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*Advances in Brief*

# Increased Telomerase Activity in Mouse Skin Premalignant Progression[1]

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## Abstract

**It has been postulated that the expression of the ribonucleoprotein telomerase is necessary to overcome cellular senescence and that malignant tumors must express telomerase to maintain their immortality. In most human adult tissues, telomerase activity is not detected. In contrast, several murine tissues express various levels of telomerase. Mouse skin however, does not show telomerase activity. Using the mouse skin chemical carcinogenesis system, a well-characterized model for studying premalignant and malignant progression, we assayed telomerase activity at various stages of premalignant papilloma progression by means of the recently developed telomeric repeat amplification protocol. We observed that at 10 weeks of promotion, only one mouse skin papilloma of 11 analyzed showed high levels of telomerase activity. The number of papillomas showing higher levels of telomerase activity increased at 20 weeks, and at 30 weeks of promotion, 100% of papillomas expressed significantly higher levels of telomerase. We learned from previous studies that early papillomas are diploid, well-differentiated lesions, whereas late papillomas are aneuploid and very dysplastic. It appears that the progressive increase in telomerase activity is associated with the increased level of genomic instability and the phenotypic progression of these premalignant tumors. It is also possible, however, that the increase in telomerase activity could be in part a consequence of an increase in the proportion of proliferating cells. Nevertheless, the mouse skin system may be a very useful *in vivo* model for the study and development of anti-telomerase therapeutic strategies.**

## Introduction

Telomeres are heterochromatic structures at the ends of eukaryotic chromosomes and consist of simple, highly conserved, repeated DNA sequences (TTAGGG in humans and mice; Refs. 1–3). Their function is to protect and stabilize the chromosome ends (4, 5). Chromosome arms lacking telomeres are prone to fusion and recombination (6–8). Because cellular DNA polymerases cannot replicate the 5′ end of the linear DNA molecule, the number of telomere repeats decreases (by 50–200 nucleotides/cell division) during aging of normal somatic cells (9–11). It has been observed that most immortal cell lines express telomerase, the ribonucleoprotein enzyme that adds telomeric sequences *de novo* (12, 13), whereas mortal cell lines do not (13). Recently, it was demonstrated that telomerase activity is detectable in cells from most human cancers (14, 15). Based on these observations, it was postulated that reactivation of telomerase expression is necessary for overcoming cellular senescence and that cancerous cells must express telomerase to maintain their immortality (15). Furthermore, it was hypothesized that in normal tissues, telomerase could be physiologically repressed to reduce the chances of cancerous growth (15). In normal human tissues, telomerase activity is only observed in germ cells, and some activity has also been detected in normal bone marrow and peripheral blood leukocytes (15, 16). All other human adult tissues appear not to show evidence of active telomerase, even after screening with the recently developed, very sensitive, PCR-mediated TRAP[3] (15, 16). In contrast to human tissues, murine tissues express various levels of telomerase (17, 18). This appears to limit the use of rodent models for studying the mechanisms of telomerase activation during tumorigenesis and testing putative anti-telomerase therapies. However, in some mouse tissues, including skin, telomerase activity is not detected (17). The mouse skin multistage carcinogenesis model is a valuable experimental system for studying precancerous changes and tumor progression. In this model, most of the squamous cell carcinomas arise from preexisting papillomas induced with a two-stage carcinogenesis protocol (19, 20). We and others have dissected several of the biochemical, molecular, and chromosomal events that occur during premalignant progression (reviewed in Ref. 19). We have shown that although papillomas arise as diploid lesions, by 40 weeks of promotion all papillomas are aneuploid and highly dysplastic, indicating a constant progression toward a malignant phenotype (21). Activation of the H-*ras* oncogene and sequential trisomization of chromosomes 6 and 7 as well as specific allelic losses are some of the nonrandom events identified as critical in the progression of papillomas to carcinomas (22–25). It remains to be determined whether other events are needed for papillomas to evolve into invasive lesions.

We considered it very important to evaluate the role and timing of telomerase activation within the context of the other events known to take place during papilloma-carcinoma progression. These studies will allow the evaluation of the mouse skin carcinogenesis system as a model for future studies of anti-telomerase therapeutic approaches. In this report, we describe the results of using the TRAP assay for semiquantitative analysis of telomerase activity in chemically induced mouse skin papillomas and carcinomas.

## Materials and Methods

**Animal Treatment and Tumors.** Papillomas and squamous cell carcinomas were induced on the dorsal skin of SENCAR mice by initiation with a single dose of 10 nmol of DMBA and repetitive applications of 2 μg TPA twice weekly. The papillomas were randomly harvested at 10, 20, and 30 weeks of promotion. Samples from squamous cell carcinomas and normal and hyperplastic skins were also collected. Part of each tumor and skins were analyzed by conventional histological techniques, and the remainder was snap frozen in liquid nitrogen and stored at −80°C until further analyzed.

**Preparation of the Cell Extracts.** Cell extracts were obtained by following a procedure described previously (15) with some minor modifications. Each sample was washed with 500 μl of buffer [10 mM Tris-HCl (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM BME] and centrifuged at 4°C at 5000 rpm for 5 min. After the washing buffer was removed, 50–200 μl of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM BME, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochlorine (AEBSF), 0.5%, 3-[(-cholamidopropyl)dimethylamino]-1-propanesulfonate, and 10% glycerol] were added, depending on the size of the tissue sample. The tubes were then placed on ice, and the samples were homogenized by hand with a Teflon pestle (Bel-Art Products, Gunnock, NJ) and placed on ice for 30 min. The lysates

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[3] The abbreviations used are: TRAP, telomeric repeat amplification protocol; DMBA, 7,12 dimethylbenz[*a*]antracene; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate, BME, β-mercaptoethanol.

were then centrifuged at 16,000 rpm for 20 min at 4°C. The supernatants were collected into 500-μl tubes and flash-frozen in a dry ice-ethanol bath. Samples of tissues and cell extracts were stored at −80°C. The protein concentrations of the cell extracts were determined with the BCA protein assay kit (Pierce, Chemical Corp. Rockford, IL), and samples were diluted to a concentration of 3 mg/ml protein.

**Telomerase Assay.** The telomerase assay was performed by a method described previously (15) with some minor modifications. The oligonucleotides used (TS and CX) have been described (15). The PCR buffer used contained 30 mM Tricine (pH 8.4), 1.5 mM $MgCl_2$, 68 mM KCl, 5 mM BME, 0.5 mM EGTA, 0.05% Tween 20, 0.05% NP40, and 0.01% gelatin. Five microliters of 10× PCR buffer were mixed with 0.1 μg of TS primer, 50 μM each deoxynucleotide triphosphates, 3 u of Taq DNA polymerase (Promega Corp, Madison, WI), 0.2 μl each 10 μCi/μl [α-$^{32}$P]dATP and [α-$^{32}$P]dCTP 3000 Ci/mmol, and sterile diethyl pyrocarbonate-treated water in a total volume of 46 μl/assay. The reaction mixtures were then placed on ice. Two microliters of the cell extracts were added to the PCR mixture, which was then layered with mineral oil and incubated for 30 min at 25°C for telomerase-mediated extension of the TS primer. Then the samples were heated to 90°C, and 0.1 μg of the CX primer was added to each tube. The samples were then subjected to PCR amplification in a thermal cycler with 31 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, and one final cycle at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. As a positive control, 2 μl of a cell extract from a sample with known telomerase activity (a rat mammary tumor line) were used, and as negative control, 2 μl of lysis buffer without any cell extract were used. These controls were assayed in parallel with every experiment. Because telomerase has an RNA component, in a separate experiment 5 μl of the positive control cell extract were incubated with 1 μl RNase A (1 mg/ml) for 30 min at 25°C, and 2 μl of the treated extract were used for the TRAP assay. As additional negative controls, cell extracts from the positive control sample without TS primer or CX primer were also used.

**Electrophoresis and Phosphorimager Visualization.** Aliquots (15 μl) of the PCR mixture were analyzed on 10% nondenaturing, 0.4-mm thick acrylamide gels (20 × 40 cm) run in 0.5 TBE buffer until the xylene cyanol had migrated 17 cm from the origin. The gels were then dried, exposed for 20 h to hyperfilm MP films (Amersham Corp, Arlington Heights, IL), and analyzed with a Molecular Dynamics phosphorimager (Sunnyvale, CA). Signal intensity was measured by area integration of three bands (the second, third, and fourth from the bottom of the gel). In optimization experiments using a telomerase-expressing cell line and increasing cell extract concentrations, we observed that the sum of the intensity of these three bands is more reliable and representative of telomerase concentration than the quantification of all the bands (data not shown). Similar conclusions were obtained by other laboratories (University of Texas, Southwestern Medical Center at Dallas, Dallas, TX).[4] After the background (from the negative control lanes) was subtracted, the signal intensity of the bands from the experimental samples was compared relative to the signal intensity of bands from the positive control that was run with each experiment. The method described is only semiquantitative, but it is sufficient for the comparative analysis of the tumors relative to the same positive control cell extract.

## Results and Discussion

Because previous reports have suggested that telomerase is important in cancer, it would be very valuable to understand the precise role of telomerase expression during the process of premalignant and malignant progression. Well-characterized experimental rodent models of carcinogenesis could contribute relevant information to achieve such a goal. However, an apparent major difference exists between rodents and humans regarding normal telomerase expression. It was observed recently that laboratory mice normally express telomerase in various tissues as detected by the very sensitive TRAP assay (17, 18). Telomerase activity in mice has been suggested to be associated with increased proliferative activity (17). Telomerase activity, however, has not been detected in mouse skin (17, 18). In agreement with those findings, in our studies of SENCAR mice we detected telomerase activity in normal mammary gland, liver, spleen, and kidney (data not shown). In normal skin, no telomerase activity was detected (Fig. 1, *Lane 1*). Some activity, however, was detected in hyperplastic skin from mice chronically treated with the tumor promoter TPA. This was observed in skins from mice that were initiated with the carcinogen DMBA, as well as from the skin of mice that were not initiated. The telomerase activity detected in hyperplastic skin could be interpreted as a consequence of increased cell proliferation or to an expansion in the number of stem cells. However, the telomerase activity detected did not increase with time of promotion since hyperplastic skins after 20 weeks of promotion did not show more activity than after 10 weeks of promotion (Fig. 1). In Fig. 1, we also show that the ladder of bands detected by the assay is due to the ribonucleoprotein telomerase because the activity can be abolished, as expected, by RNase treatment (15). The single band observed in some of the samples is probably an artifact due to primer-dimer formation, as reported previously (26).

We also analyzed telomerase activity in chemically induced mouse skin papillomas at different stages of progression. Papillomas were obtained after 10, 20, and 30 weeks of promotion with TPA. In every TRAP assay, the activity of every sample was normalized to that of an equal amount of total cellular protein. To compare relative telomerase activity between samples, aliquots from the same positive control cell extract were used for every assay. As can be observed in Fig. 2*A*, at 10 weeks of promotion only one of 11 papillomas analyzed showed

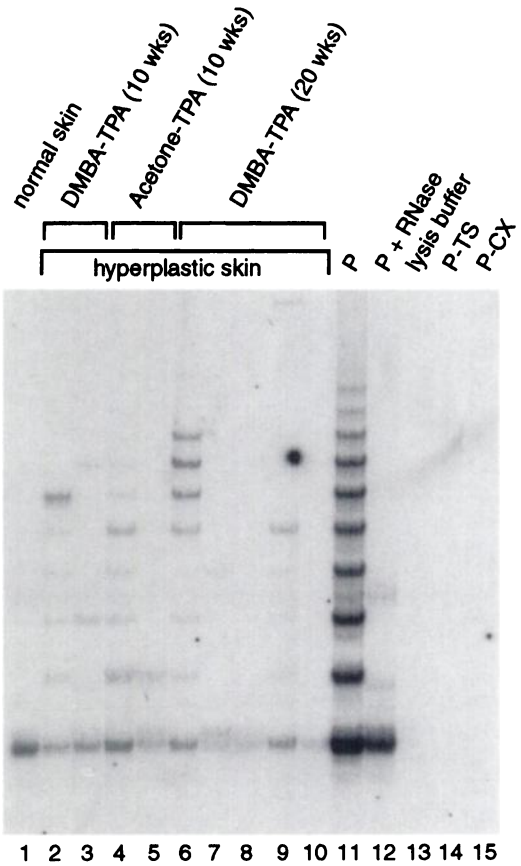


Fig. 1. TRAP assay for telomerase activity in normal and hyperplastic mouse skin. *Lane 1*, normal SENCAR mouse skin; *Lanes 2–5*, hyperplastic skins treated with the tumor promoter TPA for 10 weeks; *Lanes 2* and *3*, TPA after initiation with the carcinogen DMBA; *Lanes 4* and *5*, TPA with no initiation (acetone); *Lanes 6–10*, hyperplastic skins DMBA initiated followed by promotion with TPA for 20 weeks; *Lane 11*, positive control P; *Lane 12*, positive control treated with RNase; *Lane 13*, lysis buffer and primers, no cell extract; *Lane 14*, positive control cell extract without TS primer; *Lane 15*, positive control without CX primer. Each of the samples shown in *Lanes 1–10* was obtained from separate mice.

[4] M. Piatyszek, personal communication.

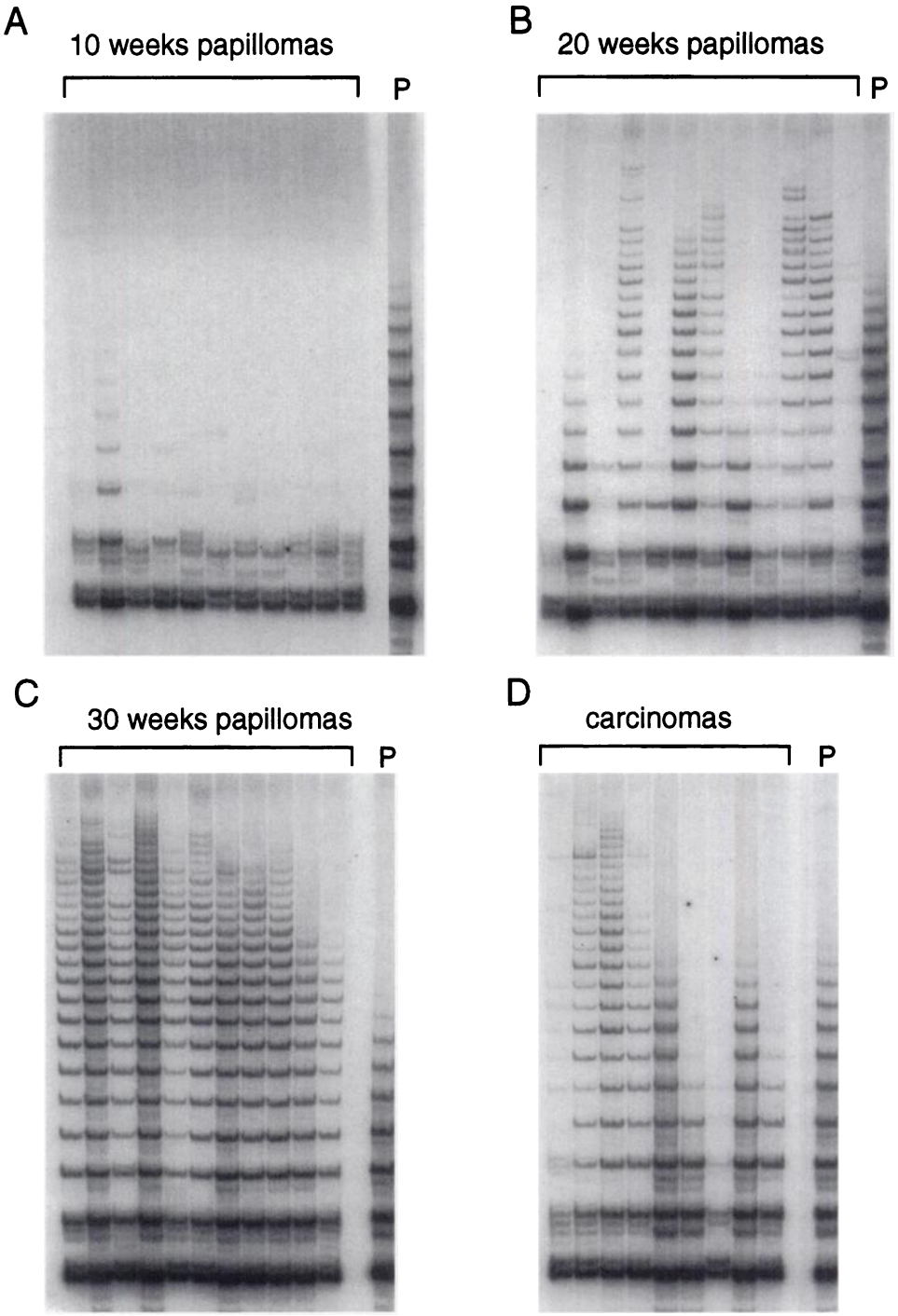


Fig. 2. TRAP assay for telomerase activity in DMBA/TPA-induced mouse skin tumors as indicated. Weeks of tumor promotion as indicated. For each experiment, an aliquot of the same positive control cell extract was used, shown as *P*.

high levels of telomerase activity. The number of papillomas showing higher levels of telomerase activity increased at 20 weeks, and at 30 weeks of promotion, 11 of 11 papillomas expressed significantly higher levels of telomerase (Fig. 2, *B* and *C*). At the malignant stage (Fig. 2*D*), telomerase activity was also detected in most tumors at variable levels. To rule out the possible presence of telomerase inhibitors in the extracts from skin or negative tumors, a sample of normal mouse skin and four representative, randomly selected tumors negative for telomerase activity were also assayed using 10- and 100-fold diluted cell extracts. We did not observe any difference with the results obtained using undiluted cell extracts (data not shown). We plotted the levels of telomerase activity (relative to the corresponding positive controls) detected at the different stages of progression (Fig. 3). As can be observed, there was a progressive increase in telomerase activity that was directly correlated with the age of the papillomas.

In previous studies using the same chemical carcinogenesis protocol, we have shown that early papillomas (10 weeks) are mostly diploid, well-differentiated lesions (21). At that stage in the study reported here, we found that most tumors did not express telomerase activity. By 20 weeks of promotion, 60% of the papillomas were moderately dysplastic, and approximately 30% of the tumors already had severe dysplastic changes (21). By then only a minority of the papillomas (30%) were purely diploid; the rest of the tumors showed signs of developing hyperdiploid subclones, and approximately one-third of the tumors already had hyperdiploid stem lines (21). At 30

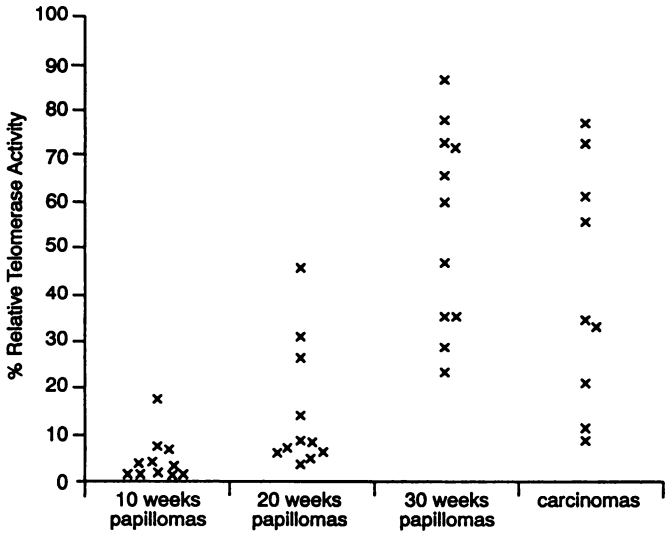


Fig. 3. Relative telomerase activity in mouse skin tumors. Activity is plotted as percentage of activity relative to the positive control of each gel from Fig. 2. Each *x* represents an individual sample. Weeks of tumor promotion are as indicated.

weeks of promotion, most of the tumors were classified as moderately or severely dysplastic papillomas, and 25% of them had reached the carcinoma *in situ* stage (21). At this stage, no diploid tumors were observed; most of the papillomas had hyperdiploid stem lines (21). Interestingly, it is at this same stage that we observed a high level of telomerase activity in 100% of papillomas analyzed.

It appears that the progressive increase in telomerase activity was associated with the increased level of genomic instability and the phenotypic progression of these premalignant tumors. At this point, we cannot rule out that the increase in telomerase activity was a mere consequence of increased cell proliferation. However, in previous studies we observed that the labeling index for proliferating cells in hyperplastic skin under protracted TPA treatment reaches a maximum of 30–35% at 4 weeks of treatment (27) and that the labeling index for papillomas appears to be similar (30–40%; Ref. 28). In the study presented here, we showed that the level of telomerase activity in hyperplastic skin was considerable lower than the high levels of telomerase activity observed in 30-week papillomas (Figs. 1 and 2). In other words, the increase in telomerase expression observed does not appear to be only the consequence of an increase in proportion of proliferating cells. Why we found a significantly lower level of telomerase expression in two of the carcinomas is presently not clear, but histologically, these tumors did not have large areas of necrosis that could explain the finding.

It is also of interest that the most characteristic type of chromosome abnormalities observed in this model are changes in chromosome number (21, 22). The most common abnormality observed is trisomy of specific chromosomes; no nonrandom chromosomal structural abnormalities have been identified in mouse skin papillomas or carcinomas (22). This could be due to the high level of telomerase expression observed in these tumors, which prevents the development of structural abnormalities that could result from telomere-telomere fusions.

Telomerase has been proposed as target for antitumor therapy (16). The mouse skin system may be a very useful model for the study and development of anti-telomerase therapeutic strategies. Among the advantages of this model are the ease of monitoring tumors and the extensive characterization of various tumor and molecular biology markers (19, 21–25). Additional studies are necessary to develop a better understanding of the mechanisms by which telomerase activity increases during papilloma progression in this model.

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