

Human Telomerase RNA and Telomerase Activity in Immortal Cell Lines and Tumor Tissues¹

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ABSTRACT

Telomerase activity has been detected in many human immortal cell lines and in tumor tissues, whereas it is generally absent from primary cell strains and from many tumor adjacent tissue samples. With the recently cloned human telomerase RNA (hTR), we used Northern analysis to follow the levels of hTR in primary, precrisis, and immortalized cells. It was surprising that the amount of hTR was high in cell strains that lacked telomerase activity, and the levels did not parallel the increases in telomerase activity, which accompanies immortalization. In addition, although the hTR levels were somewhat higher in tumor samples compared to nontumor tissues, the level of hTR in a variety of different human tumors did not predict the level of telomerase activity in the tumor. Thus, whereas hTR was detected in all samples that have telomerase activity, the presence of the RNA was not a good predictor of the presence or amount of telomerase activity.

INTRODUCTION

Chromosomal rearrangements and end associations are ubiquitous in cancer cells. The loss of telomeres, which normally provide stability to chromosome ends, may initiate or drive the genomic instability that results in abnormal chromosomes and unchecked cell growth (1-5). Telomeres are often shorter in tumor tissue than in normal adjacent tissue (reviewed in Refs. 5, 6). In many immortal organisms like yeast and *Tetrahymena*, telomere length is maintained by the unusual DNA polymerase, called telomerase. Telomerase is a ribonucleoprotein polymerase that adds telomeric sequences onto chromosome ends (7, 8). In humans, telomerase activity is present in the germline but is not detected in many normal adult tissues (9). The absence of telomerase may lead to the telomere shortening seen in somatic tissues *in vivo* (reviewed in Ref. 10). In contrast to normal tissues, telomerase activity is found in many human tumors (for review, see Refs. 6, 11-14). Tissue culture models of cell immortalization suggest that telomerase-positive cells are selected for at crisis, and that telomerase is required for the growth of most immortal cells with short telomeres (14-17). Thus, telomerase has been proposed as a target for cancer chemotherapy (15). In yeast, telomerase RNA is essential; in cells that are deleted for the RNA component, telomeres shorten and cells die after 50-70 divisions (18, 19). In addition, antisense experiments with the RNA component of human telomerase indicate that telomerase inhibition may lead to telomere shortening and cell death in human tumor cell lines (20). To gain insight into the regulation of telomerase in cell lines and tumors, we compared the

levels of telomerase activity to the levels of hTR.³ Whereas many cell lines and tumors had both increased hTR and telomerase activity, we found that hTR was present in cell lines and tissues that lacked telomerase activity, indicating that the RNA is not limiting for telomerase activity, and that the RNA component is not a good predictor of the presence of enzyme activity.

MATERIALS AND METHODS

Cell Extracts and Telomerase Assays. HEK cells were cultured in α -MEM and 10% FBS, grown on 15-cm plates, and split 1:4. Human B cells were cultured in suspension in RPMI containing 10% FBS. All cultures contained 0.03% penicillin and streptomycin. Tumor tissue was obtained from The University of Texas Southwestern Medical Center at Dallas and from North Shore University Hospital (Manhasset, New York). Cell and tissue extracts were prepared and assayed using a PCR based telomerase assay (TRAP) as described previously (4, 21) using a modified (CCCTAA)_n "CX" primer.⁴ The PCR products were run on a 12-15% acrylamide gels. In experiments with cell lines, enzyme activity was quantified by scanning gels on a Molecular Dynamics PhosphorImager, and the radioactivity in each band of the repeat ladder was determined using ImageQuant software (Molecular Dynamics). Total activity was expressed as a percentage of that in 293 cell extracts (positive control) after normalizing for protein content. Extracts were considered negative if no products were detected after 7 days of exposure.

RNA Preparation and Northern Blots. RNA was isolated from 10⁷ to 10⁸ cultured cells or 50-500 mg of tissue according to the acid guanidinium thiocyanate/phenol/chloroform extraction procedure as described by Ausubel *et al.* (22). Briefly, cells were lysed in a denaturing solution of 4 M guanidine thiocyanate-25 mM sodium citrate (pH 7.0)-0.1 M β -mercaptoethanol-0.5% Sarkosyl. The lysate was extracted with saturated phenol, chloroform, isoamyl alcohol, and sodium acetate (pH 4.0), and the RNA was precipitated from the water phase with isopropyl alcohol or ethanol. The final pellet was resuspended in diethylpyrocarbonate-treated water. The absorbance at 260 nm was measured to determine the RNA concentration, assuming that 1 absorbance unit per ml contains 40 μ g of RNA. For purifying RNA from tissues, samples were lysed by Dounce homogenization in the denaturing solution using a 1.5-ml Eppendorf tube with a fitted pestle (Kimble).

RNA was electrophoresed on 1.5% agarose-2.2 M formaldehyde gels in 20 mM 4-morpholinepropanesulfonic acid (pH 7.0)-8 mM sodium acetate-1 mM EDTA for 4-8 h at 5 V/cm (110 V using a 21-cm gel). The RNA was passively transferred onto a Nytran maximum strength membrane (Schleicher and Schuell), and the RNA was covalently attached to the membrane by baking for 1-2 h at 80°C. Blots were probed overnight in Church hybridization solution [500 mM sodium phosphate (pH 7.2)-1 mM EDTA-1% BSA-7% SDS] containing 15% formamide. Blots were rinsed 5 times in 2 \times SSC and 0.1% SDS, at room temperature, for 5 min each. One final wash was performed at the hybridization temperature for 30 min in SSC containing 0.1% SDS (for specific details regarding each probe and the concentration of SSC in the last wash, see below). Blots hybridized with the hTR component gene or ribosomal 5S RNA were probed at 65°C; blots probed with an oligonucleotide complementary to the RNase P RNA were probed at 50°C. All blots were exposed to Kodak XAR film at -70°C with an enhancing screen and to a PhosphorImager screen, and were scanned with a Fuji BAS200 PhosphorImager. Typical autoradiographic exposures for all the experiments were as follows: 3-10 days for

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³ The abbreviations used are: hTR, human telomerase RNA; HEK, human embryonic kidney; PD, population doubling; RNP, ribonucleoprotein.

⁴ N. Kim, personal communication.

blots probed with telomerase RNA, 5 h for blots probed with RNase P RNA, and less than 1 h for blots probed with ribosomal 5S RNA.

Quantitative Analysis of Telomerase RNA Abundance. To control for loading errors, telomerase RNA was normalized either to the levels of RNase P or to 5S RNA signals in the same lane. The tumor study was performed blind, with the identity and telomerase activity status of each sample unknown until after Northern analysis. Quantification of all RNAs was performed using a Fuji PhosphoImager. The RNA signal for each lane was determined by drawing a rectangle around each RNA band and integrating the signal over the area. Some of the samples initially analyzed for telomerase activity were found to have degraded RNA; these samples were omitted from the analysis presented in Fig. 3.

RESULTS

We analyzed telomerase activity and hTR amounts in primary cells and during immortalization *in vitro* in cells expressing viral oncoproteins. HEK cells transfected with SV40 T-antigen did not express telomerase activity until the culture went through crisis (15). These initial studies were done using a conventional telomerase activity assay. Recently, a more sensitive PCR-based assay was developed (4, 21). To determine whether precrisis cells were telomerase negative by analysis with this highly sensitive assay, we reanalyzed one T antigen-expressing clone (HA-1) for telomerase activity every 2–5 population doublings during the extended lifespan period during and after crisis. Representative assays are shown in Fig. 1A and are summarized in Table 1. Cell extracts were diluted and analyzed at three different concentrations to determine the level of telomerase present and to avoid false negatives due to possible inhibitors of PCR in the extract. The results confirmed our earlier findings; telomerase activity was not detectable in the primary cells or in the extended lifespan phase of growth but was present during crisis and at all points after crisis (Table 1). Using the PCR-based assay, telomerase activity was de-

tected slightly earlier than in the previous study (PD 61; Ref. 15). To determine the hTR levels in these cells before and after crisis, we used Northern blot analysis. Blots were probed with an hTR probe and then reprobbed with RNase P to normalize the signal to total RNA (Fig. 2). hTR was present at high levels even in cells that did not have detectable telomerase activity (Fig. 1A). The increase in hTR abundance was only 2-fold from PD 45 to 114 (Table 1), whereas telomerase activity increased at least 100-fold. This 100-fold estimate is based on the presence of activity in the most dilute extracts (0.1 µg protein) at PD 114 and absence of activity at PD 45 at all protein concentrations (10 µg being the highest). Thus, telomerase RNA was present in cells that did not contain detectable telomerase activity.

To examine whether the presence of hTR in telomerase-negative cell strains was a general finding, we analyzed a second model of cell immortalization. Human B cells infected with EBV analyzed by conventional telomerase assays showed telomerase activity only after crisis. We reanalyzed four B-cell clones using the TRAP assay (Fig. 1B) and found at low protein concentration only postcrisis cells were telomerase positive, while at high concentration three clones of precrisis cells were telomerase positive (Table 2). This is consistent with recent reports indicating that human hematopoietic cells express telomerase activity (21, 23–25). To estimate the levels of telomerase activity, we carried out five serial dilutions of the extracts. In the two postcrisis clones analyzed (B3 and B4), the postcrisis cells had 500–1800-fold higher levels of telomerase activity than the precrisis cells (Table 2). Northern analysis of RNA from all four clones showed no significant difference in the levels of hTR pre- and postcrisis (Fig. 2). Thus, similar to the immortalization of HEK cells, the levels of the hTR did not parallel the levels of telomerase activity, again suggesting that the RNA component of telomerase is not the limiting factor for telomerase activation in cellular immortalization.

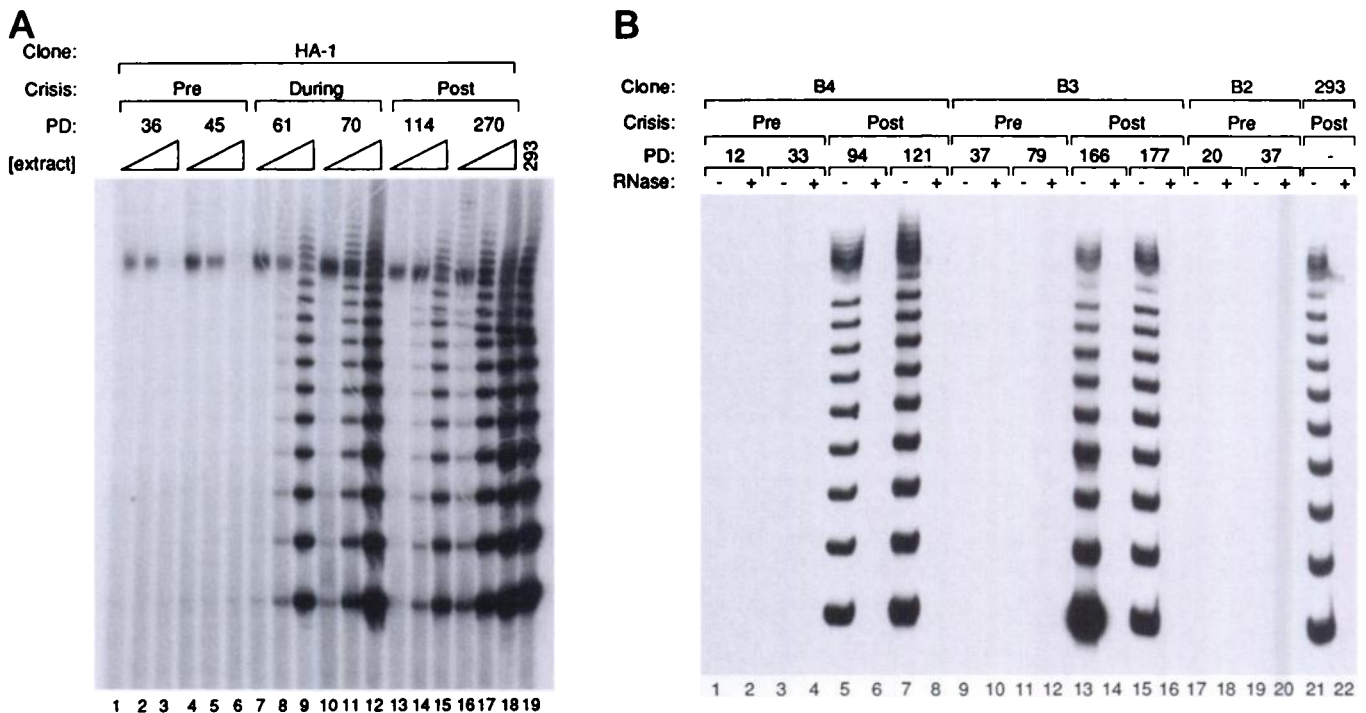


Fig. 1. Telomerase activity in transformed cells. A, HA-1 is a clonal population of HEK cells transformed by SV40 T antigen. Lysates were prepared from cells of different age (PD) during the extended lifespan (precrisis), at crisis, and after immortalization (postcrisis), and were assayed for telomerase activity by the TRAP method at 0.1, 1.0, and 10 µg protein per reaction. One-half of each reaction was loaded on the gel for analysis and quantification of enzyme activity. B, B4 and B3 are clonal populations of EBV-transformed B lymphocytes that become immortal after crisis, whereas B2 populations remain mortal and cease growth at crisis. Cell lysates prepared at the indicated PD pre- and postcrisis were assayed for telomerase activity as in A. Shown is telomerase activity in reactions containing 1 µg protein, one-half of which were loaded on the gel. +, pretreatment of the lysates with RNase. Low levels of telomerase activity were detected in precrisis B3 and B4 clones when higher concentrations of extract were assayed (see Table 2).

Table 1 *Telomerase activity and RNA levels in HEK and SV40 T-antigen-expressing clones*

Cells ^c	Stage ^d	MPD ^e	Telomerase activity ^d				Telomerase RNA ^b	
			μg protein			%293 ^f	Amount ^g	%293 ^f
			10	1	0.1			
HEK	NA ^h	5	-	-	-	-	896	20
HA1	Pre-crisis	36	-	-	-	-	ND ⁱ	ND
	Pre-crisis	45	-	-	-	-	1060	24
	Crisis	62	+	+	±	1.5	ND	ND
	Crisis	71	+	+	+	5.4	816	19
	Post crisis	115	+	+	±	1.7	2366	54
	Post crisis	271	+	+	+	12.0	ND	ND
293	Post crisis	NA ^h	+	+	+	100	4410	100

^a Telomerase activity assayed by TRAP. Amounts (μg) of protein used in each reaction are indicated.
^b The RNA component of human telomerase measured by northern analysis.
^c HEK, human embryonic kidney cells, untransformed; HA1, HEK cells transformed by SV40; 293, HEK transformed by adenovirus 5.
^d Transformed cells were assayed at different times after transformation (pre-crisis and crisis cells are mortal, whereas post-crisis cells are immortal).
^e MPD, mean population doublings.
^f Activity and RNA levels were normalized to 293 cells assayed on same gel.
^g The relative amount of human telomerase RNA (hTR) per lane.
^h NA, not applicable.
ⁱ ND, not determined.

Fig. 2. Telomerase RNA levels in HEK cells and B cells. Total RNA was made from the different cell lines, and 20 μg of each were analyzed by Northern blot analysis. All membranes were first probed with a radiolabeled random primed fragment of hTR. Blots were stripped and re-probed with an end-labeled oligonucleotide complementary to RNase P as a control for loading. A, Northern blot analysis of HEK cells transfected with SV40 T antigen. RNA was made from transfected cells (HA1) pre-crisis, at the time of crisis, and post-crisis. Mean population doubling (MPD) refers to the population-doubling time after transfection with SV40 T antigen. For controls, nontransfected HEK cells and 293 cells, an immortalized cell line derived from HEK cells transformed with adenovirus, were also analyzed. B, Northern blot analysis of human B cells infected with EBV. RNA was made from 2 different transfected clones (B3 and B4) pre-crisis, at the time of crisis, and post-crisis. Mean population doubling (MPD) refers to the population doubling time after infection with EBV. Cells from the 293 line were also analyzed as a positive control.

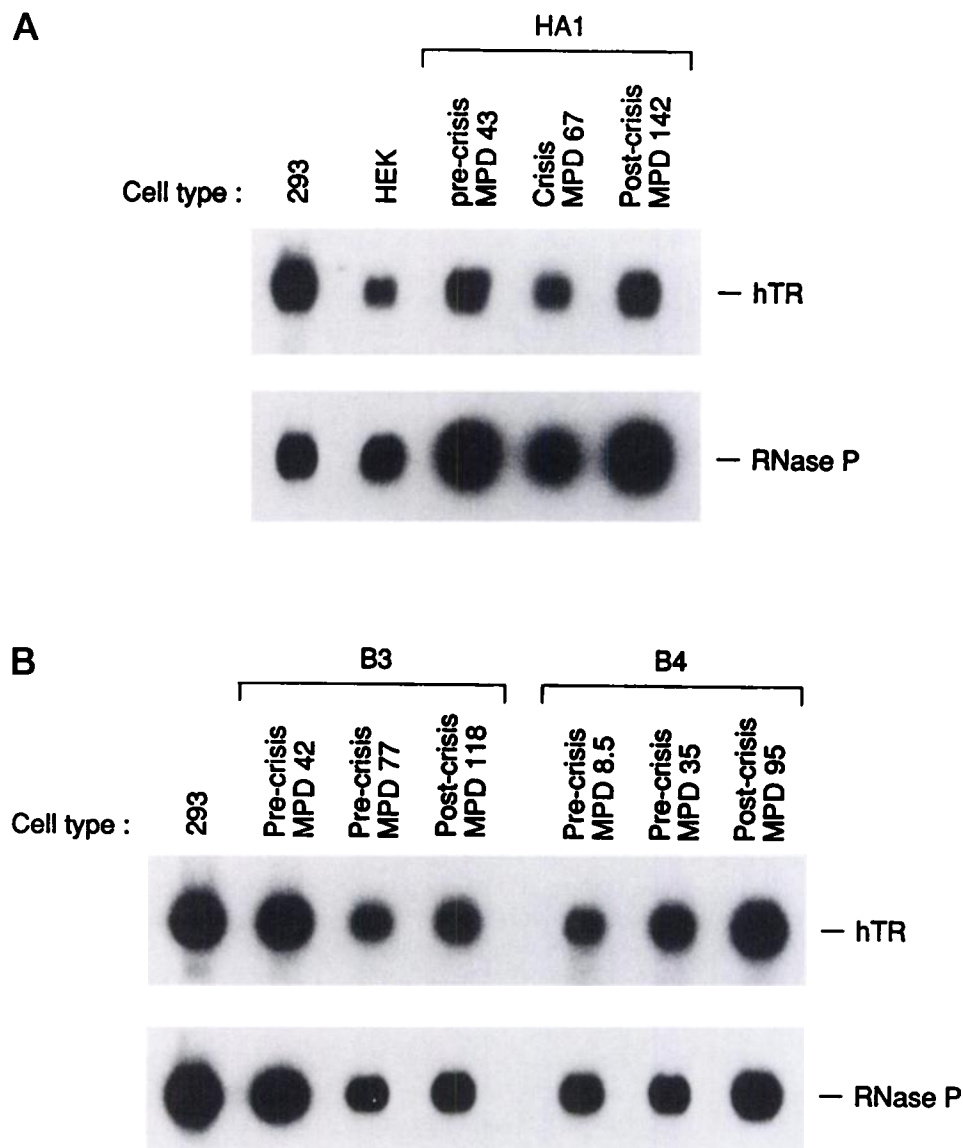


Table 2. Telomerase activity and RNA levels in B-cell clones expressing EBV

Cells ^c	Stage ^d	MPD ^e	Telomerase activity ^a					Telomerase RNA ^b		
			$\mu\text{g protein}$					%293 ^f	amount ^g	%293 ^f
			10	5	1	0.1	0.01			
B3	Precrisis	42	+	+	-	-	-	0.1	6503	132
B3	Near crisis	77	+	+	+	+	-	13.0	7056	143
B3	Postcrisis	118	+	+	+	+	+	186.0	6666	135
B4	Precrisis	8.5	+	+	-	-	-	0.1	5818	118
B4	Near crisis	35	+	+	-	-	-	0.1	7638	155
B4	Postcrisis	95	+	+	+	+	+	50.0	8179	166
B2	Precrisis	12	±	-	-	-	-	ND ^h	5162	105
B2	Precrisis	26	±	-	-	-	-	ND	4278	87
B5	Precrisis	9	-	-	-	-	-	-	4251	86
B5	Precrisis	71	-	-	-	-	-	-	4544	92
293	Postcrisis	NA ⁱ	+	+	+	+	+	100	4929	100

^a Telomerase activity assayed by TRAP. Amounts (μg) of protein used in each reaction are indicated.

^b The RNA component of human telomerase measured by northern analysis.

^c B2-B5, human B lymphocytes transformed by EBV.

^d Transformed cells were assayed at different times after transformation (precrisis and crisis cells are mortal, whereas postcrisis cells are immortal).

^e MPD, mean population doublings.

^f Activity and RNA levels were normalized to 293 cells assayed on same gel.

^g The relative amount of human telomerase RNA (hTR) per lane.

^h ND, not determined.

ⁱ NA, not applicable.

To further examine the correlation of telomerase RNA abundance and activity, we analyzed 21 human tumor samples and 5 normal tissues (Fig. 3). Extracts were made from tumors, and telomerase activity was measured. Assays were done using 6.0 μg of protein, and those extracts that were negative were tested for possible inhibitors using serial dilutions of the extracts. Telomerase activity was not detected in the normal tissues analyzed but was detected in most of the tumor samples [activity levels are designated as 0 for no activity and 1-4 (1 is the lowest level) in Fig. 3]. The levels of telomerase activity varied greatly between the different tumors. Northern analysis was carried out, and the hTR signal quantitated in a blind study on RNA isolated from all of the tumor samples (Fig. 3). The hTR was detected in all of the samples analyzed, including the normal tissues that lacked detectable telomerase activity. The level of hTR was higher in the tumor samples (median signal = 14,017) than in the normal tissue (median signal = 1, 112), although there was a high degree of variation. In some cases, the level of hTR in a telomerase-positive tumor was similar to that of telomerase-negative normal tissue (e.g., compare samples 10 and 11). Among the tumor samples, the level of hTR did not reflect the level of telomerase activity detected. Although some variability may be introduced during the surgical handling of tumor tissue before extract preparation, we have not found variability in the time of tissue freezing after removal to significantly affect telomerase activity levels (21). In addition, a recent study on mouse tumors in which all tissues were handled identically has also found tumor to tumor variations in both telomerase activity and telomerase RNA (34). Finally, the data on hTR levels in tumors and normal tissue are consistent with the study in cell lines that suggest that telomerase RNA levels do not reflect the level of telomerase activity. Thus, telomerase RNA is not a good marker for the presence of telomerase activity in a given tumor.

To compare hTR levels in tumor and normal tissues from matched samples from the same individual, we analyzed colorectal carcinoma samples. RNA was extracted from three tumors and adjacent tissue samples. hTR was present in both the tumors and the adjacent tissue, although in all cases there was a higher amount in the tumor samples (Fig. 4). Telomerase activity assays were also done at three different protein concentrations (6, 0.6, and 0.06 μg) on each sample. All of the tumor samples had telomerase activity. One of the three adjacent samples (sample 55) showed low levels of telomerase activity, and the other two were telomerase negative (data not shown). Telomerase

activity has been reported previously in tumor adjacent tissues (21) and may be due to a low percentage of contaminating tumor cells in the adjacent sample (occult micrometastasis). None of the six extracts caused inhibition of PCR in control reactions, indicating that the lack of signal was not due to inhibition of the PCR-based telomerase assay. Thus, as for the cell lines and the blind tumor study, telomerase RNA levels did not parallel telomerase activity in human colorectal carcinoma samples.

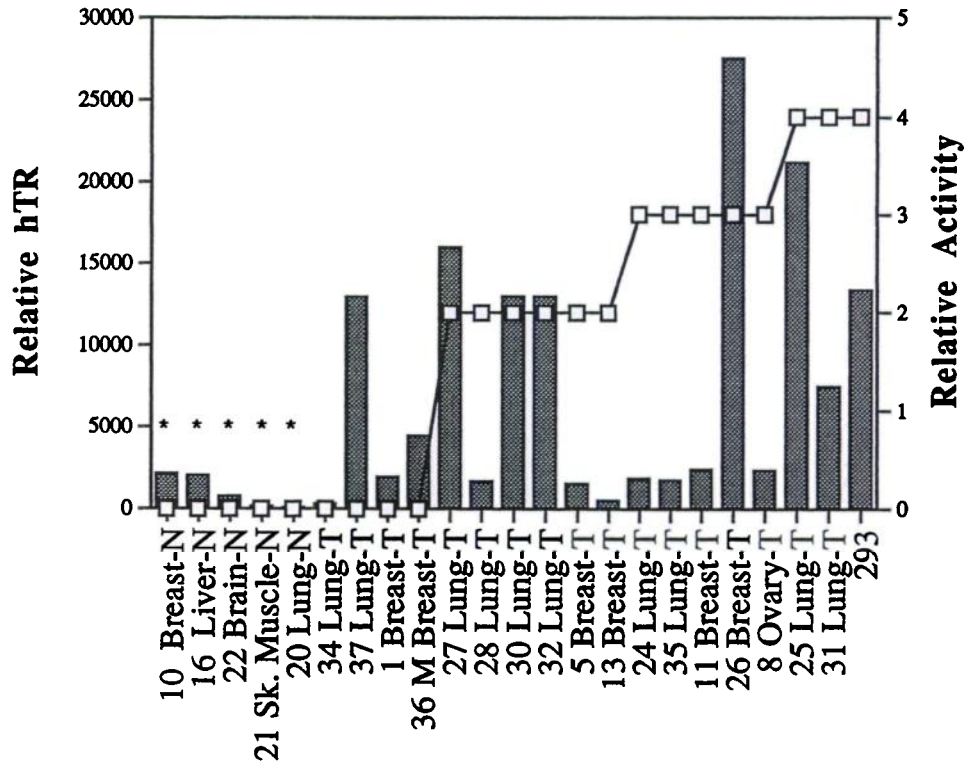
DISCUSSION

Telomerase activity is up-regulated in a variety of immortal cell lines and in tumors in both human and mouse (reviewed in Refs. 6, 11, 13, 26). To understand the regulation of telomerase during tumorigenesis, we analyzed the levels of the recently cloned hTR component during telomerase activation. In both cell lines and in tumors, we found that hTR levels did not always parallel the level of telomerase activity. Although there was an increase in the hTR level when tumor and normal tissues were compared, the amount of telomerase activity did not always parallel the amount of hTR. The activity and RNA levels in tumors may vary at the cellular level. Some large tumors that have little RNA may only express telomerase RNA (and perhaps activity) in a portion of the cells. This intratumor heterogeneity might contribute to the variable level of RNA seen in tumors. An *in situ* assay will be required to test this hypothesis.

Similar results showing the presence of telomerase RNA in normal human tissues that are telomerase negative were recently reported (20). In addition, a lack of correlation between telomerase RNA and activity was recently found during progression of multistage tumorigenesis in transgenic mice (34). Taken together, these results indicate that the telomerase RNA component is not a good predictor of telomerase activity.

The presence of telomerase RNA in cell cultures that lack telomerase activity suggests that telomerase is regulated at several different levels. Telomerase RNA was present in EBV- and SV40 T antigen-expressing cell clones that did not have detectable telomerase activity. Recent evidence from transgenic mice suggests that viral oncogenes or their cellular effects may directly up-regulate telomerase RNA but not telomerase activity (34). Thus, the increase in hTR in the HA1 clones may represent a direct or indirect effect of the viral oncogenes.

Fig. 3. hTR levels in tumor (T) versus normal (N) tissues. Total RNA was made from different tissues, and 25 µg of each were analyzed by Northern blot analysis. *, normal tissues tested; other tissues are from tumors. The graph shows the relative amounts of telomerase RNA (Relative hTR) in different tumors normalized to the amount of 5 S RNA. Telomerase activity levels are plotted on the right as 0 (for no activity) or 1–4 to indicate the relative level of telomerase activity detected. Extracts that showed activity at 0.06 µg were designated 4 for telomerase activity. Extracts that had weak activity at higher concentrations (6 µg) were designated 1 for weak activity, 2 for strong activity at 6 µg, or 3 for strong activity at 0.6 µg.



The lack of activity in cells that contain high levels of hTR may be due to the absence of a protein component of telomerase or to specific down-regulators of telomerase present in primary cells and tissues (27). In *Tetrahymena*, overexpression of telomerase RNA does not lead to an increase in the steady-state amount of telomerase RNA, suggesting that the RNA component must be bound by a limiting factor, possibly the protein components to be stabilized against degradation *in vivo*. (28) Thus, it will be of interest to determine whether telomerase proteins are present in cells that lack telomerase activity or whether telomerase RNA is packaged as a RNP in these cells. Telomerase proteins components have been isolated from the ciliate

Tetrahymena (29) but not yet from human cells; thus, it is not yet possible to determine whether they are limiting for telomerase activity *in vivo*.

Cell fusion experiments between telomerase-positive immortal cells and telomerase-negative immortal or normal cells have shown that telomerase activity is down-regulated in at least some cases, suggesting a *trans*-repression activity may be present in telomerase-negative cells (27, 30, 31). This *trans*-acting repression could act directly on the availability of one or more telomerase polypeptides or it may represent a direct repressor of the telomerase enzyme activity. Mixing extracts from telomerase-positive cells with those from telomerase-negative cells did not inhibit telomerase activity in the positive extracts (15, 30, 32). Thus, lack of activity is not due to a simple, diffusible telomerase inhibitor. However, the presence of a tightly associated specific telomerase inhibitor has not been excluded.

Telomerase activity is commonly found in a variety of cancers (4, 33). Telomere shortening and eventual cell death were seen in yeast cells deleted for telomerase RNA component and in human cells expressing antisense RNA (18–20). Thus, telomerase appears to be required for the growth of at least some immortal cell types. This requirement has raised the possibility that telomerase inhibitors may be useful in cancer chemotherapy (reviewed in Ref. 13). Understanding the details of telomerase activity and component regulation is important to evaluate the potential of new therapeutic and diagnostic approaches to cancer.

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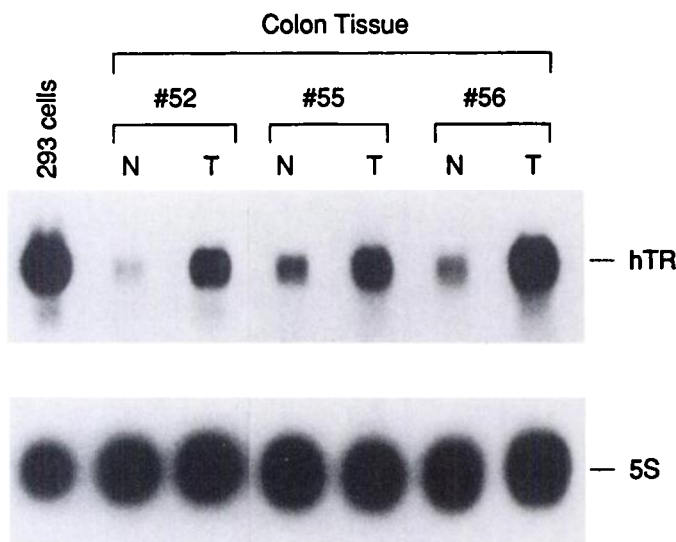


Fig. 4. Northern blot analysis of human colon tumor (T) samples and normal (N) adjacent tissue from three patients. The Northern blots were probed with hTR and then with 5 S RNA gene, and the levels of hTR were normalized to the 5 S RNA levels in the same lanes.

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