice (Fig. 4 A). Quantitative analysis of the telomeric signal intensity for individual HSC nuclei using a confocal microscope revealed a significant reduction in telomere length (~30%; $P = 0.001$) for HSCs after two rounds of transplantation (Fig. 4 B). This reduction in telomeric signal intensity during serial HSC transplantation agrees reasonably well with the reduction in TRF length for donor-derived bone marrow cells from secondary recipients assessed using FIGE (Fig. 3).

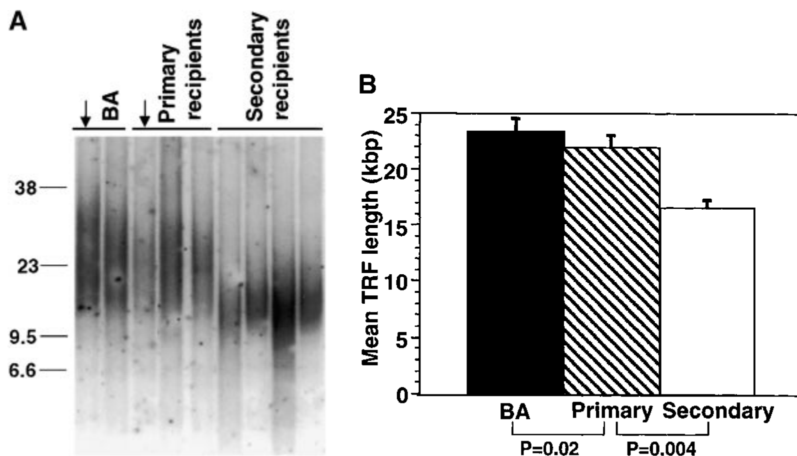

Figure 3. FIGE analysis of TRF length of bone marrow cells during serial HSC transplantation. (A) Southern blot analysis of TRF length by FIGE. In this sample analysis, all primary recipients and two secondary recipients, represented by the rightmost two lanes, were reconstituted with 100 pure HSCs. The other two secondary recipients were reconstituted with 2×10^6 bone marrow cells. Whole bone marrow cells were isolated from adult BA mice and recipient mice and digested with restriction enzymes as described (see Materials and Methods). 0.5 μg of each digested DNA sample was resolved in a 0.75% agarose gel by FIGE (pulse conditions, 0.8 V forward and 0.4 V reverse for 12 h). The bone marrow sample of the donor mouse in each round of transplantation is indicated by an arrow (↓) at the top. The gel was dried and the DNA hybridized to a ^{32}P-end labeled telomeric oligomer as described (see Materials and Methods, and reference 23). Sizes of molecular weight standards (in kb) are shown on the left. (B) Measurement of mean TRF length during serial transplantation. The mean TRF length was calculated as described previously (references 23, 29) for a total of 9 sibling adult BA mice, 11 primary recipients, and 10 secondary recipients and averaged for all experiments. Error bars (standard deviation) and P values (Student's t test) are shown.

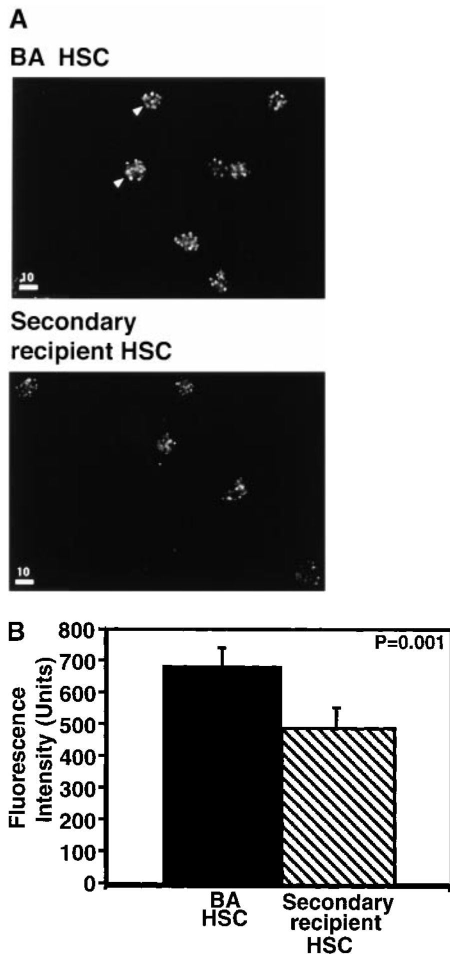

Figure 4. FISH analysis of telomere length of HSCs from adult BA mice and secondary recipients. (A) Confocal microscope image of individual HSC nuclei after hybridization to a fluorescent telomeric probe. 1,000 HSCs were isolated from the pooled bone marrow from two BA mice and two secondary recipients by FACS®, cytospun onto glass slides, and fixed as described (see Materials and Methods). The telomeres were detected by FISH using an FITC-conjugated PNA telomeric oligomer as described by Lansdorp et al. (reference 33). Individual interphase nuclei are indicated by arrowheads. Image was collected using a 60× objective. The size scale (in μm) is indicated in the lower left. (B) Fluorescent signal intensity measurements of HSC nuclei. After FISH, the fluorescent telomeric signal intensity was calculated and corrected for background for 30 well-isolated individual HSC nuclei from both adult BA mice and secondary recipients. The mean fluorescent signal intensity and corresponding standard deviation are shown. *P* value was calculated by Student's *t* test.

Discussion

This study investigates the potential role of telomere shortening on the finite in vivo replicative capacity of HSCs during transplantation. We show that telomere length in *mus musculus* hematopoietic cells is relatively short and can be readily measured by FIGE. HSCs were found to be more frequently in cycle for several months after transplantation and the relative abundance of HSCs is restored to normal levels after one or two rounds of serial transplantation. We also show that telomeres shorten extensively in most, if not all, donor hematopoietic cells, including HSCs, during serial HSC transplantation.

Our analysis of telomere length in BA mice (C57Bl/Ka-Thy1.1 mice), the donor strain used for HSC transplantation, as well the related C57Bl/6J mice, shows that the TRFs for these strains range in size from ~10 to 40 kb (Figs. 1 and 3). While this is a substantially lower size range for TRFs than what has generally been reported for other inbred mice strains (12–14), studies that have directly assessed TRF length in the C57Bl/6J mice have also found that the TRF size range for these mice (~20–75 kb; 12, 13, 15) is less than the size of TRFs from other inbred strains. In addition, considerable variation in telomere length among different inbred strains (12, 13, 15), different lab colonies (13), and even individual mice of the same mouse strain (13) has also been shown to occur. Thus, the results from our analysis of TRF length in mice agree reasonably well with what has been published previously.

As might be predicted, the number of cycling HSCs did increase during hematopoietic reconstitution of the transplant recipients, and was inversely proportional to the initial number of transplanted HSCs (Fig. 2). Surprisingly, the cycling activity of the donor HSCs remained elevated for at least 4 mo after transplant. The myeloid and lymphoid lineages were both reconstituted (data not shown) and the donor HSC pool was restored to normal levels within the first 8 wk after transplantation (Table I); thus, the reason for this sustained cycling activity of the donor HSC pool is not clear. It may reflect a long-term stress response of the animal to a lethal dose of irradiation, long-term impairment of immune protection during hematopoietic reconstitution, and/or a slightly reduced ability of the donor-derived hematopoietic cells to thrive in a foreign host.

After two rounds of serial transplantation, the frequency of donor HSCs showed little change from that observed in young adult BA mice (Table I). However, evidence for a decrease in HSC concentration after bone marrow transplantation has been reported in earlier studies, in which HSC concentration was calculated by either limit dilution analysis (37) or analysis of covariance in recipients of mixtures of whole bone marrow from two genetically distinguishable donor strains (38). Although the reason for this discrepancy is unknown, it is likely accounted for in part by differences in methodology. The transplantations in these earlier studies included competitor cells and determined donor HSC frequency relative to the total number of marrow cells in the recipient mice, whereas our transplants were done without competition and donor HSC frequency calculations were performed relative to the donor marrow population. Also, the methods used to determine HSC number in these earlier studies are based on functional assays, unlike our method of direct analysis of HSCs based on cell surface phenotype, and therefore cannot clearly distinguish between a decline in functional retransplantation ability of HSCs and actual HSC number.

Telomere length analysis of donor-derived bone marrow cells (Fig. 3) and HSCs (Fig. 4) shows substantial telomere shortening during serial HSC transplantation. The difference in mean TRF length (ΔTRF) of bone marrow cells after one round of HSC transplantation is ~1.5 kb (Fig. 3

B). This agrees reasonably well with the predicted reduction in telomere size assuming that ΔTRF is mainly due to the minimum number of extra population doublings (∼12–13) required for expansion of the fraction of the transplanted HSC population which engraft to the size of the HSC pool in adult mice (∼3–5 $\times 10^4$ cells; references 3, 4), and that the rate of telomere shortening during division of the transplanted HSCs is 50–100 bp per population doubling, as observed for other mouse cells (14, 39, 40). However, the extent of telomere shortening during the second round of HSC transplantation (ΔTRF ≈ 5.5 kb) is considerably greater than ΔTRF during the first round of transplantation (Fig. 3). One possible explanation for this is that the intrinsic properties of the donor HSC population may have changed, after the second transplantation, to effect an increase, by whatever mechanism(s), in the rate of telomere shortening. However, we favor a model, as outlined in Fig. 5, in which the increase in ΔTRF is primarily accounted for by an increase in the rate of HSC turnover during self-renewal and/or hematopoietic reconstitution. This notion is supported by the following observations. (a) Telomere shortening is mainly, if not entirely, dependent on cell division. Telomere shortening has only been observed in dividing cell populations or tissues, and direct comparison of changes in telomere length over time in actively replicating cells and very slowly replicating cells reveals telomere shortening in the former, but little or no shortening in the latter (41). (b) We find that the telomere length in the recipient bone marrow is shorter in mice reconstituted with 100 times fewer HSCs (see Results), which further supports a mechanism involving increased cell division. (c) The frequency of cycling HSCs increases in response to transplantation (Fig. 2), as well as several stressors including cytoxan/G-CSF–induced HSC mobilization (42) and the aging process (43), and the reconstitutive capacity of HSCs is inversely correlated with the cycling activity (35, 36, 42, 43). Direct transplantation of S/G2/M HSCs demonstrated that these cycling HSCs engraft quantitatively less well than G0/G1 HSCs (36), although they retain multipotent hematopoietic activity at the single cell level (42, 43). The reason for this behavior is unknown, but may possibly reflect a reduced ability of cycling HSCs to home to and settle in the bone marrow, the only site of large-scale hematopoiesis. Another possible explanation for the increased telomere shortening during the second round of transplantation is a reduction in telomerase activity in HSCs during transplantation, which in turn could lead to an accelerated rate of telomere loss. This possibility is amenable to testing.

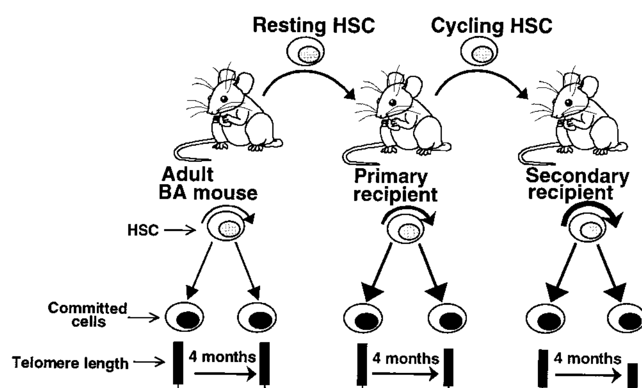

Figure 5. Hypothetical model to explain the reduction in telomere length during serial transplantation of HSCs. In adult mice, HSCs are predominantly in a nonstressed, resting state (indicated by the small arrows) where they function mainly to sustain homeostatic levels of hematopoietic cells. Since telomere shortening is primarily dependent on cell division (reference 41), there is no discernible telomere shortening over a time period of a few months in adult BA mice (data not shown). After transplantation into primary recipients, the cycling activity of the donor HSCs increases (thicker arrows), primarily to allow reconstitution of all hematopoietic lineages including the HSC pool. Consequently, a modest amount of telomere shortening occurs. The cycling activity of the donor HSCs is still elevated when they are once again isolated for a second round of transplantation. This results in a considerable drop in reconstitutive capacity because of poor engraftment in the secondary recipients and a further increase in the rate of HSC turnover (thickest arrows) so that regeneration of the HSC pool and hematopoietic reconstitution can be completed once again. It is also possible that, after transplantation, HSCs from primary recipients may be more prone to cell death or replicative exhaustion than HSCs taken directly from adult mice, which could also influence the rate of HSC division in secondary recipients. Thus, during hematopoietic reconstitution in the secondary recipients, telomeres in the HSCs undergo an even greater degree of shortening.

The precipitous drop in telomere length that we observe during serial transplantation of HSCs in mice is consistent with the shortening of telomeres that others have observed in the donor cells from bone marrow transplant recipients (31), and in candidate HSCs during aging in humans (30). It will be important to test whether telomere shortening or other events, such as the increase in cell cycle activity in response to transplantation, ultimately limits the replicative life span of HSCs during serial transplantation. To test these nonexclusive possibilities, it will be important to carry out serial transplantation of HSCs to exhaustion in mice to determine if the telomere length becomes critically short. Also, it will be informative to test transgenic mice that overexpress murine terminal reverse transcriptase in HSCs to assess whether telomere length is maintained in these mice and what effect this has on the long-term transplantation capacity of HSCs.

We thank L. Jerabek and V. Braunstein for their excellent technical assistance, P. Fallon and T. Knaak for their operations of the FACS® machines, and L. Hidalgo for animal care.

R.C. Allsopp holds a National Research Service Award, National Institutes of Health postdoctoral fellowship. This work was supported by National Institutes of Health grants CA42551 and DK53074 (to I.L. Weissman).

Submitted: 27 November 2000
Revised: 15 February 2001
Accepted: 20 February 2001

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