

Telomerase Activity in Human Development Is Regulated by Human Telomerase Reverse Transcriptase (hTERT) Transcription and by Alternate Splicing of hTERT Transcripts¹

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ABSTRACT

The correlation between telomerase activity, telomere lengths, and cellular replicative capacity has led to the theory that maintenance of telomere lengths by telomerase acts as a molecular clock to control replicative capacity and senescence. Regulation of this molecular clock may have applications in the treatment of cell aging and tumorigenesis, although little is presently known about the regulation of telomerase activity. To investigate possible mechanisms of regulation, we examined telomerase activity and the expression of the human telomerase RNA subunit and the human telomerase reverse transcriptase protein (hTERT) during human fetal heart, liver, and kidney development. The human telomerase RNA subunit is expressed in all three tissues at all gestational ages examined. hTERT expression correlates with telomerase activity in the liver, where both are expressed at all ages surveyed, and in the heart, where both are present until the 11th gestational week but not thereafter. However, although telomerase activity in the kidney is suppressed after the 15th gestational week, the hTERT transcript can be detected until at least the 21st week. Reverse transcription-PCR using primers within the reverse transcriptase domain of hTERT show the presence of multiple alternately spliced transcripts in these tissues, corresponding to full-length message as well as spliced messages with critical reverse transcriptase motifs deleted. Of note, telomerase activity in the kidney is only present at those gestational ages when full-length hTERT message is expressed (until ~ week 15), with spliced transcripts continuing to be expressed at later stages of development. The tissue-specific and gestational-age dependent expression of hTERT mRNA seen in human development suggests the presence of at least two regulatory mechanisms controlling the activity of telomerase: transcriptional control of the *hTERT* gene and alternate splicing of hTERT transcripts.

INTRODUCTION

Linear chromosome ends, known as telomeres, are composed of hexameric repeats and associated proteins (1). Telomeres are essential to maintaining chromosomal integrity (1). The end-replication problem, the inability of conventional DNA polymerases to replicate the end of a linear chromosome during lagging strand synthesis, requires cells to use other methods to maintain telomere integrity (2, 3). Telomerase, a multisubunit ribonucleoprotein capable of adding telomeric DNA to the end of linear chromosomes, fulfills this requirement in organisms as diverse as tetrahymena, ciliates, yeast (4), mice (5), and humans (6). In humans, the active telomerase enzyme is composed of at least two components: hTR,³ which contains the template for reverse transcription (7), and hTERT [previously referred to as

hTRT (8), hEST2 (9), and hTCS1 (10)], which is thought to be the enzyme's catalytic subunit. Other telomerase-associated proteins have been found in ciliates, such as p80 [Ref. 11; which has a human homologue known as hTLP1 (12, 13)] and p95 (Ref. 11; which has no known human homologue), although hTR and hTERT alone are sufficient to reconstitute telomerase activity *in vitro* (14, 15).

The hTERT transcript undergoes alternative splicing in several tissues and cell lines (10). The function of these alternate transcripts is unknown, although the positions of the splice sites suggest that many of the alternate transcripts do not code for functional reverse transcriptases. A diagram of the hTERT protein highlighting variably spliced segments and regions critical for enzymatic activity is shown in Fig. 1.

In humans, telomerase is active during embryonic development (16, 17), in adult germ-line tissues (16) and in most cancers (6) but not in most adult somatic tissues. In the human fetus, telomerase activity is present in many tissues early in gestation, with tissue-specific repression of activity during development (17). Repression of telomerase activity correlates with the histological differentiation of tissue stem cells. This correspondence between the presence of undifferentiated cells and telomerase activity parallels the loss of telomerase activity in immortal cells induced to differentiate by chemical agents (18, 19).

Human cells that lack telomerase activity progressively lose telomeric DNA with each round of cell division (20–23). Shortening of telomeric DNA has been found to correlate with the onset of cellular senescence (24, 25). The telomere hypothesis of cell aging and immortalization (26) suggests that continued shortening of telomeres via cell replication, without the action of telomerase to replace lost telomeric DNA, leads to a critical shortening of telomeres, which signals cellular senescence. The telomere hypothesis is strongly supported by recent studies in which the gene encoding hTERT was transfected into normal human cells that lack hTERT and telomerase activity but express hTR. This transfection results in the expression of hTERT, reconstitution of telomerase activity (27, 28), maintenance of telomeres, and extension of replicative capacity (29, 30).

The telomere hypothesis dictates that replicating cells lacking a method to maintain telomeres must eventually senesce. Most human tumors that express telomerase activity rely on maintenance of telomere lengths to retain cellular proliferation and an immortal state. Thus, the mechanisms regulating telomerase activity are of great interest. This study examines the expression of telomerase enzymatic activity, hTR mRNA, and hTERT mRNA during human development. We present data supporting at least two potential mechanisms for regulation of telomerase activity: transcriptional regulation of the hTERT gene and alternate splicing of hTERT transcripts.

MATERIALS AND METHODS

Cell Lines and Strains. Immortal cell lines used were HL-60 human promyelocytic leukemia cells (31) and K-562 human erythroid leukemia cells (32). Mortal cell strains used were WI-38 human lung fibroblast cells (9) and two primary human myometrial cell strains, MA45 and MA46, developed in our lab.

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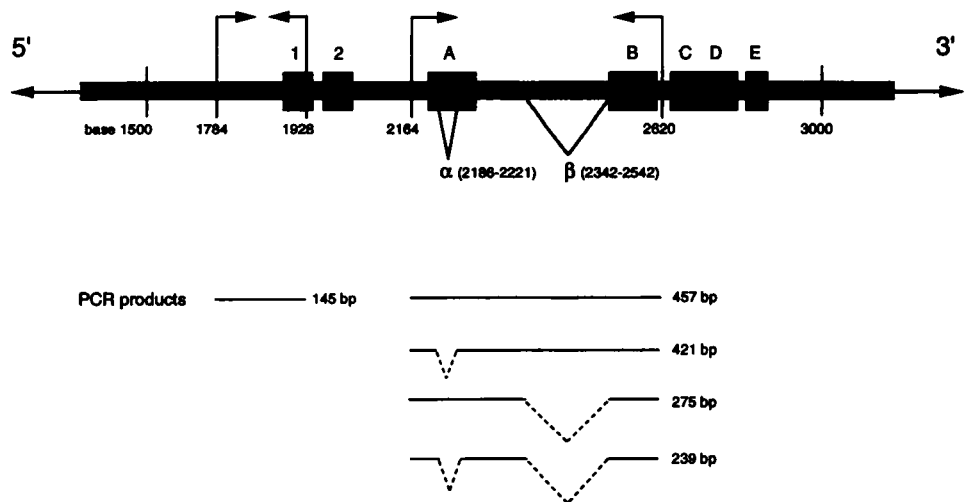
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³ The abbreviations used are: hTR, human telomerase RNA; hTERT, human telomerase reverse transcriptase; TRAP, telomerase repeat amplification protocol; TS, telomere substrate; RT-PCR, reverse transcription-polymerase chain reaction.

Fig. 1. Diagram of the hTERT mRNA. Regions of the hTERT mRNA coding for conserved reverse transcriptase motifs 1, 2, A, B, C, D, and E (36) are highlighted. Alternate splicing of the hTERT mRNA may result in deletions within the protein. The α splice site causes a 36-base (12-amino acid) deletion within the conserved reverse transcriptase motif A. The β splice site results in a 182-base deletion resulting in a nonsense mutation, truncating the protein before the conserved reverse transcriptase motifs B, C, D, and E. PCR primers TERT-1784S/TERT-1928A amplify a 145-base region near the 5' end of the reverse transcriptase domain. PCR primers TERT-2164S/TERT-2620A amplify a region of the hTERT mRNA within the reverse transcriptase domain, which includes both the α and β splice sites. The size of PCR products produced by the 2164/2620 primer pair depends upon alternate splicing of the hTERT transcript in the sample. In this report, we refer to hTERT mRNA that is complete from base 2164 to base 2620 (457 bp) as full length. The base numbering of the hTERT mRNA in this report is based upon the sequence of GenBank Database entry accession number AF015950 (8).



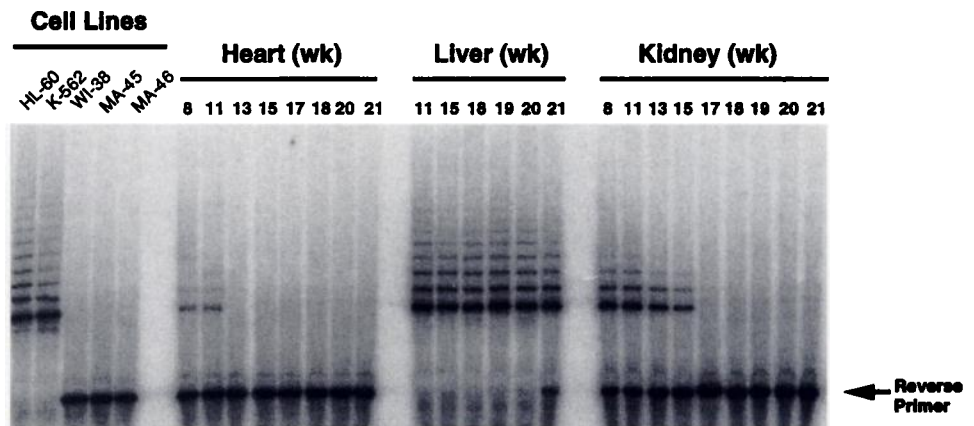
Each myometrial cell strain was created from myometrial tissue obtained from a uterus after hysterectomy. Approval to use human tissue was obtained from the Stanford University Panel on Human Subjects in Medical Research, and informed consent was obtained before acquiring tissue. Briefly, myometrial tissue was cut into 1-mm³ pieces, incubated in 2 mg of collagenase per ml PBS overnight at 37°C and then in DNase I (0.2 mg/ml) for 5 min. Cells were collected by centrifugation, resuspended in DMEM with 10% fetal bovine serum, and plated in culture flasks. Cells from the third passage (~10 population doublings after creation of the cell line) were used in these studies.

Fetal Tissues. Tissues were obtained after informed consent from patients and with the approval of the Stanford University Panel on Human Subjects in Medical Research. Fetal tissues were obtained from nine elective terminations of pregnancy. Gestational age was determined by crown-rump length when possible, otherwise by foot length. The accuracy of dating in this manner is ± 3 days. Heart, liver, and kidney tissues were obtained by dissection, washed twice in ice-cold PBS, flash frozen in a liquid nitrogen, and stored at -80°C.

Telomerase Assays. Telomerase activity was analyzed in samples using the PCR-based TRAP assay described previously (6) with the following modifications. Three hundred cells were analyzed in the TRAP reaction for each cell line specimen, and a sample containing 1 μ g of protein was analyzed in the TRAP reaction for each tissue specimen. Unlabeled TS oligonucleotide (AATCCGTCGACGAGATT) was used as the substrate for telomere extension. ³²P end-labeled reverse primer (GCGCGGCTAACCCCTAACCTAACCTA-ACC) was used for telomerase product amplification. Amplification was carried out in a total volume of 5 μ l under 15 μ l of liquid wax (MJ Research, Boston, MA) with an initial 94°C heating for 90 s, followed by 30 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s. Amplified products were electrophoresed on 5% polyacrylamide-urea gel and visualized by use of a PhosphorImager Scanner (Molecular Dynamics, Sunnyvale, CA).

PCR Analysis of hTR and hTERT. Total RNA was collected from samples using Tri-Reagent (Sigma Chemical Co., St. Louis, MO). 0.4 μ g total RNA, hexamer, and reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) was used to create cDNA for each sample. The cDNA samples were amplified in a 5- μ l reaction mixture containing 0.25 μ Ci of [α -³²P]dCTP (Amersham Co., Arlington Heights, IL), 50 μ M deoxynucleoside triphosphate, 0.625 unit KlenTaq I (Ab Peptides, St. Louis, MO), and 0.2 μ M of primers. hTR cDNA was amplified using TR-46S (CTAACCTAACTGAGAAGGGCGTAG) and TR-148A (GAAGGCGGCGAGCCGAGGCTTTTCC) oligonucleotides with an initial heating at 94°C for 90 s, followed by 28 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 30 s. Primers 5899 (CAGGCATCACCATTGGCAATGAGC) and 5900 (CGGATGTCCACGTCACACTTTCATGA) were added at 72°C of cycle 7 for the β -actin internal control. The first hTERT cDNA amplification used TERT-1784S (CGGAAGAGTGTCTGGAGCAA) and TERT-1928A (GGATGAAGCGGAGTCTGGA) oligonucleotides (see Fig. 1) with an initial heating at 94°C for 90 s, followed by 33 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 30 s. Primers 774 (GGGAATTCAAAACCTGGAACGGTGAAGG) and 775 (GGAAGCTTATCAAAGTCCTCGGCACACA) were added at 72°C of cycle 13 for the β -actin internal control. The second hTERT cDNA amplification used TERT-2164S (GCCTGAGCTGTACTTTGTCAA) and TERT-2620A (CGCAAACAGCTTGTCTCCATGTC) oligonucleotides with an initial heating at 94°C for 90 s, followed by 35 cycles of 95°C for 25 s, 68°C for 50 s, and 72°C for 50 s. Primers 774 and 775 were added at 72°C of cycle 15 for the β -actin internal control. Amplified products were electrophoresed on 5% polyacrylamide-urea gel and visualized by use of a PhosphorImager Scanner (Molecular Dynamics, Sunnyvale, CA).

Fig. 2. Telomerase activity in cell lines and fetal tissues. Telomerase activity measured by the TRAP assay. The presence of a 6-base ladder is evidence for telomerase activity in a given sample. Telomerase activity in fetal heart, liver, and kidney is seen to be expressed in a tissue-specific and gestational age-dependent manner. The position of 24-base ³²P end-labeled reverse primer, which is unincorporated into PCR products, is noted.



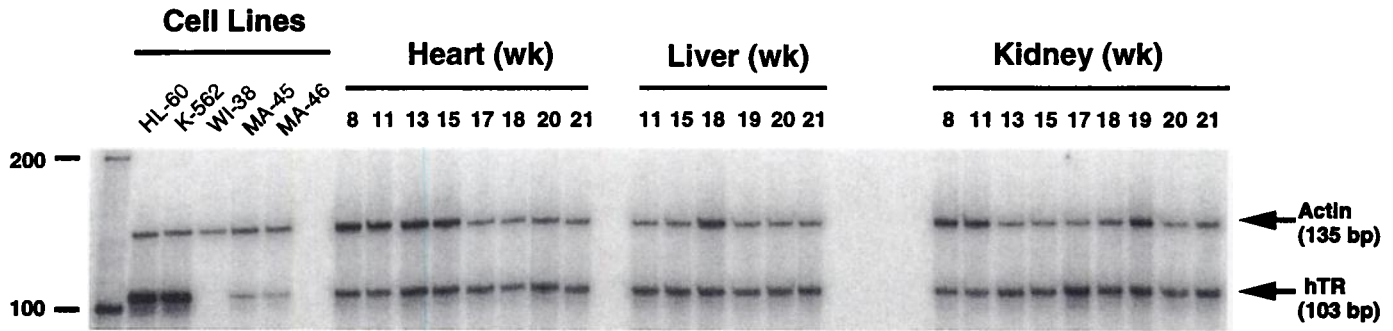


Fig. 3. hTR in cell lines and fetal tissues. hTR RNA expression measured by RT-PCR with primers TR-46S and TR-148A. The hTR PCR product and β -actin internal control are labeled. Of all specimens, only the WI-38 cell line does not express hTR mRNA.

RESULTS

Telomerase Activity in Cell Lines and Fetal Tissues. To examine the relationship between telomerase activity and expression of hTR and hTERT, we used the TRAP assay to measure telomerase activity in cell lines, cell strains, and in human fetal heart, liver, and kidney during early gestation. The TRAP assay measures telomerase activity in sample lysates by telomerase-mediated extension of the TS oligonucleotide, followed by amplification, electrophoresis, and visualization of extended products. Because human telomerase adds sequential 6-bp TTAGGG repeats onto the TS oligonucleotide, samples with telomerase activity produce a 6-base incremental ladder of TRAP products. Samples without telomerase activity do not display this characteristic ladder.

TRAP assays revealed that both cell lines (HL-60 and K-562 cells) displayed telomerase activity, and the cell strains (WI-38, MA45, MA46) did not (Fig. 2). Telomerase activity in the fetal tissues was similar to that reported in previous studies (16, 17). Telomerase activity was present in liver during all gestational ages sampled, but enzymatic activity in kidney was present only until the 15th week of gestation but not thereafter, and the activity in heart was observed only until the 11th gestational week.

Expression of hTR and hTERT in Cell Lines and Fetal Tissues. Because hTR and hTERT are believed to be the core components of the telomerase enzyme (14, 27, 29), we examined whether one or both of these subunits were limiting components for the expression of telomerase activity. All fetal tissues expressed hTR mRNA (Fig. 3), and thus hTR transcription was not correlated with telomerase activity. Both telomerase-positive cell lines contained hTR mRNA, as did two of three telomerase-negative cell strains.

hTERT expression in these samples was initially measured by RT-PCR using primers for a region of the transcript upstream of the reverse transcriptase domain (TERT-1784S and TERT-1928A).

hTERT mRNA was present in both telomerase-positive cell lines but in none of the telomerase-negative cell strains, suggesting that expression of hTERT may be the limiting subunit in expression of telomerase activity (Fig. 4). This pattern was also seen in the fetal heart and liver. In heart, hTERT mRNA and telomerase activity were expressed until the 11th week, but thereafter, neither hTERT mRNA nor telomerase activity were present. In the fetal liver, hTERT mRNA and telomerase enzymatic activity were detected in all samples through week 21. However, the correlation between hTERT mRNA and telomerase activity was not apparent in the kidney samples. Although hTERT mRNA expression in the kidney was present until at least the 21st week of gestation, telomerase enzymatic activity ceased after the 15th week (see Fig. 2).

Alternate Splicing of hTERT. To examine hTERT mRNA expression in greater detail, we performed RT-PCR using a primer set for the reverse transcriptase domain of the transcript (TERT-2164S and TERT-2620A). Two potential splice sites are within the region spanned by this primer set (Ref. 10; see Fig. 1). The α splice site causes a 36-bp deletion (bases 2186–2221) corresponding to the conserved reverse transcriptase motif A, which is required for an active reverse transcriptase. The β splice site results in a 182-bp deletion (bases 2342–2524) resulting in a nonsense mutation, truncating the protein before the conserved reverse transcriptase motifs B, C, and D, which are also required for an active reverse transcriptase.

PCR with the 2164/2620 primer set revealed alternate splicing of the *hTERT* gene in the two telomerase-positive cells lines and in human fetal tissues (Fig. 5). Four PCR products were found in both of the telomerase-positive cell lines. These four products represent the full-length hTERT transcript (457 bp), the α deletion transcript (421 bp), the β deletion transcript (275 bp), and the α and β deletion transcript (239 bp). Only the full-length hTERT transcript would be expected to code for an active reverse transcriptase. As expected, no

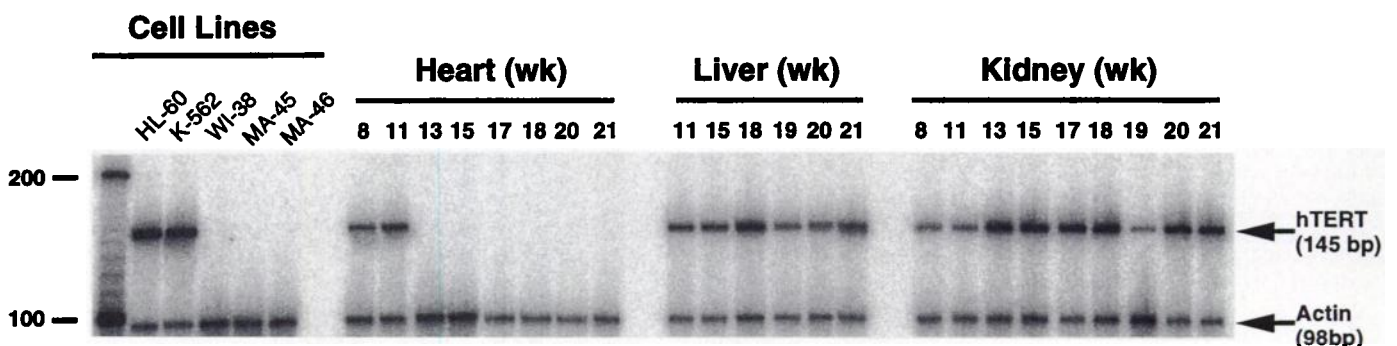


Fig. 4. hTERT in cell lines and fetal tissues. hTERT RNA expression measured by RT-PCR with primers TERT-1784 and TERT-1928. The hTERT PCR product and β -actin internal control are labeled. hTERT expression correlates with the presence or absence of telomerase activity (see Fig. 2) in all samples except the fetal kidney specimens.

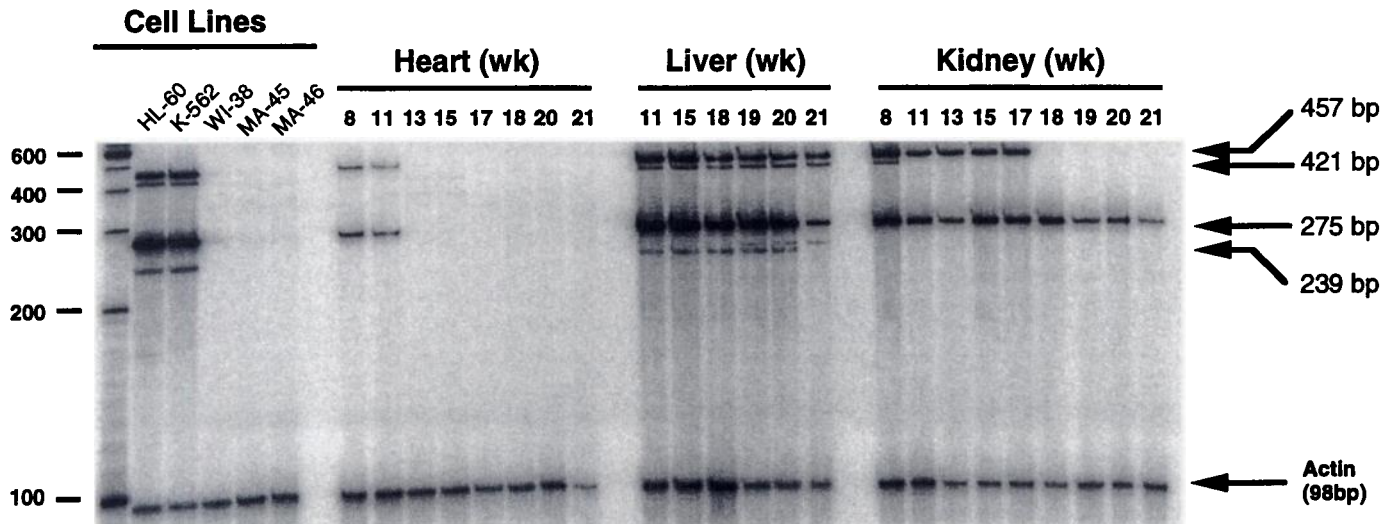


Fig. 5. Alternate splicing of hTERT in cell lines and fetal tissues. hTERT RNA expression measured by RT-PCR with primers TERT-2164 and TERT-2620. Four alternate transcripts of hTERT and the β -actin internal control are labeled. The presence of full-length hTERT transcript correlates with the presence or absence of telomerase activity (see Fig. 2) in all samples except the 17-week kidney.

PCR products were seen in any of the three telomerase-negative cell strains. Fetal heart contained the full-length and β deletion transcripts early in gestation, with all expression disappearing after 11 weeks (compare with Fig. 4). Fetal liver contained all four transcripts throughout early gestation. Fetal kidney expressed different transcripts, depending upon gestational age. In the 8-week specimen, full-length transcript is seen along with the α deletion and β deletion transcripts. Shortly thereafter, the α deletion transcript is no longer expressed, which leaves the full-length and β deletion transcripts until the end of the 4th gestational month. Only the β deletion transcript is seen in the older fetal kidney specimens. Sequences of PCR products were confirmed by ABI model 373 automated sequencing. Notably, when telomerase activity is absent in kidney development, there is a lack of full-length hTERT transcript. We have additionally examined human ovarian, endometrial, and myometrial neoplasms and matching normal tissues, and in no instance has telomerase activity been expressed without full-length hTERT message.⁴

DISCUSSION

The mechanisms by which telomerase is regulated is of great interest. Past studies have reported that neither TLP1 nor hTR expression correlates with telomerase activity (7, 8, 12, 13). However, three recent reports have presented evidence that expression of the hTERT protein correlates with telomerase activity (8–10).

In our study, the loss of telomerase activity in fetal heart and kidney appears to occur via separate mechanisms. In the heart, the lack of all hTERT transcripts after the 11th gestational week would lead to an absence of the catalytic component of the telomerase complex and cessation of enzymatic activity. However, hTERT transcripts persist during kidney development long after telomerase enzymatic activity disappears. With the exception of the 17-week sample, telomerase activity in the kidney correlates with the presence of full-length hTERT transcript. After the 17th week of kidney development, only the β deletion hTERT transcript is present. The β deletion transcript encodes for a truncated protein lacking vital RT motifs, which theoretically should not produce an active enzyme. Indeed, in *in vitro*

transcription and translation experiments, the β deletion transcript does not reconstitute an active telomerase enzyme (14). Thus, the disappearance of telomerase activity in fetal human tissues appears to occur both by repressing transcription of the *hTERT* gene (as seen in heart development) and by alternate splicing of the hTERT transcript (as seen in kidney tissue) in a tissue-specific and gestational age-dependent manner.

The discrepancy seen in the 17-week kidney sample (the presence of full-length hTERT without telomerase activity) remains unexplained. The 17-week kidney may be transitioning toward the disappearance of telomerase activity. Because two separate specimens from the 17-week kidney were used to assay telomerase activity and the expression of hTERT mRNA, it is possible that one segment contained hTERT and telomerase activity and the other contained neither. It is also possible that although the full-length hTERT is transcribed in the 17-week kidney sample, it might not be translated to produce an active telomerase complex. A final, unlikely explanation is that the 17-week kidney sample contained so little telomerase activity that it could not be detected in the TRAP assay.

It is evident that different tissues have different profiles of hTERT transcript expression. The developing liver contains a greater variety of hTERT transcripts than heart or kidney. This may be due to the presence of hematopoietic precursors in the liver during gestation, because hematopoietic progenitor cells have been shown to have telomerase activity (33). Thus, different lineages of cells expressing telomerase activity may generate the different hTERT transcripts seen in the liver specimens.

This study raises several important questions. Perhaps most interesting is what role the seemingly inactive variants of hTERT mRNA play in the cell, especially because the spliced variants account for a significant fraction of the total hTERT transcripts in all tissues examined. It is tempting to postulate that translated proteins from spliced transcripts could serve as regulators of telomerase activity. Alternative splicing may also represent an additional check point by which cells can suppress telomerase activity if hTERT message is transcribed. It will be crucial to learn if alternate hTERT-derived proteins are expressed in the cell and whether hTERT proteins lacking critical reverse transcriptase motifs can associate with hTR or TLP1 to modulate telomerase activity.

⁴ Gary A. Ulaner, Ji-Fan Hu, Thanh H. Vu, Haritha Oruganti, Linda C. Giudice, and Andrew R. Hoffman, manuscript in preparation.

Also of interest is whether tumors regulate telomerase via mechanisms similar to those used in development. It is likely that the tumor cell must overcome both transcriptional repression of the *hTERT* gene and alternate transcript splicing before an active enzyme can be assembled. Could preneoplastic and neoplastic tissues contain spliced but not full-length *hTERT* transcripts? This change would represent overcoming one hurdle to reexpression of telomerase activity, but not all hurdles. If this is the case, then analysis of *hTERT* variants in preneoplastic and neoplastic tissues could be useful in grading tumor phenotypes.

Maintenance of telomeres without detectable telomerase activity has been reported (34, 35). Whether alternate mechanisms of telomere maintenance are active in human development has not been investigated.

The data presented here support the presence of at least two mechanisms regulating telomerase activity in human development: transcriptional regulation of the *hTERT* gene and alternate splicing of *hTERT* transcripts. Whether other mechanisms exist and how tumors overcome these regulatory mechanisms to reactivate telomerase are of great interest. Further knowledge of telomerase regulation will enhance our understanding of cell aging and tumorigenesis.

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