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Telomere Shortening and Tumor Formation by Mouse Cells Lacking Telomerase RNA

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Summary

To examine the role of telomerase in normal and neoplastic growth, the telomerase RNA component (*mTR*) was deleted from the mouse germline. *mTR*$^{-/-}$ mice lacked detectable telomerase activity yet were viable for the six generations analyzed. Telomerase-deficient cells could be immortalized in culture, transformed by viral oncogenes, and generated tumors in nude mice following transformation. Telomeres were shown to shorten at a rate of 4.8 $\pm$ 2.4 kb per *mTR*$^{-/-}$ generation. Cells from the fourth *mTR*$^{-/-}$ generation onward possessed chromosome ends lacking detectable telomere repeats, aneuploidy, and chromosomal abnormalities, including end-to-end fusions. These results indicate that telomerase is essential for telomere length maintenance but is not required for establishment of cell lines, oncogenic transformation, or tumor formation in mice.

Introduction

Telomeres protect chromosomes, preventing fusions, recombination, and degradation (reviewed in Greider, 1996). Telomerase is a ribonucleoprotein DNA polymerase that plays a key role in telomere synthesis (Greider and Blackburn, 1985; Yu et al., 1990; Singer and Gottschling, 1994). Telomerase adds telomere repeats onto chromosome ends to compensate for sequence loss during DNA replication (reviewed in Blackburn, 1991). The RNA component of telomerase provides the template for telomere repeat synthesis (Greider and Blackburn, 1989; Yu et al., 1990). The human and mouse telomerase RNA components have been cloned (Blasco et al., 1995; Feng et al., 1995), and one protein associated with mammalian telomerase was identified recently (Harrington et al., 1997; Nakayama et al., 1997).

In most human primary cells, telomerase activity is not detected, and telomeres shorten with each cell division. If cells are transfected with a viral oncogene, senescence can be bypassed, and most of the cells will die at a point defined as "crisis." In the established cultures that are recovered after crisis, telomerase activity is detected, and telomere length is stabilized or lengthened. The appearance of telomerase-positive cells after crisis may be due to the selective growth advantage of cells that have stochastically activated telomerase at crisis (Counter et al., 1992; reviewed in Autexier and Greider, 1996).

Telomerase activity is detectable in 80% to 90% of human tumor samples, while the normal adjacent tissue usually lacks measurable activity (reviewed in Harley et al., 1994; Kim et al., 1994, Shay and Bacchetti, 1997). Although the mechanism of activation of telomerase in tumors is not known, the correlation between telomerase activation during cellular immortalization and activation in tumors led to the view that telomerase is required for tumor growth in vivo, and telomerase inhibition was proposed as a new approach to cancer therapy (Harley, 1990; Counter et al., 1992).

Mouse tumors, like human tumors, express elevated levels of telomerase activity (Bednarek et al., 1995; Chadeneau et al., 1995; Blasco et al., 1996; Broccoli et al., 1996). In transgenic mouse models of tumorigenesis, telomerase is activated late in tumor progression, while the RNA component is upregulated early (Blasco et al., 1996; Broccoli et al., 1996). To test whether telomerase is required for tumor formation and cell viability, we generated mice deleted for the gene encoding the telomerase RNA component. We describe here the effects of the absence of telomerase activity on telomere length, cellular viability, immortalization, neoplastic transformation, and tumor formation in nude mice.

Results

Generation of Viable Mice Homozygous Null for the *mTR* Locus

To generate a mouse deficient for telomerase activity, we deleted the germline copy of the mouse telomerase RNA gene (*mTR*) (Thomas and Cappechi, 1987). Genomic Southern blots probed with the transcribed region of the mouse telomerase RNA gene identified a single band, suggesting that *mTR* is a single copy gene (data not shown). To delete the *mTR* gene from the mouse germline, we constructed the plasmid pPNT-mTRΔ, which allows replacement of the entire *mTR* gene with the neomycin resistance gene (Figure 1A; see Experimental Procedures). Using standard protocols, WW6

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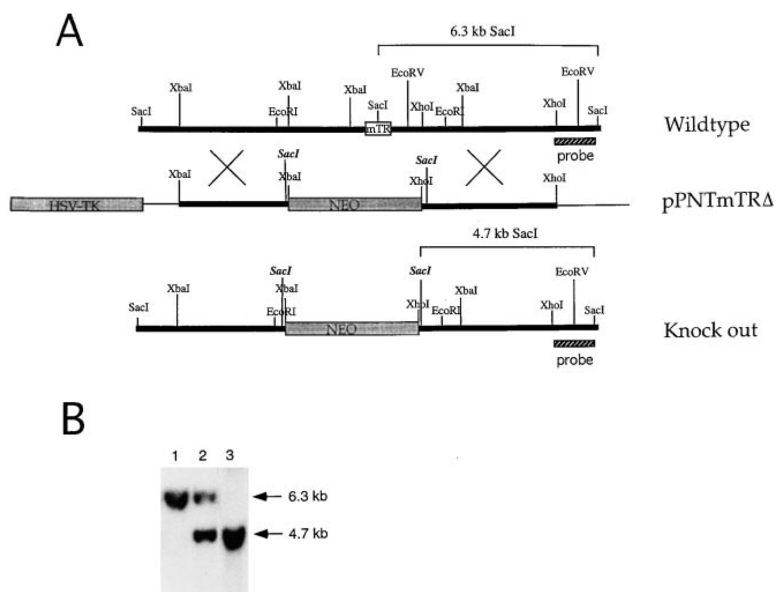

Figure 1. Genomic Replacement of the *mTR* Locus

(A) The restriction map of the wild-type *mTR* locus, the pPNT-mTRΔ plasmid, and the targeted deletion locus are shown. Recombination (shown as large Xs) between the targeting vector and the wild-type locus will eliminate the HSV TK gene and produce the knockout allele shown below. Two SacI restriction sites (shown as *SacI*) were engineered to allow the wild type and knockout genomic loci to be distinguished. The 1.2 kb XhoI-SacI fragment labeled probe will identify a 6.3 kb wild-type band and a 4.7 kb deletion allele band in SacI genomic digests.

(B) Southern blot analysis of progeny from $mTR^{+/-}$ intercrosses. Genomic DNA isolated from the tails of the progeny of a $mTR^{+/-}$ cross was digested with SacI. Southern blots were probed with the 1.2 kb XhoI-SacI probe. The bands corresponding to the wild type (6.3 kb) and to the mutant (4.7 kb) are indicated.

mouse embryonic stem (ES) cells (Ioffe et al., 1995) heterozygous for the *mTR* gene replacement were identified. The $mTR^{+/-}$ ES cells were used to generate chimeric progeny that transmitted the neomycin resistance gene through the germline (Ramirez-Solis et al., 1993). The chimeric mice were backcrossed to C57BL/6J mice before intercrossing them to generate the first generation (G1) *mTR*-deficient mice. Intercrosses between $mTR^{+/-}$ heterozygous mice generated viable $mTR^{-/-}$ offspring.

A probe flanking the *mTR* locus detected a 6.3 kb SacI restriction fragment in wild-type mice and a 4.7 kb SacI restriction fragment at the targeted locus (Figure 1B). Disruption of the *mTR* locus was verified on three additional levels. First, Southern blots of tail DNA probed with the *mTR* transcribed region showed a band in wild-type but not in $mTR^{-/-}$ animals (data not shown). Second, Northern blots of total RNA isolated from mouse embryonic fibroblasts (MEFs) showed that the *mTR* transcript was not detectable in total RNA derived from $mTR^{-/-}$ mouse embryos (data not shown). Finally, analysis of $mTR^{-/-}$ animals showed no detectable telomerase activity (see below).

The generation of $mTR^{-/-}$ mice indicated that *mTR* is not essential for embryonic development. To determine whether the $mTR^{-/-}$ mice are fertile, first generation (G1) $mTR^{-/-}$ mice were crossed to each other. Second generation (G2) $mTR^{-/-}$ mice were obtained, indicating that *mTR* is not essential for fertility. Additional generations of $mTR^{-/-}$ mice were obtained by crossing two animals that were deleted for the *mTR* locus and designated G3, G4, G5, and G6. In all cases, in our nomenclature, the parents of a given $mTR^{-/-}$ generation were both from the previous generation. Thus, a G3 $mTR^{-/-}$ animal is derived from crossing two G2 $mTR^{-/-}$ mice.

$mTR^{-/-}$ Mice and Embryonic Fibroblasts Lack Telomerase Activity

To determine whether deletion of *mTR* abolished telomerase activity as expected, five independently derived MEF cultures from $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ G1 embryos were assayed for telomerase activity. $mTR^{+/+}$ and $mTR^{+/-}$ MEFs had detectable telomerase activity, while MEFs derived from $mTR^{-/-}$ embryos showed no detectable activity as assayed by the sensitive telomeric repeat amplification protocol (TRAP) assay (Kim et al., 1994) (Figure 2A).

To test whether telomerase activity was present in adult mouse tissues, S100 extracts from brain, liver, thymus, and spleen from $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ littermates were assayed using the TRAP assay. Telomerase activity was detected in liver and thymus from both $mTR^{+/+}$ and $mTR^{+/-}$ mice but not detected in these tissues from $mTR^{-/-}$ mice (Figure 2B and data not shown). For all the genotypes, no activity was detected in adult brain or spleen, which were shown previously to be telomerase negative (Chadeneau et al., 1995; Prowse and Greider, 1995).

The TRAP assay for telomerase activity is based on the PCR amplification of telomerase products containing the sequence $(TTAGGG)_n$ (Kim et al., 1994). Only repeats of this sequence will be amplified in the PCR reaction. To determine whether some other sequence might be synthesized by telomerase in the absence of the *mTR* gene, we used a direct assay to visualize sequences added onto telomeric primers (Prowse et al., 1993). Using two different telomerase substrates, $(TTAGGG)_3$ and MP 1 (Blasco et al., 1995), the expected ladder of primer elongation products was generated with extracts prepared from $mTR^{+/+}$ MEFs but not from extracts prepared from $mTR^{-/-}$ MEFs (data not shown). The absence of telomerase activity in $mTR^{-/-}$ MEFs and tissues indicates that the *mTR* gene is essential for mouse telomerase activity in vivo.

To determine if $mTR^{-/-}$ cells retain the ability to express telomerase activity, we reintroduced the wild-type gene into $mTR^{-/-}$ MEF cultures by transient transfection (see Experimental Procedures). Cell cultures transfected with a plasmid containing 5 kb surrounding the genomic *mTR* locus (Blasco et al., 1995) had detectable telomerase activity 48 hr after the transfection. In contrast,

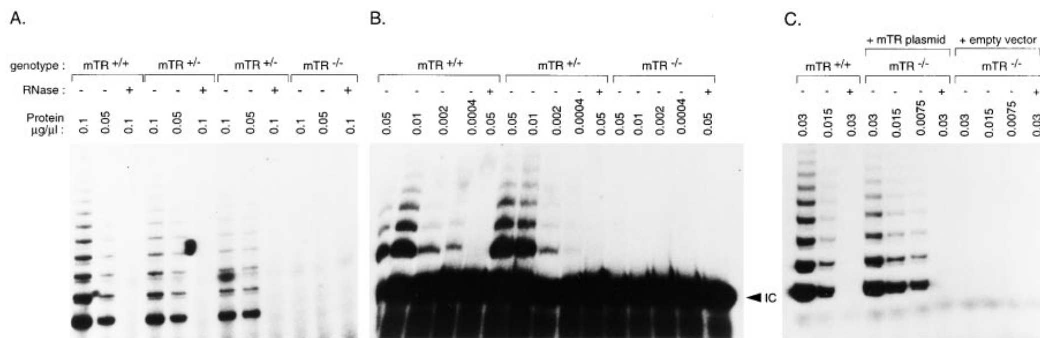

Figure 2. Telomerase Activity in $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ Fibroblasts

(A) S100 extracts were prepared from early passage mouse embryonic fibroblasts, $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ and assayed for telomerase activity. All the extracts were pretreated (+) or not (−) with RNase A before the telomerase assay. The protein concentration in μg/μl in the PCR step of the enhanced TRAP assay is indicated in the figure.

(B) TRAP assay of telomerase activity in $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ thymus extracts. S100 extracts were pretreated (+) or not (−) with RNase A as indicated in the figure. The final protein concentrations in the TRAP assay are indicated. The arrow indicates the position of the internal control (IC) for PCR efficiency.

(C) Rescue of telomerase activity in an $mTR^{-/-}$ cell line. A plasmid containing 5 kb of mouse genomic DNA that contained the *mTR* gene and upstream sequences (Blasco et al., 1996) or an empty vector (Bluescript) was transfected into a $mTR^{-/-}$ cell line (KO-3 at passage 23). Forty-eight hours after transfection, S100 extracts were prepared and assayed for telomerase activity. All the extracts were pretreated (+) or not (−) with RNase before the telomerase assay. The protein concentration is given in μg/μl in the PCR step of the enhanced TRAP assay.

$mTR^{-/-}$ cultures transfected with an empty vector had no detectable telomerase activity (Figure 2C). Restoration of telomerase activity in vivo with ectopically expressed *mTR* indicates that it is the absence of the telomerase RNA that is responsible for the loss of telomerase activity in $mTR^{-/-}$ cells. Further, it suggests that either the telomerase protein components are present in the cell even in the absence of *mTR* or the expression of *mTR* rapidly induces the protein components to form an active mouse telomerase complex.

Establishment of Telomerase Null Embryonic Fibroblast Cultures

The presence of telomerase activity in immortalized human and mouse cell cultures and the frequent activation of telomerase in human and mouse tumors suggested that telomerase activity might be required for the growth of immortalized cells (reviewed in Autexier and Greider, 1996). To test this hypothesis, we compared the ability of telomerase-negative ($mTR^{-/-}$) and telomerase-positive ($mTR^{+/+}$) MEFs to generate established cell lines using the 3T3 protocol (Todaro and Green, 1963) (see Experimental Procedures). Primary MEF cultures were generated from $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ G1 embryos and from $mTR^{-/-}$ G2, G3, G4, and G6 embryos (see Experimental Procedures). Primary cultures of $mTR^{-/-}$ MEFs were morphologically indistinguishable from $mTR^{+/+}$ and $mTR^{+/-}$ MEFs (data not shown). The initial growth rates of $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ MEFs from the first generation and $mTR^{-/-}$ MEFs from generations G2, G3, G4, and G6 were similar (Figure 3A). At a population doubling level (PDL) of approximately 10, normal primary MEFs enter a slow growth phase that has been described in mouse cells as senescence or crisis (Todaro and Green, 1963; Conzen and Cole, 1995; Rittling, 1996). Spontaneous escape from this senescence/crisis phase occurs in normal rodent cells at a frequency of 2×10^{-6} (Kraemer et al., 1986). Serial passage of G1 $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ MEFs as well as G2, G3, G4, and G6 $mTR^{-/-}$ MEF cultures showed the typical slow growth phase around 10 PDL characteristic of senescence/crisis (Figure 3A and data not shown). The cells adopted a flat and extended morphology with prominent cytoplasmic enlargement during the senescence/crisis period (data not shown). After several passages of slow growth, all the cultures regained a rapid growth rate, characteristic of established cells. To date, the MEF cultures derived from G1 embryos have

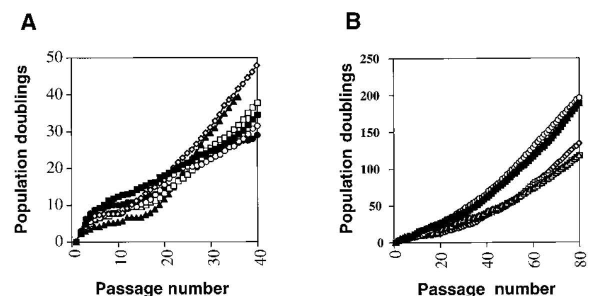

Figure 3. Establishment and Growth of *mTR* and $mTR^{-/-}$ MEFs in Culture

(A) The growth characteristics of MEF cultures from wild type, G1, G2, G3, G4, and G6 $mTR^{-/-}$ embryos are shown. The cultures were derived from individual embryos and passaged according to the 3T3 protocol (see Experimental Procedures). The following mouse embryonic fibroblasts were studied: Wt-6 (closed squares); $mTR^{-/-}$ KO-G1 (open diamonds); KO-G2 (open circles); KO-G3 (open squares); KO-G4 (closed circles); and KO-G6 (closed triangles). The number of population doublings was calculated as follows: $\Delta PDL = \log(n_f/n_0)/\log 2$, where n_0 is the initial number of cells and n_f is the final number of cells at each passage.

(B) Long-term growth of $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ established cell lines. MEF cultures were derived from $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ G1 embryos and established in culture following the 3T3 protocol. $mTR^{+/+}$ Wt-4 (closed squares); $mTR^{+/-}$ H-1 (open triangles); $mTR^{+/-}$ H-5 (hatched squares); $mTR^{+/-}$ H-9 (open diamonds); $mTR^{-/-}$ KO-3 (open circles).

Table 1. Colony Formation Assay

Cell Type[a]	Total Colonies 1[b]	Total Colonies 2[c]
Wt-1	22	13
KO G2-2	26	14
KO G3-4	31	4
KO G4-10	22	14
KO G4-4	13	16
KO G4-8	13	8
KO G6-9	22	19
KO G6-11	9	42

[a] The genotype (Wt for *mTR*$^{+/+}$ and KO for *mTR*$^{-/-}$), generation (G2–G6), and culture number of the MEF cells plated in the assay are given. Three independently derived cultures were used from mTR$^{-/-}$ G4 embryos and two independent cultures from G6 embryos.

[b] In the first experiment, colonies were identified by visual inspection of plates after 2 weeks of growth.

[c] In the second experiment, colonies were identified in the microscope to avoid counting large senescent cells as colonies.

undergone more than 200 PDL, and those derived from the later generations (G2–G6) have divided for at least 40 PDL (Figure 3B and data not shown).

To quantitate the ability of *mTR*$^{+/+}$ and *mTR*$^{-/-}$ cell cultures to generate established lines, we used a colony formation assay. The ability of primary cell cultures to produce colonies when plated at low density is related to the proliferative potential of cells and the ability to escape senescence/crisis (Kraemer et al., 1986; Conzen and Cole, 1995; Serrano et al., 1996). In contrast to serial passage, a colony formation assay provides a quantitative estimate of the immortalization efficiency. MEFs derived from *mTR*$^{-/-}$ G1–G4 and G6 mice formed colonies with a similar efficiency to MEFs derived from *mTR*$^{+/+}$ mice (Table 1). Thus, telomerase-negative cells can overcome senescence/crisis at a rate similar to telomerase-positive cells.

Telomere Length in Telomerase Null Cells

Telomerase activity is thought to be required for telomere length maintenance in mammals, as it is in yeast. In human primary fibroblasts that lack detectable telomerase activity, telomeres shorten by about 100 bp per cell division (Harley et al., 1990; Counter et al., 1992). The terminal restriction fragment (TRF) containing the telomeric repeats in human cells is typically between 10 and 15 kb in length. Some mouse species such as Mus spretus have a similar TRF length and rate of telomere shortening in primary fibroblasts (Prowse and Greider, 1995). The laboratory mouse species Mus musculus used to generate the *mTR*$^{-/-}$ strain has TRFs that have been estimated on pulsed-field gels to range from 40 to 150 kb (Kipling and Cooke, 1990; Starling et al., 1990). However, recent experiments using fluorescence in situ hybridization (FISH) suggest that the TTAGGG repeats on mouse chromosomes range from only 10 to 60 kb (Zijlmans et al., 1997). The difference between the estimates of telomere length determined using FISH and that determined by Southern blot is likely due to the scarcity of restriction sites in the subtelomeric repetitive DNA on the mouse chromosomes. Thus, the very long TRFs may consist mostly of subtelomeric repetitive DNA and only a relatively short terminal portion of telomeric repeats (Kipling et al., 1991; Zijlmans et al., 1997).

To determine if telomere shortening occurs when telomerase activity is absent, we used both the Southern blot and the FISH techniques. For the former, primary MEF cultures were derived from G1 *mTR*$^{+/+}$ animals and from the *mTR*$^{-/-}$ G1, G2, G3, G4, and G6 generation embryos. Genomic DNA was digested with MboI, resolved on a pulsed-field gel, blotted, and probed with a $(TTAGGG)_n$ probe to detect the terminal fragments (see Experimental Procedures). Primary cell cultures derived from individual mice from the different generations had very different TRF sizes, as expected from the hypervariable nature of mouse TRFs (Kipling and Cooke, 1990). Thus, from this analysis it was not possible to distinguish whether telomeres were shorter in the G6 compared to the wild-type animals (Figure 4A).

To look further for telomere shortening, we analyzed MEF cultures from wild-type and *mTR*$^{-/-}$ G1, G3, and G4 animals. DNA was isolated from cells during early, mid, and late passage of these cultures. Although there again was significant variability in the fragment size in cultures derived from individual embryos, it was apparent that the telomere bands shortened during growth in culture (Figure 4B). As a control, we probed the same blot with the major satellite repetitive DNA element (Kipling and Cooke, 1990) and found no decrease in the size of the repetitive sequence bands with increased cell generation in culture. Thus, the decrease of fragment size seen in Figure 4B is not due to a general change in repetitive DNA or to an artifact of gel running. We conclude that the telomere fragments shorten during cell doublings in culture in the absence of telomerase.

To obtain more quantitative information on telomere shortening, we used FISH analysis (Lansdorp et al., 1996; Zijlmans et al., 1997). The signal intensity of telomeric DNA visualized by FISH can be related to the approximate number of kilobase pairs of $(TTAGGG)_n$ repeat sequence present on a given chromosome end. One telomere fluorescence unit (TFU) is defined as the signal that represents 1 kb of $(TTAGGG)_n$ repeat (Zijlmans et al., 1997; see Experimental Procedures). Primary MEF cultures were derived from wild-type and *mTR*$^{-/-}$ G2, G4, and G6 animals, and the fluorescence intensity on all chromosome ends from 25 or more metaphase samples at each generation was determined (Figure 5 and Table 2). These cultures revealed a significant decrease in TTAGGG hybridization signal with increasing *mTR*$^{-/-}$ generation. The rate of telomere shortening was 4.8 ± 2.4 kb per mouse generation. This value was calculated by comparing the mean of the TFU distributions for the p arm, the q arm, and for all ends between wild type and *mTR*$^{-/-}$ G2, G4, and G6. Representative FISH images from wild-type *mTR*$^{-/-}$ G2, G4, and G6 MEF cultures are shown in Figure 6. Strikingly, no TTAGGG signal was detected on chromosome ends in 5% of the chromosomes in G6 cultures (Figure 6D and Table 2).

The lack of detectable TTAGGG signal on some chromosomes suggests that telomere function, which normally caps or stabilizes chromosome ends, might be lost on those ends. Consistent with this, increased frequencies of aneuploid cells and apparent end-to-end

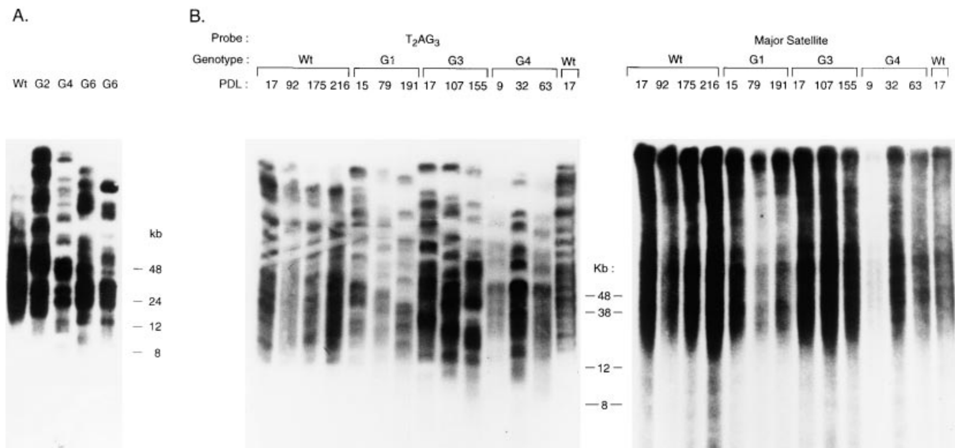

Figure 4. TRF Length in $mTR^{-/-}$ Mouse Embryonic Fibroblasts

(A) Early passage MEFs derived from different generations of the $mTR^{-/-}$ embryos were grown, and the samples were embedded in agarose plugs and pulsed-field gels were run as described (see Experimental Procedures). The wild-type (Wt) mice had been backcrossed to C57BL/6J for five generations, while the G2, G4, and G6 $mTR^{-/-}$ cells had not been backcrossed.

(B) MEF cell cultures were established from wild type or different generations of mice bred in the absence of telomerase ($mTR^{-/-}$ G1, G3, or G4). The cells were passaged in culture for the number of PDL indicated, and genomic DNA was prepared and resolved on a pulsed-field gel. After probing with $(TTAGGG)_n$, the blot was stripped and reprobed with the mouse major satellite probe to control for gel artifacts loading and integrity of the DNA sample.

associations of chromosomes including Robertsonian fusions (see, for example, Garagna et al., 1995; Nanda et al., 1995) were observed in metaphase spreads from different generations of $mTR^{-/-}$ cultures (Figures 6B–6D; Table 2). The loss of telomere signal and the concomitant appearance of chromosomal rearrangements indicates that the absence of telomerase activity leads to telomere shortening and chromosome instability.

Malignant Transformation and Tumor Formation in the Absence of Telomerase Activity

To test the requirement for telomerase during mouse tumorigenesis, we compared cells derived from $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ embryos for their ability to become neoplastically transformed and then to form tumors in nude mice. Late passage, established G1 $mTR^{-/-}$ and $mTR^{+/+}$ MEF cell lines were transfected with a plasmid expressing ras^{v12} alone, T antigen (TAg) alone, or ras^{v12} plus TAg. The $mTR^{-/-}$ and $mTR^{+/+}$ cells formed similar numbers of foci after transfection, indicating that the absence of *mTR* does not inhibit oncogenic transformation. In addition, ras^{v12} was sufficient for foci formation (data not shown), further substantiating that the late passage cells derived in the 3T3 experiment were established cell lines. Two independent foci for each cell type were subcloned, and 10^5 cells from each clone were injected into two different sites on a nude mouse. Before injection, each transformed cell line was assayed for telomerase activity using the TRAP assay. As expected, foci derived from $mTR^{+/+}$ MEFs had telomerase activity,

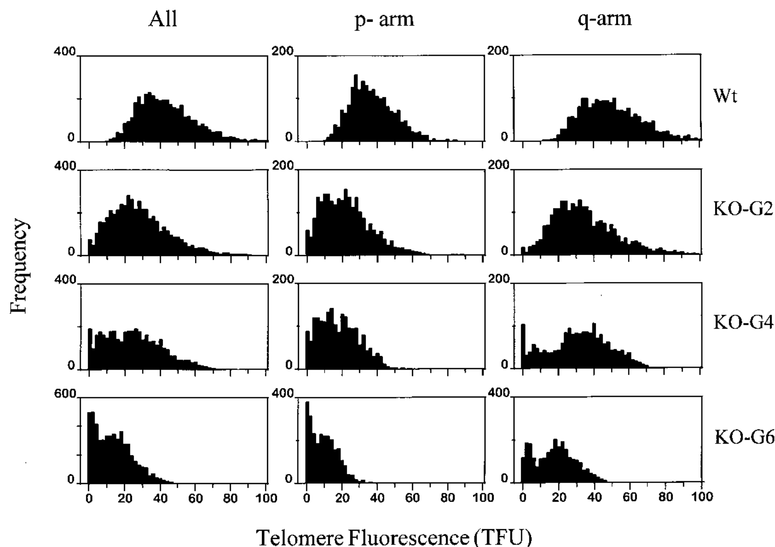

Figure 5. Frequency Distribution of Telomere Fluorescence in Metaphase Spreads Prepared from Embryonic Fibroblasts

Early passage MEF cultures were prepared from $mTR^{+/+}$ (Wt) and $mTR^{-/-}$ second generation (KO-G2), fourth generation (KO-G4), and sixth generation (KO-G6). The fluorescence intensity of individual telomeres and, separately, the p-arm telomeres and q-arm telomeres are shown for all 25–31 metaphases analyzed at each generation. Telomere fluorescence intensity is expressed so that 1 telomere fluorescence unit (TFU) corresponds to 1 kb of $(TTAGGG)_n$ sequence, according to results obtained from similarly hybridized and analyzed plasmids with a defined $(TTAGGG)_n$ length (Zijlmans et al., 1997). The differences in mean fluorescence intensity were significant ($p < 0.001$, Mann-Whitney rank sum test) between each group.

Table 2. Summary of Cytogenetic Observations and Quantitative FISH Data in Primary Embryonic Fibroblasts from $mTR^{+/+}$ and $mTR^{-/-}$ Mice

	Metaphases Analyzed	Aneuploidy (% Cells)[a]	Telomeres without Detectable TTAGGG Repeats (%)[b]	End-to-End Associations/Fusions (Frequency per Metaphase)[c]	TFUs of Individual Telomeres (Mean ± SD)		
					All	p-arm	q-arm
Wt	26	0	0	0	43.8 ± 16.8	37.2 ± 12.7	50.5 ± 17.8
KO-G2	31	23	58 (1.1)	0.26	29.0 ± 18.0	22.8 ± 14.7	35.1 ± 18.8
KO-G4	25	28	96 (2.4)	0.56	25.0 ± 16.6	18.1 ± 11.4	31.9 ± 18.0
KO-G6	27	56	282 (5.3)	1.93	13.9 ± 10.6	9.7 ± 7.7	18.0 ± 11.5

Wt = Wild type; KO-G2, KO-G4, and KO-G6 represent $mTR^{-/-}$ mice of second, fourth, and sixth generations, respectively.
[a] Expressed as percentage of metaphases with other than 40 chromosomes (each Robertsonian fusion was counted as two chromosomes).
[b] The total number of telomeres lacking TTAGGG signal is shown. The number in parentheses is the percent of all ends. For this calculation, each metaphase chromosome was expected to show four fluorescent spots. If only two spots were observed, the corresponding telomere was assumed to lack detectable TTAGGG at one end. We estimate that those ends lacking telomere signals have <0.2 kb TTAGGG repeats.
[c] Expressed as total number of end-of-end associated chromosomes per metaphase, including dicentric chromosomes, telomere associations, and Robertsonian fusions.

whereas those derived from $mTR^{-/-}$ MEFs had no detectable telomerase activity (data not shown). All transformed cell lines had a similar morphology and growth rates in culture. When injected, both G1 $mTR^{-/-}$ and $mTR^{+/+}$ transformed cell lines generated tumors in nude mice, while untransformed control cells did not (Figure 7A). These results indicate that telomerase activity per se is not essential for transformed cells to form tumors in nude mice.

Because established cell lines may have multiple genetic changes and may have activated an alternative telomere maintenance pathway, we also used primary MEFs to determine the role of telomerase in cell transformation and tumor growth. Early passage MEF cultures from G1 $mTR^{+/+}$ and $mTR^{+/-}$ and G1, G2, G3, G4, and G6 $mTR^{-/-}$ mice were infected with a retrovirus carrying a construct that directs the expression of the adenovirus E1A, ras^{v12}, and the puromycin drug resistance gene (see Experimental Procedures). After infection, foci were selected, and 10^5 cells were injected at a total of four sites in nude mice to assay tumor formation. Tumors were generated from the $mTR^{+/+}$ and all of the $mTR^{-/-}$ G1, G2, G3, G4, and G6 derived cells after a similar latency. Although there was significant variation in tumor size, cells derived up to the sixth generations of $mTR^{-/-}$ animals were capable of tumor formation (Figures 7B and 7C). These data indicate that telomerase null cells can form tumors in nude mice.

Discussion

Telomerase activity has been proposed to be required for cell immortalization and tumor progression in humans and mice (reviewed in Autexier and Greider, 1996). To evaluate the role of telomerase in mammalian development, cell growth, and tumor formation, we deleted the gene encoding the telomerase RNA component from the mouse germline. Cells and tissues from $mTR^{-/-}$ mice lacked detectable telomerase activity. The $mTR^{-/-}$ mice are viable through at least six generations. Quantitative FISH analysis showed that progressive loss of $(TTAGGG)_n$ telomere sequence repeats occurred with each generation and that chromosomal rearrangements were frequent in the later generations of mice.

The rate of telomere shortening in mouse cells appears to be similar to that reported for humans cells. In $mTR^{-/-}$ mice, telomeres shortened at a rate of 4.8 ± 2.4 kb per mouse generation. From one mouse generation to the next, there are about 62 cell divisions in the male and 25 in the female germline (Drost and Lee, 1995). On a per cell division basis, a loss rate of 50 to 100 base pairs, as seen in human and M. spretus primary cells (Harley et al., 1990; Allsopp et al., 1992; Prowse and Greider, 1995), would generate a 3-to-6-kb loss in the male germline and a 1-to-3-kb loss in the female germline. Our experimental data fit this prediction remarkably well. This agreement between the rate of telomere shortening in $mTR^{-/-}$ mice and primary fibroblasts supports the hypothesis that telomere shortening in primary cells is due to the lack of telomerase activity as proposed (Cooke and Smith, 1986; Harley et al., 1990; Hastie et al., 1990).

Aneuploidy and chromosomal end fusions were frequent in cultures from $mTR^{-/-}$ mice in G2 to G6 cultures, providing striking evidence that the ability of telomeres to protect chromosome ends is lost when telomerase is absent and telomere shortening occurs. The appearance of Robertsonian fusions is of particular interest, since this type of rearrangement has been predicted to result from loss of telomere function. Robertsonian chromosomal rearrangements in mice typically lack pericentromeric telomeric DNA and contain variable amounts of minor satellite sequences, indicating the elimination of substantial amounts of chromosomal DNA during their formation (Garagna et al., 1995; Nanda et al., 1995). The Robertsonian fusions observed in $mTR^{-/-}$ mice also lack pericentromeric telomeric DNA but are not expected to show loss of minor satellite DNA. Further analysis of the molecular composition of the metacentric fusion area and the stability of $mTR^{-/-}$ Robertsonian fusion chromosomes will provide important insights into the generation of these chromosome fusions.

Human cells will stop dividing or senesce after 40 to 80 cell divisions, and permanent lines are only generated if senescence is bypassed by oncogenic virus or mutation. Immortal cells can be selected after a growth crisis in the oncogene-expressing culture. In contrast, mouse and other rodent cell cultures undergo a period of slow growth and then spontaneously generate established cell lines (Todaro and Green, 1963). The period of slow growth in MEFs has been referred to in the literature as either senescence (Meek et al., 1977; Harvey and Levine,

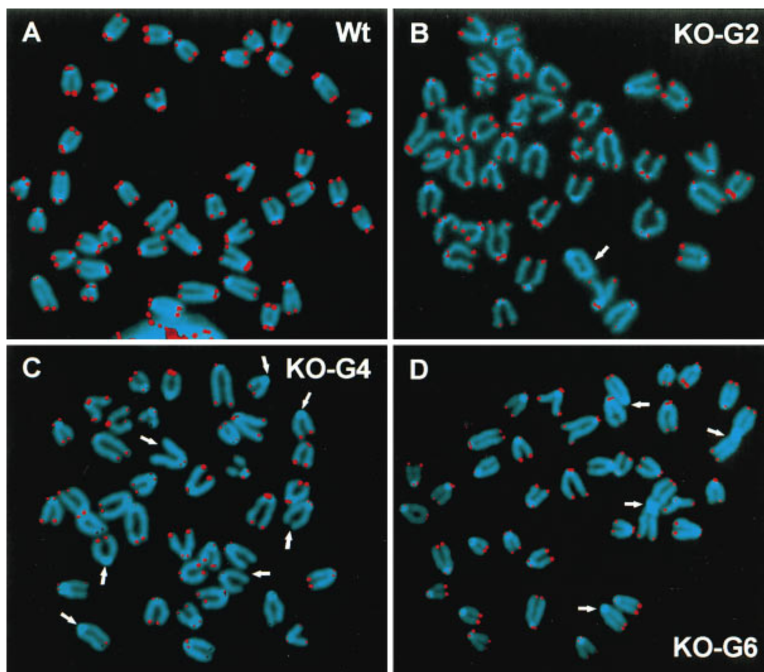

Figure 6. Telomere Fluorescence of Metaphase Chromosomes from Embryonic Fibroblasts

Fibroblasts from *mTR*$^{+/+}$ (Wt) and indicated generations of *mTR*$^{-/-}$ null mice (KO, G2–G6) were hybridized with a Cy3 labeled $(CCCTAA)_3$ PNA probe. Arrows indicate telomeres without detectable TTAGGG. Images were obtained using a Zeiss Axioplan fluorescence microscope coupled to a charge-coupled device (CCD) camera. Separate images of DAPI-stained chromosomes and telomeres hybridized with $(CCCTAA)_3$ PNA probe were merged and assigned pseudocolors using Adobe Photoshop (Adobe Systems, Mountain View, California).

(A) Metaphase from Wt cells showing distinct bright signal intensities for all telomeres.

(B) A KO-G2 cell showing decreased overall fluorescence and occasional absence of telomere fluorescence signal.

(C) A KO-G4 metaphase showing strong heterogeneity in the telomere signal intensities. Many telomeres show little if any fluorescence (arrows), yet bright telomere staining can be seen on some chromosome ends.

(D) Metaphase from a KO-G6 cell. Note the overall weaker telomere fluorescence and the increased frequency of telomeres without detectable TTAGGG. Two Robertsonian fusions in this metaphase were observed with no detectable TTAGGG at the fusion point (arrow).

1991; Ikram et al., 1994) or crisis (Kraemer et al., 1986; Rittling, 1996). We found no difference in the ability to overcome senescence/crisis between the G1 *mTR*$^{+/+}$, *mTR*$^{+/-}$, and G1 through G6 *mTR*$^{-/-}$ primary cultures using both the 3T3 growth protocol and the colony formation assay. Strikingly, some chromosomes in *mTR*$^{-/-}$ G2 and later primary MEF cultures lacked detectable telomeric repeats. While the induction of senescence may differ in human and mouse cells, our data in mouse cells suggest that telomere length is not a primary determinant of senescence/crisis in mouse cells and that telomerase activity is not required in mouse cells to allow escape from the senescence/crisis point.

A striking finding from this work is that oncogenically transformed telomerase null mouse cells with severely shortened telomeres can form tumors in nude mice. To generate a sizable tumor from a single cell in a mouse requires only about 40 to 50 cell divisions. Yet, *mTR*$^{-/-}$ G6 MEFs that have already undergone over 300 cell divisions in vivo still formed tumors. Thus, while telomerase is activated in mouse tumors in vivo (Bednarek et al., 1995; Chadeneau et al., 1995; Blasco et al., 1996; Broccoli et al., 1996), it appears not to be required for growth during the 40 or more divisions necessary for tumor formation. This raises the question of why telomerase is activated in mouse tumors if it is not essential for tumor growth. Although initial work with human cells suggested that telomerase was required for tumor cell viability after critical telomere shortening, recent studies of mouse tumors concluded that telomerase was activated even in the absence of critical telomere shortening (Broccoli et al., 1996). At the time of their report, it was not clear how many divisions a mouse cell could undergo without telomerase before viability would be compromised. Our data indicate that telomerase null mouse cells can be established in culture and grow for more than 200 cell divisions without loss of viability and that *mTR*$^{-/-}$ G6 cells that have gone though an estimated 300

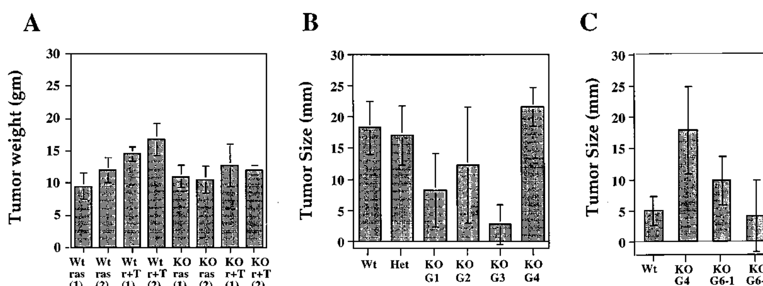

Figure 7. Tumor Formation in Nude Mice by *mTR*$^{-/-}$ Transformed Cells

(A) Tumor formation in nude mice from TAg and ras^{v12} transformed *mTR*$^{+/+}$ and G1 *mTR*$^{-/-}$ cells. Each transformed cell line (5×10^5 cells) was injected at a total of four different sites (two sites in each of two mice), collected, and weighed 20 days after injection. Tumor size for each cell line is plotted as the average of four tumors arising from four different injection sites (two each on two different mice).

(B) Tumor formation in nude mice injected with E1A and ras^{v12} transformed cells from wild type and *mTR*$^{-/-}$ G1, G2, G3, and G4 embryos. Transformed cells were selected after infection with the retroviral vector, and 1.5×10^6 cells were injected into nude mice at four independent sites. The average size of the tumor 47 days after injection is plotted.

(C) A second experiment was done similar to that described in (B) using wild type, *mTR*$^{-/-}$ G4 cells, and two different isolates of *mTR*$^{-/-}$ G6 cells. A total of 1.5×10^6 cells was injected at four independent sites, and the size of the tumor is shown 55 days after injection.

cell divisions or more are viable. This further supports the notion that telomerase activation during mouse tumorigenesis may not be a response to critical telomere shortening. Perhaps telomerase activation is linked to some other pathway that is essential for tumor growth and is passively coselected during tumorigenesis. In fact, evidence is accumulating that telomerase activity may be best correlated with a high rate of proliferation in those cells that express it (Blasco et al., 1996; Broccoli et al., 1996; Kyo et al., 1997; Shay and Bacchetti, 1997).

Telomerase-negative cells that escape senescence/crisis might maintain telomere length by a recombinational mechanism. Yeast cells defective for telomere maintenance but possessing a wild-type recombination system generate rare "survivors" after extended growth in culture. In these cells, gene conversion allows lengthening of telomere repeat tracts. When the yeast *RAD52* gene is deleted, gene conversion is severely reduced, and survivors are not generated (Lundblad and Blackburn, 1993; McEachern and Blackburn, 1996). Some human cell lines that lack detectable telomerase activity are thought to maintain telomeres via such a recombination-mediated pathway (Bryan et al., 1995). Although no evidence of greatly elongated telomeres was seen in early passage $mTR^{-/-}$ G2 through G6 MEFs using FISH, it will be interesting to follow telomeres during establishment and long-term growth in culture using this technique to determine if recombination plays a role in the selection of cells at senescence/crisis.

Although mice provide a powerful tool for understanding cancer progression, there are some significant differences between human and mouse biology that may have implications for tumor growth. First, human telomeres are on average shorter than mouse telomeres. Telomere shortening and telomerase activation might play a role in human tumor growth and viability while having little effect on mouse tumorigenesis. A second major difference between human and mouse tumorigenesis is that mouse cells spontaneously immortalize, while human cells do not. Whether this difference represents an additional level of growth control in human cells remains to be established. Despite these differences between human and mouse cells, the observation that in mice telomerase activation occurs after less than 40 divisions, while the enzyme is not required for tumor growth after more than 300 divisions, raises the possibility that not all telomerase-positive human tumors may require telomerase. It is possible that for some human tumors, telomerase may be required for growth; however, additional evidence besides the presence of high levels of telomerase will be needed to verify this in the light of the data presented here. Further analysis of the *mTR*-deficient mice may lead to new insights into the role of telomeres in cell biology and in tumorigenesis.

Experimental Procedures

mTR Knockout Construct

For the *mTR* genomic deletion, site-directed mutagenesis was used to introduce two new unique SacI restriction sites (*SacI*, Figure 1A) into a 3.3 kb XbaI genomic fragment and a 4.0 kb XhoI genomic fragment (Figure 1A). These sites allow the correct homologous recombination events to be identified by digestion of genomic DNA with SacI. Using a 1.2 kb probe (indicated in Figure 1), SacI-digested DNA generated a 6.3 kb wild-type band or a 4.7 kb band from the correctly targeted deletion allele. The site-directed mutagenesis was carried out using the Amersham Sculptor In Vitro Mutagenesis system and the sites were confirmed by sequencing. The mutagenized fragments were cloned into the targeting vector pPNT (Tybulewicz et al., 1991) to generate the plasmid pPNT-mTRΔ. The plasmid contained a neomycin resistance gene for replacement of the *mTR* gene and the HSV TK gene for counterselection in ganciclovir of randomly integrated constructs (Thomas and Cappechi, 1987).

ES Cell Culture and Blastocyst Injection

To generate ES cells with targeted integration, the pPNT-mTRΔ plasmid was linearized with NotI, and the DNA was electroporated into WW6 ES cells (Ioffe et al., 1995) by standard protocols (Ramirez-Solis et al., 1993). Cells were selected in G418 (100 to 150 μg/ml) and ganciclovir (2 μM), and positive clones were picked after 9 to 10 days of selection into duplicate 48-well microtiter plates. Of the 449 neomycin/ganciclovir resistant clones initially screened, four contained a correctly targeted *mTR* locus (Tel-1, Tel-2, Tel-3, and Tel-4). The four clones were independently injected into C57BL/6J blastocysts, and the blastocysts were implanted into pseudopregnant mice. The chimeric progeny were identified by their mosaic coat color. The mice derived from the Tel-1 microinjections were mated to C57BL/6J to test for germline transmission of the *mTR* gene replacement, and heterozygous $mTR^{+/-}$ progeny were identified. To obtain further generations of telomerase null mice, the $mTR^{-/-}$ mice were mated to each other. All of the mice described in this report were derived from Tel-1 ES cells.

Isolation and Growth of Mouse Embryonic Fibroblasts

MEFs were prepared from day 13.5 embryos derived from crosses between either $mTR^{+/-}$ mice or $mTR^{-/-}$ mice from different generations. The whole embryo was minced and dispersed in 0.25% trypsin and incubated for 2 hr at 4°C. Cells were plated in 10 cm plates containing DMEM plus 10% FBS and incubated at 37°C until confluent. The first passage was designated PDL 2. MEFs were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Serial cultures were done according to the 3T3 protocol (Todaro and Green, 1963). In brief, 10^6 cells were plated on a 10 cm dish; after 3 days, the cells were counted and 10^6 cells were replated. The population doubling number was calculated using the formula $\Delta PDL = \log(n_f/n_0)/\log 2$, where n_0 is the initial number of cells and n_f is the final number of cells. For colony formation assay, 3.5×10^3 cells were distributed on a total of ten 6 cm diameter dishes and incubated in DMEM plus 10% FBS. After 8 days, the dishes were stained with Giemsa, and the number of visible colonies with >1.5 mm diameter was scored. For the reintroduction of *mTR* into $mTR^{-/-}$ cells, a $mTR^{-/-}$ established cell line, KO-3 at passage 45, was transfected using calcium phosphate procedures (Ausubel et al., 1993) with 20 μg of a 5 kb genomic fragment containing the *mTR* gene (Blasco et al., 1995) or an empty Bluescript vector. Forty-eight hours after transfection, cells were collected from the plate, and S100 extracts were prepared and assayed for telomerase activity.

Telomerase Assays

S100 extracts were prepared from tissues and from cell cultures as described previously (Blasco et al., 1995). Telomerase activity was measured using a modified version (Blasco et al., 1995) of the TRAP assay (Kim et al., 1994) as well as the conventional telomerase assay (Prowse et al., 1993). For the modified TRAP assay, a telomerase reaction was first carried out under optimal conditions of the enzyme, and a portion of the products were then amplified by PCR (Blasco et al., 1996). When indicated, an internal control for PCR efficiency was included in the TRAP assay (TRAPeze kit, Oncor).

Pulsed-Field Gel Electrophoresis and TRF Analysis

Cells were isolated and embedded in agarose plugs following instructions from the vendor (CHEF agarose plug kit from Bio-Rad). DNA embedded in agarose plugs was digested with MboI and electrophoresed through 1% agarose gels in 0.5× TBE maintained at 12°C, using a CHEF DR-II pulsed-field apparatus (Bio-Rad). Separation was for 23 hr at 6 V/cm at a constant pulse time of 5 s. The gel was blotted and probed with a 1.6 kb fragment containing the

sequence $(TTAGGG)_n$ (a gift from T. de Lange; see Hanish et al., 1995) and later with the mouse major satellite repeat (a gift from D. Kipling).

Fluorescence In Situ Hybridization

Primary embryonic fibroblasts from $mTR^{+/+}$ (wild type) and $mTR^{-/-}$ second generation (KO-G2), fourth generation (KO-G4), and sixth generation (KO-G6) were harvested from early passage cultures following treatment with colcemid (0.1 μg/ml) for 4 to 5 hr. Cells were trypsinized and spun for 8 min at 120 × g. After hypotonic swelling in sodium citrate (0.03 M) for 25 min at 37°C, the cells were fixed in methanol:acetic acid (3:1). After two to three additional changes of fixative, the cell suspension was dropped on wet, clean slides and dried overnight. FISH with Cy-3 labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) and quantitative analysis of digital images was performed as described (Zijlmans et al., 1997).

Retroviral Infections

Phoenix cells (5 × 10^6) were plated in a 10 cm dish, incubated for 24 hr, and then transfected by calcium phosphate precipitation with 25 μg of a retroviral plasmid pLPC-EIR (a gift from S. Lowe). This construct is based on a Maloney retrovirus expressing puromycin phosphotransferase from the LTR and coexpressing E1A 12S cDNA and oncogenic ras^{v12} cDNA from the CMV promoter via an internal ribosome entry site. After 48 hr, the virus was collected, and retroviral infections of the target fibroblasts were carried out as described (Serrano et al., 1997). Sixteen hours after the infection, cells were selected in 2 μg/ml puromycin for 2 days and collected for transformation assays and tumor formation studies in nude mice.

Nude Mouse Tumor Assays

BALB/c nude mice (Taconic Farms) were injected at each of the two sites with 0.5 × 10^6 cells transformed with TAg and ras^{v12}. For the E1A and ras^{v12} transduced foci, 1.5 × 10^6 cells were injected to each site. Mice were monitored daily, and tumor growth was measured by caliper every 3 days. Tumors were removed from all of the animals in a given experiment when some animals had substantially large tumors.

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