

Telomerase Activation in Mouse Mammary Tumors: Lack of Detectable Telomere Shortening and Evidence for Regulation of Telomerase RNA with Cell Proliferation

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Activation of telomerase in human cancers is thought to be necessary to overcome the progressive loss of telomeric DNA that accompanies proliferation of normal somatic cells. According to this model, telomerase provides a growth advantage to cells in which extensive terminal sequence loss threatens viability. To test these ideas, we have examined telomere dynamics and telomerase activation during mammary tumorigenesis in mice carrying a mouse mammary tumor virus long terminal repeat-driven *Wnt-1* transgene. We also analyzed *Wnt-1*-induced mammary tumors in mice lacking p53 function. Normal mammary glands, hyperplastic mammary glands, and mammary carcinomas all had the long telomeres (20 to 50 kb) typical of *Mus musculus* and did not show telomere shortening during tumor development. Nevertheless, telomerase activity and the RNA component of the enzyme were consistently upregulated in *Wnt-1*-induced mammary tumors compared with normal and hyperplastic tissues. The upregulation of telomerase activity and RNA also occurred during tumorigenesis in p53-deficient mice. The expression of telomerase RNA correlated strongly with histone H4 mRNA in all normal tissues and tumors, indicating that the RNA component of telomerase is regulated with cell proliferation. Telomerase activity in the tumors was elevated to a greater extent than telomerase RNA, implying that the enzymatic activity of telomerase is regulated at additional levels. Our data suggest that the mechanism of telomerase activation in mouse mammary tumors is not linked to global loss of telomere function but involves multiple regulatory events including upregulation of telomerase RNA in proliferating cells.

Telomerase is a cellular RNA-dependent DNA polymerase that can maintain the tandem arrays of telomeric repeats at eukaryotic chromosome ends (reference 26; reviewed in references 6 and 25). Human and mouse telomerases elongate the 3' ends of chromosomes with strings of TTAGGG repeats (49, 50). This motif is dictated by a short template sequence within the RNA components of mammalian telomerases (7, 23). When telomerase is absent, chromosomes show progressive terminal sequence loss with cell divisions (23, 46, 57), probably reflecting the fact that the chromosomal replication machinery cannot duplicate DNA ends (61). The maintenance of the telomeric repeat array and its associated protein complex is essential for chromosome stability. Uncapped chromosomes are sensitive to degradation and fusion and can activate DNA damage checkpoints (40, 45, 54). Because of its ability to replenish lost telomeric sequences, telomerase is thought to be required for long-term cell proliferation.

Human telomerase has been implicated in cellular immortalization and tumorigenesis. High levels of telomerase activity have been demonstrated in immortalized cell lines (16, 17, 34, 49) and in the majority of human cancers (references 19 and 34; reviewed in reference 29). In normal human cells, telomerase activity is lower (9, 18, 31) or not detectable (19, 34). These variations in telomerase activity correlate with the dynamics of human telomeres. Telomere shortening at a rate of 50 to 200 bp per population doubling is a general phenomenon

in normal human somatic cells (reference 28; reviewed in reference 29). In contrast, immortalized cell lines with high telomerase activity have stable telomeres or show telomere elongation (16, 17, 19, 39). One explanation for the increased telomerase activity in cancer is that restored telomeres confer a selective growth advantage during tumor progression (reviewed in reference 20). According to this model, telomere shortening in the early stages of tumor development results in loss of telomeric function, possibly because of a failure to engage the telomere-binding protein, hTRF (13). This in turn leads to the activation of DNA damage checkpoints, followed by cell cycle arrest and possibly apoptosis. In tumors that have lost the ability to detect uncapped chromosome ends, telomere malfunction might lead to extreme genome instability. Cells with high levels of telomerase activity may not experience the decreased viability associated with telomere malfunction and eventually dominate the later stages of tumorigenesis.

In order to test the idea that telomere attrition drives selection of telomerase-positive tumor cells, we have focused on telomere dynamics and telomerase activation in a mouse tumor model. Mouse telomerase activity has been demonstrated in a number of immortalized cells, tumors, and normal tissues (12, 50, 51). However, unlike human somatic cells in which the telomeres are composed of 5 to 10 kb of TTAGGG repeats (2, 3, 21), murine (*Mus musculus*) tissues have telomeres that are remarkably long, with telomeric tracts in the 20- to 50-kb range (37, 58). Since there is no indication for accelerated telomeric decline in the murine soma (37), it is expected that the telomeric tracts will not be depleted during tumor development, thereby obviating the need for increased telomere maintenance during tumorigenesis.

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We have employed a murine system in which mammary tumors are induced by a mouse mammary tumor virus long terminal repeat-driven *Wnt-1* transgene, which results in ectopic expression of the *Wnt-1* proto-oncogene in mammary tissue and salivary glands (60). *Wnt-1* transgenic animals have hyperplastic mammary glands and are predisposed to the development of mammary adenocarcinomas. The stochastic appearance of these tumors is consistent with the idea that tumor formation requires alterations in addition to expression of *Wnt-1* in the mammary glands. Candidate cooperating genes (such as *int-2*, *fgf-3*, and *fgf-4*) have been identified elsewhere (41, 43, 55). *Wnt-1*-induced tumorigenesis is strongly accelerated in animals lacking a functional *p53* gene, possibly because of increased genomic instability (22). *p53*-deficient tumors display multiple karyotypic abnormalities, including aneuploidy, dicentric chromosomes, amplifications, and deletions (22).

Here we report that the telomerase activity in *Wnt-1* tumors was increased 10- to 20-fold relative to that measured in normal and hyperplastic mammary glands. Telomerase RNA levels were elevated twofold in the tumors and correlated with the expression of histone H4 mRNA in each of the tissues. The results indicate that telomerase activation in these tumors includes at least two regulatory events, one of which involves upregulation of the telomerase RNA with cellular proliferation. The average telomere array size was not appreciably altered during tumorigenesis. Since telomerase activation does not appear to be selected through global telomere shortening, upregulation of telomerase in tumors may serve a role other than maintenance of telomere length.

MATERIALS AND METHODS

Cell lines and mouse strains. The mouse myeloma cell line J558 (ATCC TIB6) was cultured in Dulbecco modified Eagle medium supplemented with 10% bovine calf serum, antibiotics, and glutamine. The transgenic mice used in this study have been previously described (22, 60). Normal mammary glands described in Table 1 were derived from SJL mice. *Wnt-1* transgenic mice used in this study have been backcrossed to the SJL strain for at least 10 generations; all hyperplastic mammary glands and mammary tumors described in Table 1 were harvested from these animals. For the *p53*-related studies, *Wnt-1* transgenic mice were crossed to *p53*-deficient mice of 129/Sv genetic background (22) and the resulting progeny were interbred. *Wnt-1* transgenic offspring of *p53*^{+/+}, *p53*^{+/-}, and *p53*^{-/-} genotypes were monitored for mammary tumors. Tumors of 1.5 to 2.0 cm in diameter were harvested. A portion of the tumor was fixed and subjected to histopathological analysis to confirm the malignancies, and the remainder was frozen for the analysis performed here.

Protein extracts. All procedures were conducted at 4°C. Cell extract from the J558 cell line was prepared as previously described with 3-[(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate] (CHAPS) detergent buffer (34). Extracts from frozen solid mammary tissues were prepared by disrupting 100 to 250 mg of tissue in 0.25 to 1 ml of CHAPS buffer with a mechanical homogenizer (9, 34). The suspension was mixed gently for 30 min and centrifuged at 100,000 × *g* in a Beckman TL 100.3 rotor for 30 min. For samples from which DNA plugs for pulsed-field gradient electrophoresis (see below) were prepared, the lysate was centrifuged at setting 4 in an Eppendorf microcentrifuge for 15 min to collect the nuclei before centrifugation of the supernatant at 100 K. For a subset of the preparations, one-half of the S100 was flash frozen while the remaining supernatant was first dialyzed against 50 mM KCl–20 mM HEPES(*N*-2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.9)–0.2 mM EDTA–0.2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]–1 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–20% glycerol for 2 h. In general, dialysis did not affect the telomerase activity but did remove nonspecific inhibitors of the reactions involved in the telomeric repeat amplification protocol (TRAP) present in some of the samples. Protein concentrations were determined by the Bradford assay (Bio-Rad) and bovine serum albumin as a standard. Western blot (immunoblot) analysis using an antibody which recognizes the heterogeneous nuclear ribonucleoprotein particle D group (32) was used to confirm protein concentrations and establish the absence of protein degradation.

Telomerase assay. Telomerase activity was detected by the PCR-based TRAP assay (34) with modifications and quantitation procedures as described previously (9). To measure telomerase activity, extract from each sample was first titrated between 0.1 and 1 μg to determine the protein range at which the TRAP products were proportional to the amount of protein. As was previously noted for human cell extracts (9, 63), the TRAP assay of mouse telomerase is quanti-

tative when 0.05 to 1.0 μg of protein is assayed per reaction. TRAP assay products were visualized, and quantitation was done with a PhosphorImager and ImageQuant software (Molecular Dynamics). Telomerase activity was determined by summing the amount of signal present in TRAP assay products and correcting for background. The summed signal was then normalized to the amount of protein used to yield specific telomerase activities. The relative specific telomerase activity was determined by comparing the normalized TRAP product signals of experimental extracts with the normalized TRAP product signal obtained by using J558 extract from an assay performed in parallel. The level of specific telomerase activity in J558 was set to 100% in each assay, and the relative specific telomerase activities of the experimental extracts are expressed as a percentage of the specific telomerase activity found with the J558 standard. For each sample, the relative specific telomerase activity was determined from two to six independent assays. Similar amounts of protein were used in J558 and experimental extracts, precluding the need for extensive extrapolation. However, assays using different protein concentrations within the linear range of the dose-response curve of the assay resulted in similar values for the relative specific telomerase activity of any given sample. Samples with low levels of telomerase activity were mixed with the J558 standard to determine whether they contained an inhibitor of telomerase. None of the samples discussed in this study contained an inhibitor of the J558 telomerase standard.

Pulsed-field gel electrophoresis and genomic blotting. Nuclear pellets (see above) were washed three times in phosphate-buffered saline, cast in low-melting-point agarose plugs, and treated as previously described (1, 37). Restriction endonuclease digestions with *Mbo*I, *Rsa*I, and *Bam*HI were carried out according to manufacturers' recommendations. DNA was resolved on 1% agarose–0.5× Tris-borate-EDTA gels with a contour-clamped homogeneous electric field–DR11 apparatus (Bio-Rad) for 20 h at 180 V with a constant pulse time of 5 s at 13°C. The DNA was subjected to acid depurination and transferred to Hybond N membranes (Amersham) by standard procedures. The telomeric oligonucleotide (TTAGGG)₄ was 5' end labeled with [γ-³²P]ATP and T4 polynucleotide kinase. Hybridizations were carried out as described previously (38) at 65°C. The filters were washed in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C prior to exposure to autoradiographic film.

RNA analysis. RNA was extracted from solid tissues by disrupting the tissue on ice with a mechanical homogenizer in 10 volumes of 6 M urea–3 M LiCl as described previously (5). RNAs (15 μg) were fractionated on 1% agarose-formaldehyde gels and transferred to Nytran membranes (Schleicher and Schuell) in 20× SSC. The mouse telomerase RNA probe (mTR [7]) and the histone H4 mRNA probe (kindly provided by N. Heintz, Rockefeller University) were labeled by the random priming reaction with 50 ng of isolated insert, [α-³²P]dCTP and [α-³²P]dGTP, and Klenow enzyme. Antisense oligonucleotides complementary to the mouse 7SK RNA polymerase III transcript (5' CAGCCAGAT CAGCCGAATCAACCCTG 3' and 5' TGGACCTTGAGAGCTTGTGG AGG 3' [48]) were 5' end labeled with [γ-³²P]ATP and T4 polynucleotide kinase. Membranes were hybridized sequentially with the mTR and H4 probes as described previously (14) at 65°C with intervening removal of the probe by boiling in 2 mM sodium phosphate buffer (pH 7.2)–15 mM NaCl–0.5% SDS. Final washes were at 65°C in 40 mM sodium phosphate buffer (pH 7.2)–1 mM EDTA–1% SDS. Hybridization and washes with the 7SK probes were carried out as described above for the (TTAGGG)₄ oligonucleotide (above). Membranes were exposed to autoradiographic film for the generation of the data in Fig. 3 and to PhosphorImager screens (Molecular Dynamics) for quantitation.

RESULTS

Mouse telomeres do not shorten detectably during tumorigenesis. Telomeric restriction fragments were analyzed by pulsed-field gradient gel electrophoresis of DNA from normal and hyperplastic mammary glands and mammary tumors. In order to detect telomeric loci, the DNAs were digested with frequently cleaving restriction endonucleases (*Mbo*I or *Rsa*I) and probed with a telomere-specific oligonucleotide (Fig. 1). As expected, on the basis of previous reports of *M. musculus* telomeres (37, 58), the bulk of the telomeric fragments migrate in the 15- to 100-kb range with a peak of hybridization intensity around 25 to 30 kb. These DNA fragments have been shown to originate from the physical ends of chromosomes, on the basis of the telomere-specific in situ hybridization pattern of TTAGGG repeats (24, 47) and the sensitivity of these loci to exonuclease BAL 31 (37, 58). The largest telomeric fragments (50- to 150-kb range) in part originate from centromere-proximal telomeres and contain pericentromeric satellite sequences (36). These regions show a high degree of polymorphism between inbred strains as well as between individual mice (36, 37, 58) (Fig. 1).

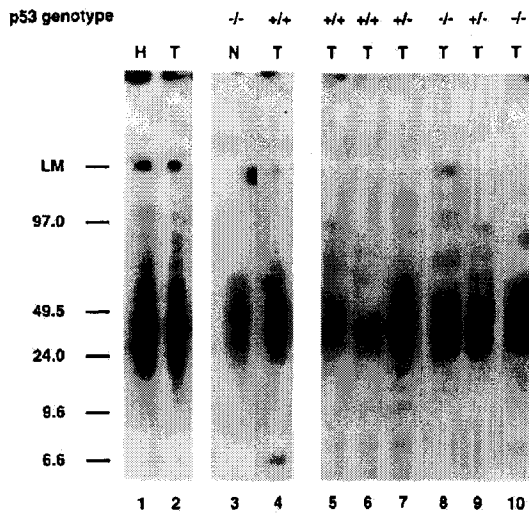


FIG. 1. Absence of telomere shortening during mouse mammary tumorigenesis. DNAs from normal mammary glands (N), hyperplastic mammary glands (H), and mammary tumors (T) were cleaved with either *Mbo*I (lanes 1 to 4) or *Rsa*I (lanes 5 to 10), resolved on contour-clamped homogeneous electric field gels, and annealed to a (TTAGGG)₄ probe. The DNAs in lanes 1 and 2 are derived from the same *Wnt-1* transgenic mouse. The *p53* genotype of the mice is indicated above the lanes for those samples that originated from crosses involving mice with *p53* deficiencies. The lanes contained similar amounts of DNA with the exception of lane 6, which was slightly underloaded. The fragments in the 4- to 10-kb range are thought to be derived from chromosome-internal telomere-related sequences on the basis of their discrete size and their apparent identical size in different mice. The migration and molecular size (kilobases) of marker DNAs are indicated (LM is the limit of mobility).

Telomere size was found to be very similar in four normal mammary glands from control SJL mice, in four *Wnt-1*-induced hyperplastic mammary glands, and in four mammary tumors from *Wnt-1* transgenic mice. In each case, the bulk of telomeric fragments migrated in the 25- to 30-kb range (Fig. 1 and data not shown). This was true for DNA samples cleaved with *Mbo*I as well as for those cleaved with *Rsa*I and *Bam*HI. A side-by-side comparison of the telomeric restriction frag-

ment patterns of a hyperplastic mammary gland and a mammary carcinoma derived from a single mouse is shown in Fig. 1. No alteration in telomere length was detectable in this or other tumor samples. In addition, we failed to detect changes in the length of the telomeric fragments or their annealing to TTAGGG repeat probes in three normal mammary glands and eight mammary carcinomas from mice with heterozygous or homozygous *p53* deletions (Fig. 1 and data not shown).

In human cells, increased chromosome instability occurs when the bulk of the telomeres have shortened to less than 2 kb of TTAGGG repeats (16). The data on the transgenic mice presented here are incompatible with such a dramatic decline and argue against transitory changes in telomere length during tumorigenesis in this system. Although we cannot rule out loss of a few kilobases from one or more telomeres, this level of shortening is unlikely to have compromised telomere function since at least 20 kb of TTAGGG repeats remains at the chromosome ends.

Elevated telomerase activity in mammary tumors. Telomerase activity was detectable by the PCR-based TRAP assay (34) in each of 15 normal mammary glands, 8 hyperplastic mammary glands, and 24 mammary tumors (Fig. 2). In each case, the activity resulted in the 6-nucleotide (nt) ladder typical of telomerase, and in each case pretreatment of the extract with RNase A inhibited the formation of TRAP products (Fig. 2 and data not shown).

Quantitative analysis was carried out to determine the relative enzyme activity in the extracts. We have previously shown that differences in telomerase activity between human tissue extracts can be determined in a quantitative manner with the TRAP assay and an internal standard (9). Similarly, we found that the telomerase levels in mouse cell extracts can be quantitated in relation to a standard (see Materials and Methods section for details). By this method, the specific telomerase activity (TRAP assay products per microgram of protein) in each extract is compared with a standard extract derived from the murine J558 cell line (Fig. 2). The relative specific activity (averaged from two to five independent assays) is expressed as a percentage of the specific telomerase activity found in the J558 standard (Table 1). For each sample, an RNase A digestion is included to confirm that the TRAP products are attrib-

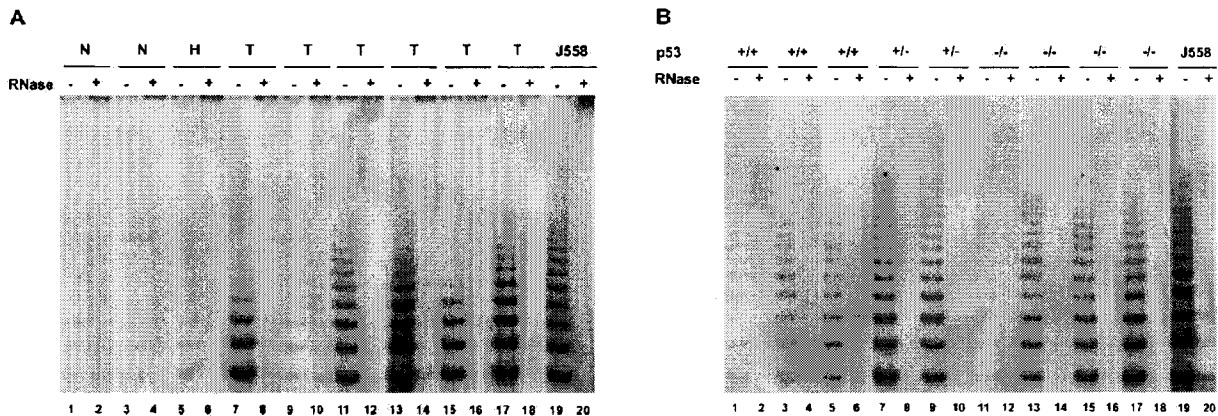


FIG. 2. Telomerase activity in normal and hyperplastic mammary glands and in mammary tumors. Telomerase activity was determined in tissue extracts by a modified version of the TRAP assay (see Materials and Methods for details) and 0.2 to 0.8 μ g of protein. The murine myeloma cell line J558 is assayed in parallel as a standard (lanes 19 and 20). (A) Telomerase activity in normal SJL mammary glands (N, lanes 1 to 4), *Wnt-1* transgenic hyperplastic mammary glands (H, lanes 5 and 6), and *Wnt-1* transgenic mammary carcinomas (T, lanes 7 to 18). (B) Telomerase activity in *Wnt-1*-induced mammary tumors from mice with different *p53* genotypes. Lanes 1 to 18 contain TRAP products obtained with mammary tumor extracts. The genotype of the mice carrying the tumors is indicated above the lanes. Even-numbered lanes in both panels contain TRAP products obtained with extracts that are treated with RNase A.

TABLE 1. Activation of telomerase in *Wnt-1* TG mammary tumors

Type and designation of sample	Telomerase activity ^a
Normal mammary gland	
MG 2B	4.4 (2)
MG 4B	7.6 (2)
MG 5B	2.7 (2)
MG 6B	5.9 (2)
MG 7B	1.2 (2)
MG 8B	3.0 (2)
MG 10B	1.5 (2)
MG 1-1	1.6 (2)
MG 2	5.6 (2)
MG 4	3.9 (3)
MG 2200	7.8 (2)
MG 2300	2.5 (3)
Median	3.1 (<i>n</i> = 12)
Hyperplastic mammary gland	
hMG 2	3.1 (2)
hMG 3	13.8 (4)
hMG 4	3.0 (2)
hMG 11	2.1 (2)
hMG 14	1.8 (2)
hMG K4	11.6 (3)
hMG 1328	9.5 (3)
hMG 1546	2.1 (2)
Median	3.1 (<i>n</i> = 8)
Mammary tumor	
MT 1	51 (4)
MT 2	43 (5)
MT 3	33 (2)
MT 4	5 (3)
MT 5	45 (5)
MT 6	30 (2)
MT 15B	59 (3)
MT 16B	89 (2)
MT 17B	34 (2)
MT 18B	63 (3)
MT K4	357 (2)
MT 1328	73 (5)
MT 1329	59 (3)
MT 1563	56 (2)
Median	54 (<i>n</i> = 14)

^a Telomerase activity is expressed as relative specific activity normalized to a mouse J558 standard. Average percent activity was determined from two to five assays as indicated in parentheses for each sample.

utable to telomerase. As an additional control, duplicate extracts were prepared from 12 mammary tissue samples and found to give reproducible results (data not shown). It should be noted, however, that these methods do not control for possible variations in nuclease and protease activities, which could potentially reduce the recovery of active telomerase in the extracts.

Quantitative analysis indicated that the telomerase activity in normal and *Wnt-1* transgenic hyperplastic mammary glands was low, ranging from 1 to 14% of the J558 standard (Table 1). The median values for 12 normal mammary glands and 8 hyperplastic glands were identical (3.1%) (Table 1). In comparison with the normal and hyperplastic glands, the telomerase activity was significantly elevated in the *Wnt-1* transgenic mammary tumors (Fig. 2A and Table 1). The majority (13 of 14) of the tumors had telomerase levels that were greater than 30% of the standard (Table 1), and the median specific activity

was 54%, which is 10- to 20-fold higher than in the nonmalignant tissues (Table 1). These data indicate that telomerase is upregulated in the majority of the *Wnt-1*-induced mouse mammary carcinomas and that this upregulation occurs at a stage after the formation of hyperplastic tissues. Elevated telomerase activity has also been documented in human breast carcinoma (30, 34).

Telomerase activation in p53-deficient mice. Telomerase activity was detectable by TRAP assay in all tested normal mammary glands from p53-deficient mice, in malignant mammary glands from *Wnt-1* transgenic mice lacking a functional p53 gene, and in tumors arising in p53 heterozygous mice carrying the *Wnt-1* transgene (Fig. 2A and Table 2). Normal mammary gland samples from p53^{-/-} animals not carrying the *Wnt-1* transgene were assayed and revealed a low basal level of telomerase (approximately 9.3% [Table 2]). Similar to what was observed in mice with functional p53 genes, the telomerase activity in the p53-deficient mice is consistently elevated in the *Wnt-1*-induced mammary tumors (Table 2). These results indicate that p53 function is not required for the suppression of telomerase in normal tissues or for the upregulation of the enzyme activity during mouse mammary tumorigenesis.

The *Wnt-1* transgenic/p53-deficient animals used in this study were derived from crosses of SJL mice carrying the *Wnt-1* transgene to 129/Sv mice lacking a functional p53 gene. The resulting F₂ litters also contained *Wnt-1* transgenic animals with a p53^{+/+} genotype. The telomerase activity in *Wnt-1*-induced tumors from five such p53^{+/+} mice was approximately twofold lower than that in tumors from their p53^{-/-} littermates (24 versus 54% [Table 2]), suggesting a subtle effect of p53 status on the activation of telomerase. However, the tumor telomerase levels in the p53^{+/+} animals from this cross are also approximately twofold lower than the telomerase activity in *Wnt-1*-induced tumors in SJL mice (24 versus 54%; compare Tables 1 and 2). Since SJL mice have functional p53 genes, this difference would suggest a strain-specific variation in the level of telomerase upregulation during mammary tumorigenesis. Further analysis is required to confirm these minor effects of genetic background and p53 status on the regulation of telomerase.

Correlation between telomerase RNA and histone H4 mRNA. Changes in the steady-state levels of the 430-nt mouse telomerase RNA (mTR) (7) were determined by RNA blotting in 6 normal mammary glands, 3 hyperplastic glands, and 11 tumors (Fig. 3A). The mTR signal was normalized to the signal obtained with probes for 7SK, a highly abundant and stable RNA polymerase III transcript that is expressed at the same level in normal and transformed mouse cells (11). Normalization of the mTR signals to the RNA component of RNase P gave the same results (data not shown). Telomerase template levels were similar in the normal and hyperplastic mammary glands and were elevated in the tumors, consistent with the increased telomerase activity in the mammary carcinomas (see Table 3). Upregulation of mTR did not appear to be affected by the p53 status of the animals. Although both the telomerase activity and the mTR levels are enhanced in the tumors, linear regression analysis indicated that there was no direct correlation between the enzymatic activity and the abundance of the telomerase RNA in individual samples (Table 3). In addition, the data indicate that the enzymatic activity is upregulated more strongly (10-fold) during tumorigenesis than the telomerase RNA (2-fold) (Table 3), suggesting that the activation of telomerase involves multiple levels of regulation. Differential regulation of telomerase activity and telomerase RNA has also recently been noted in mouse skin and pancreatic islet tumors (8).

TABLE 2. Activation of telomerase in *Wnt-1* TG mammary tumors in mice with different p53 genotypes

Type and designation of sample	Telomerase activity ^a
<i>p53</i> ^{-/-} normal mammary gland	
MG 1 ^{-/-}	9.3 (6)
MG 2 ^{-/-}	4.5 (2)
MG 3 ^{-/-}	18.3 (5)
Median.....	9.3 (<i>n</i> = 3)
<i>p53</i> ^{+/+} mammary tumor	
W2.....	17 (3)
W10.....	11 (2)
W30.....	36 (3)
W134.....	54 (3)
W151.....	24 (3)
Median.....	24 (<i>n</i> = 5)
<i>p53</i> ^{-/-} mammary tumor	
W98.....	100 (3)
W121.....	94 (2)
W154.....	56 (3)
W177.....	14 (3)
W184.....	55 (4)
Median.....	54 (<i>n</i> = 5)

^a Telomerase activity is expressed as relative specific activity normalized to a mouse J558 standard. Average percent activity was determined from two to six assays as indicated in parentheses for each sample.

The steady-state level of telomerase RNA showed a close correlation with histone H4 mRNA (Fig. 3 and Table 3), a marker for cell proliferation. Histone H4 mRNA is specifically expressed in proliferating cells, with the peak levels occurring in S phase (reviewed in reference 59). The observed correlation between H4 mRNA and mTR is not simply a consequence of elevated expression of both markers during tumorigenesis. The tumors showed a wide range of histone H4 mRNA levels,

TABLE 3. Comparison of levels of telomerase RNA, histone H4 mRNA, and telomerase activity

Sample ^a	mTR ^b	H4 mRNA ^b	Telomerase ^c
Normal and hyperplastic mammary gland ^e			
MG 2	0.29	0.50	5.6
MG 4	0.46	0.36	3.9
MG2200	0.49	0.97	7.8
MG 1 ^{-/-}	0.21	0.78	9.3
MG 2 ^{-/-}	0.42	0.78	4.5
MG 3 ^{-/-}	0.75	1.5	18
hMG 1328	0.45	0.42	9.5
hMG 1546	0.31	0.72	2.1
hMG K4	0.31	0.61	12
Median	0.45	0.61	7.8
Mammary tumor ^d			
MT 1328	1.0	1.0	73
MT 1329	0.56	0.48	59
MT 1563	1.3	2.3	56
MT K4	1.2	1.5	357
W2	0.99	1.3	17
W10	0.49	0.48	11
W134	0.98	1.2	54
W151	0.31	0.54	24
W98	0.60	1.3	100
W154	1.2	1.3	56
W177	0.66	0.80	14
Median	0.98	1.1	56

^a For genotypes of the mice from which these samples are derived, see Tables 1 and 2.

^b Telomerase template RNA (mTR) and histone H4 mRNA levels were normalized to 7SK RNA (see legend to Fig. 3).

^c Relative specific telomerase activities (see Tables 1 and 2).

^d On the basis of Student's *t* test, the mTR, H4 mRNA, and telomerase activity levels are significantly increased in the tumor samples ($P < 0.001$).

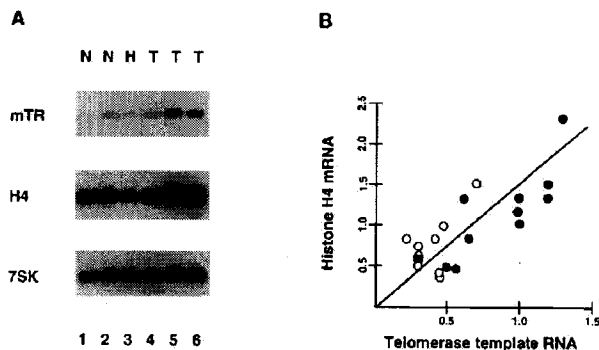


FIG. 3. Correlation of telomerase template RNA with histone H4 mRNA. (A) Total RNA from normal mammary glands (N, lanes 1 and 2), a hyperplastic mammary gland from a *Wnt-1* transgenic mouse (H, lane 3), and three *Wnt-1* transgenic mammary tumors (T, lanes 4 to 6) was blotted and sequentially probed for the 430-nt telomerase RNA (mTR), the ~400-nt histone H4 mRNA (H4), and the 330-nt 7SK RNA (7SK). (B) Relationship between the normalized levels of mTR and histone H4 mRNA in normal and hyperplastic mammary glands (open circles) and mammary tumors (filled circles). Steady-state levels of H4 mRNA and mTR were quantitated from RNA blots similar to the blot shown in panel A and normalized to 7SK RNA (Table 3). The expression levels on each axis are given in arbitrary units. Linear regression analysis indicates that mTR and histone H4 mRNA are closely correlated ($P < 0.0001$).

and the mTR levels in these samples fluctuated in concert (Fig. 3B). Thus, the correlation of mTR and H4 mRNA suggests that the RNA component of telomerase is upregulated in response to proliferation. Our data are consistent with a regulation of the telomerase activity that involves both induction of the telomerase RNA with cell proliferation and an additional event(s) that affects other components of the enzyme.

DISCUSSION

Mechanism of telomerase activation in mouse tumors. The work presented here aimed to test the link between telomere attrition and activation of telomerase during tumorigenesis. To address this issue, we focused on tumorigenesis in *M. musculus*. This animal has extraordinarily long telomeres in its somatic tissues, estimated to contain TTAGGG repeat arrays in excess of 20 kb, which should allow for considerable expansion of malignant cell populations without activation of telomere maintenance functions. Analysis of telomeres in transgenic animals confirmed the presence of long telomeric repeat arrays and indicated no detectable reduction in telomere length in mammary tumors induced by a mouse mammary tumor virus long terminal repeat-driven *Wnt-1* transgene. On the basis of our data, general telomere attrition does not appear to constitute a proliferative hurdle in these tumors. Nevertheless, the

RNA component and the activity of telomerase were clearly elevated during the formation of mammary carcinomas. A recent report similarly documented activation of telomerase in mouse skin carcinomas and in pancreatic islet tumors (8).

Our data demonstrate a strong correlation ($P < 0.0001$) between the expression of telomerase RNA and histone H4 mRNA, a marker for cell proliferation. This result suggests that at least one component of telomerase, its template RNA, is expressed at higher levels in cells that are actively progressing through the cell cycle. The correlation between mTR expression and cell proliferation is corroborated by immunostaining for proliferation cell nuclear antigen, a factor associated with DNA polymerase δ . Consistent with the two-fold increase in H4 mRNA and telomerase RNA, the frequency of proliferating cell nuclear antigen-positive nuclei was elevated two- to threefold in tumors compared with hyperplastic mammary glands (33). The connection between telomerase RNA and cell proliferation is also consistent with the higher levels of mTR in newborn versus adult mouse tissues and the increased expression of mTR in immortalized fibroblasts (7). Whether telomerase RNA is actually induced in S phase or upregulated during the G_0 -to- G_1 transition remains to be determined. In favor of the latter possibility, previous analysis showed that telomerase activity is present throughout the cell cycle of *Xenopus* eggs (44) and a number of mammalian cells (25). We note that the hyperplastic mammary glands do not appear to express more mTR, histone H4 mRNA, and telomerase activity than normal mammary glands. The lack of telomerase activation and proliferation in these tissues is consistent with the differentiated histology of hyperplastic mammary glands and their relatively low levels of proliferating cell nuclear antigen-cdk2 complexes and other parameters of cycling cells (52).

In addition to upregulation of telomerase RNA with cell proliferation, our data suggest that the tumor-specific activation of telomerase is regulated at a second level. While telomerase RNA was increased twofold in the tumors, their telomerase activity was 10-fold higher than in nonmalignant tissues. The target of this second regulatory mechanism could be a protein component of the enzyme or a telomerase inhibitor. Elucidation of this issue awaits cloning of the protein components of telomerase. We note that our data do not exclude that this level of regulation is also linked to the proliferative state of the cells. Telomerase activity is upregulated upon mitogenic stimulation of human peripheral blood lymphocytes (31), increased during in vitro culturing of primary mouse cells (12), and repressed during terminal differentiation of human and mouse cell lines (56). However, in a number of studies the correlation of telomerase activity with proliferation is less clear (16, 17, 34), suggesting additional levels of regulation or cell-type-dependent regulatory pathways. Multiple levels of telomerase regulation are compatible with findings with *Saccharomyces cerevisiae* in which a large number of genes affect telomere dynamics (reviewed in reference 64).

Telomerase function. It is not clear what role telomerase fulfills in proliferating *M. musculus* cells. If the enzyme is needed to maintain telomeres at a 20- to 50-kb length, this raises the question of why *M. musculus* requires telomeres that are approximately 10-fold longer than the 1- to 2-kb telomeres that appear fully functional in some human and mouse cells (4, 15, 16, 53). Another possibility is that telomerase activity is required in proliferating cells to add a 3' overhang to the blunt ends created by leading strand synthesis (42). However, in two budding yeasts loss of telomerase activity through inactivation of their telomerase RNA genes does not diminish short-term cell viability (46, 57), suggesting that 3' telomere termini are

either not required or can be generated by another mechanism. Another, as yet unexplored, possibility is that a telomerase protein is required as a structural component of the telomeric complex (9, 20).

While a structural role for telomerase at telomeres is in keeping with the moderate upregulation of telomerase RNA in proliferating cells, it does not explain the 10- to 20-fold increase in telomerase activity observed in tumors. Since there is no demonstrable telomere shortening during tumorigenesis, it is unlikely that telomerase activation functions to counter gross telomeric decline. However, as discussed below, it is possible that the genomic blots failed to detect one or more shortening "clock telomeres" that require activated telomerase for their maintenance. A second possibility is that the activated tumor telomerase serves to heal damaged chromosomes.

Structure of mouse telomeres. It is pertinent to this study to consider what is known about telomere structure in *M. musculus*. An important assumption in our interpretation of the findings is that all telomeres of this species end in long uninterrupted stretches of precise TTAGGG repeats. This view is consistent with the absence of restriction endonuclease cleavage sites in the terminal 20 to 50 kb, with the strong hybridization of mouse telomeres to telomeric sequences, and with the presence of TTAGGG repeats on terminal fragments that have been shortened by ~20 kb through digestion with exonuclease BAL 31 (37, 58). Is it possible that one or more of these telomeres have a different structure? For instance, if one of the chromosome ends carries a much shorter telomeric stretch, the decline of this telomere could drive telomerase activation during tumorigenesis. Quantitative in situ detection methods will be required to establish whether normal *M. musculus* cells harbor such clock telomeres.

We have also considered the possibility that *M. musculus* chromosomes might have long subtelomeric regions of TTAGGG-related repeats, similar to the blend of variant repeats found at the base of human telomeres (2, 10). Such sequences would explain the absence of restriction endonuclease recognition sites and the strong hybridization of the terminal fragments to TTAGGG repeat probes. Yet, these repeats would probably not function to protect chromosome ends. For instance, the mammalian telomeric protein TRF fails to bind these sequences (13, 65), and de novo formation of telomeres requires precise TTAGGG repeats (27). A frequently occurring variant in the subtelomeric TTAGGG-related repeats has a T→G transversion (TGAGGG) (10), introducing *MnII* (GAGG) and *HphI* (GGGTGA) recognition sites in the arrays. Indeed, these enzymes remove about 2 kb from human terminal restriction fragments, consistent with the estimates for the region of mixed repeats at the base of human telomeres (2). Yet, both *MnII* and *HphI* yield telomeric fragments in *M. musculus* that are larger than 20 kb (35, 58), arguing against extensive degenerate telomeric arrays in these telomeres. Thus, the available evidence is consistent with the presence of exceedingly long stretches of tandem TTAGGG repeats at *M. musculus* chromosome ends.

p53 and telomere dynamics. Two main conclusions can be drawn from the analysis of mice lacking a functional *p53* gene. First, with regard to telomere dynamics and telomerase regulation, these mice were very similar to the transgenic mice with functional *p53* genes. Although minor regulatory changes cannot be excluded, we tentatively conclude that the induction of mTR with cell proliferation and the activation of telomerase during tumorigenesis do not require p53 function. During immortalization of human cells, telomerase activation is also independent of p53 function (62). Second, our results are pertinent to the genomic instability observed in mammary tumors

originating in p53-deficient mice (22). The chromosomal abnormalities seen in these tumor cells include numerical changes, amplifications, and dicentric, which are associated with telomere shortening in human cells (reviewed in reference 20). However, our results fail to reveal significant telomere shortening in the *Wnt-1* transgenic *p53*^{-/-} mammary tumors, suggesting that the genome instability in these *p53*^{-/-} tumors is not due to loss of telomeric DNA.

Comparison of murine and human telomeres. Telomere dynamics in mice and humans differ. Our results show that the bulk of the telomeres do not shorten significantly during mouse tumorigenesis. Therefore, it is unlikely that the telomere-dependent tumor suppressor mechanism proposed for human cells functions as such in mice. However, in other aspects telomere metabolism in murine and human cells may be more similar. Specifically, the regulation of telomerase in normal and tumor tissues appears comparable in both systems. In mice and humans, telomerase RNA can be detected in somatic cells, and in both systems, the enzyme is consistently activated in tumors. These parallels suggest that insights in telomere dynamics and telomerase regulation in mice may bear on the role of telomerase in human cancer.

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