

# *hEST2*, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization

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## Summary

Telomerase, the ribonucleoprotein enzyme that elongates telomeres, is repressed in normal human somatic cells but is reactivated during tumor progression. We report the cloning of a human gene, *hEST2*, that shares significant sequence similarity with the telomerase catalytic subunit genes of lower eukaryotes. *hEST2* is expressed at high levels in primary tumors, cancer cell lines, and telomerase-positive tissues but is undetectable in telomerase-negative cell lines and differentiated telomerase-negative tissues. Moreover, the message is up-regulated concomitant with the activation of telomerase during the immortalization of cultured cells and down-regulated during in vitro cellular differentiation. Taken together, these observations suggest that the induction of *hEST2* mRNA expression is required for the telomerase activation that occurs during cellular immortalization and tumor progression.

## Introduction

The linear chromosomes of eukaryotic cells offer the biological advantages of rapid recombination, assortment, and genetic diversification. However, linear DNA is inherently more unstable than circular forms. To address this difficulty, the eukaryotic chromosome has evolved to include a DNA-protein structure, the telomere, which

caps chromosome ends and protects them from degradation and end-to-end fusion (Blackburn, 1984; Zakian, 1995).

The DNA component of telomeres consists of tandem repeats of guanine-rich sequences that are essential for telomere function (Blackburn, 1984, 1991; Zakian, 1995). These repeats are replicated by conventional DNA polymerases and by a specialized enzyme, telomerase (Greider, 1995), first identified in the ciliate *Tetrahymena* (Greider and Blackburn, 1985). The telomerase enzyme is essential for complete replication of telomeric DNA because the cellular DNA-dependent DNA polymerases are unable to replicate the ultimate ends of the telomeres due to their requirement for a 5' RNA primer and their unidirectional mode of synthesis. Removal of the most terminal RNA primer following priming of DNA synthesis leaves a gap that cannot be replicated by these polymerases (Olovnikov, 1971; Watson, 1972). Telomerase surmounts this problem by de novo addition of single-stranded telomeric DNA to the ends of chromosomes (Greider and Blackburn, 1985, 1989; Yu et al., 1990; Greider, 1995).

The telomerase enzymes that have been characterized to date are RNA-dependent DNA polymerases that synthesize the telomeric DNA repeats by using an RNA template that exists as a subunit of the telomerase holoenzyme (Greider, 1995). The genes specifying the RNA subunits of telomerases have been cloned from a wide variety of species, including humans (Feng et al., 1995; Greider, 1995), and have been shown in several instances to be essential for telomerase function in vivo (Yu et al., 1990; Yu and Blackburn, 1991; Singer and Gottschling, 1994; Cohn and Blackburn, 1995; McEachern and Blackburn, 1995). In addition, three proteins have been identified to date that are associated with telomerase activity. p80 and p95 were purified from the ciliate *Tetrahymena* (Collins et al., 1995), and the gene encoding a mammalian homolog of p80, TP1/TLP1, has also been cloned (Harrington et al., 1997; Nakayama et al., 1997). The specific mechanism by which these proteins participate in telomerase function has not been defined.

Most recently, two related proteins, Est2p from the yeast *Saccharomyces cerevisiae* and p123 from the ciliate *Euplotes aediculatus*, were identified as the catalytic subunits of telomerase in their respective species (Counter et al., 1997; Lingner et al., 1997). *EST2* was first identified as a gene required for telomere maintenance in yeast (Lendvay et al., 1996) and is essential for telomerase activity (Counter et al., 1997; Lingner et al., 1997). Both the yeast and *Euplotes* proteins harbor several sequence motifs that are hallmarks of the catalytic regions of reverse transcriptases; substitution of several such residues in Est2p abolishes telomerase activity (Counter et al., 1997; Lingner et al., 1997). The mammalian homolog of these telomerase subunits has not yet been reported.

As might be expected from the known enzymatic properties of telomerase, perturbing the function of this enzyme in the ciliate *Tetrahymena*, through the overexpression of an inactive form of the telomerase RNA, or

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in yeast, through the mutation of genes encoding either the catalytic protein or template RNA subunit, leads to progressive telomere shortening as cells pass through successive cycles of replication (Yu et al., 1990; Singer and Gottschling, 1994; McEachern and Blackburn, 1995; Lendvay et al., 1996; Counter et al., 1997; Lingner et al., 1997). This loss of telomeric DNA is ultimately lethal if it is not overcome. The lethality seems to be triggered when telomeres have been truncated below a critical threshold level. Hence, in the absence of compensating mechanisms, yeast cell lineages that lack telomerase activity have a lifespan dictated by the lengths of their telomeres.

In humans, telomerase activity is readily detectable in germline cells and in certain stem cell compartments. However, enzyme activity is not detectable in most somatic cell lineages (Harley et al., 1994; Kim et al., 1994; Broccoli et al., 1995; Counter et al., 1995; Hiyama et al., 1995). Consistent with this, telomeres of most types of human somatic cells shorten with increasing organismic age and with repeated passaging in culture, similar to the situation seen in protozoan and yeast cells that have been deprived experimentally of a functional telomerase enzyme (Harley et al., 1990; Hastie et al., 1990). Eventually, the proliferation of cultured human cells will halt at a point termed senescence (Hayflick and Moorhead, 1961; Goldstein, 1990), apparently before the telomeres of these cells have become critically short.

Cultured normal human cells can circumvent senescence and thereby continue to proliferate when transformed by a variety of agents. In such cultures, telomere shortening continues until a subsequent point is reached that is termed crisis, where telomeres have become extremely short (Counter et al., 1992, 1994a; Shay et al., 1993; Klingelutz et al., 1994). Crisis, perhaps best described in SV40-transformed cells, is characterized by karyotypic instability, particularly the types of instability observed in chromosomes lacking functional telomeres, and by significant levels of cell death (Sack, 1981). The crisis phenotype is reminiscent of that observed in yeast and *Tetrahymena* cells in which telomerase function has been experimentally perturbed.

The simplest interpretation of these data is that the lifespan of telomerase-negative human cells, like that of their yeast and ciliate counterparts, is ultimately limited by the length of telomeres. Rare human cells that have acquired the ability to grow indefinitely emerge from crisis populations with a frequency of  $10^{-6}$ – $10^{-7}$  (Huschtscha and Holliday, 1983; Shay and Wright, 1989). This implies that a mutational event is required to confer the immortal phenotype on these cells. The immortal cells that escape crisis are characterized by readily detectable levels of telomerase activity and by stable telomeres (Counter et al., 1992, 1994a; Shay et al., 1995; Whitaker et al., 1995; Gollahon and Shay, 1996; Klingelutz et al., 1996). This suggests that activation of telomerase can overcome the limitations imposed by telomere length on the lifespan of cell lineages.

Activation of telomerase also appears to be a major step in the progression of human cancers. Unlike normal human cells, cancer cells can be established as permanent cell lines and thus are presumed to have undergone immortalization during the process of tumorigenesis.

Moreover, telomerase activity is readily detected in the great majority of human tumor samples analyzed to date (Counter et al., 1994b; Kim et al., 1994; Shay and Bacchetti, 1997).

Taken together, these various observations have been incorporated into a model that proposes that the limitation on prolonged cell replication imposed by telomere shortening serves as an important antineoplastic mechanism used by the body to block the expansion of precancerous cell clones. According to such a model, tumor cells transcend the crisis barrier and emerge as immortalized cell populations by activating previously unexpressed telomerase, enabling them to restore and maintain the integrity of their telomeres (Counter et al., 1992, 1994b; Harley et al., 1994).

A major question provoked by this model is the mechanism used to resurrect telomerase expression during tumor progression. Expression of the telomerase-associated protein TP1/TLP1 does not reflect the level of telomerase activity (Harrington et al., 1997; Nakayama et al., 1997). It is also clear that the levels of the human telomerase RNA component, *hTR*, cannot completely explain the regulation of telomerase activity. Although the levels of *hTR* and its mouse counterpart, *mTR*, increase with tumor progression (Feng et al., 1995; Blasco et al., 1996; Broccoli et al., 1996; Soder et al., 1997), the amounts of these transcripts do not always correlate with enzymatic activity. Indeed, *hTR* or *mTR* transcript levels can be significantly higher in telomerase-negative cells and tissues than in telomerase-positive cancer cells (Avilion et al., 1996; Bestilny et al., 1996; Blasco et al., 1996). Similarly, even though telomerase levels increase 100- to 2000-fold during the immortalization of human cells, the level of *hTR* message increases, at most, two-fold (Avilion et al., 1996). Therefore, derepression of the *hTR* and TP1 subunits cannot easily be invoked to explain the appearance of telomerase activity in the great majority of human tumor samples. Thus far, the rate-limiting step in telomerase activation has remained elusive.

We report here the isolation of a gene that represents the human homolog of the yeast and ciliate genes encoding the telomerase catalytic subunits. Evidence presented here also provides an important clue indicating how telomerase activity is activated during cellular immortalization and malignant tumor progression.

## Results

### Identification of the *hEST2* Gene

Using the sequences derived from the catalytic subunit of the *S. cerevisiae* telomerase, Est2p, and from the Euplotes p123 protein (Lendvay et al., 1996; Counter et al., 1997; Lingner et al., 1997), we identified a human homolog (GenBank accession number AA281296) in the National Center for Biotechnology Information expressed sequence tag database. As shown in Figure 1 (underlined sequence), the conceptual translation of this DNA sequence shows clear relatedness to both the yeast and ciliate telomerase sequences, as evidenced by identical or similar amino acid residues scattered throughout the entire length of the expressed sequence tag.

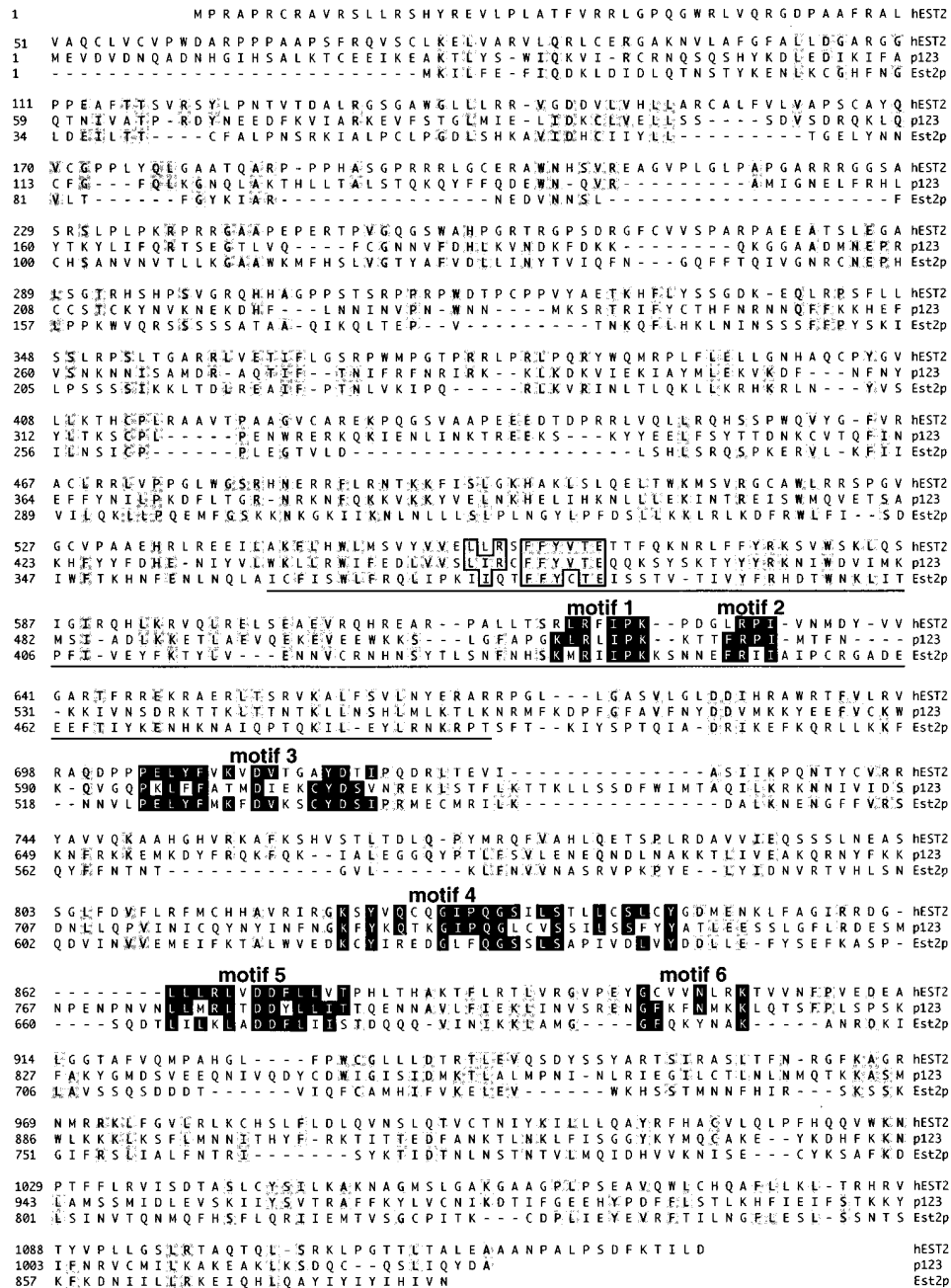


Figure 1. *hEST2* Encodes a Human Homolog of *Est2p* and *p123*

Alignment of the predicted amino acid sequence of *hEST2* with the yeast *Est2p* and *Euplotes p123* homologs. Amino residues within shaded and closed blocks are identical between at least two proteins. Identical amino acids within the RT motifs (Poch et al., 1989; Xiong and Eickbush, 1990) are in closed boxes, an example of a telomerase-specific motif in an outlined shaded box, and all other identical amino acids in shaded boxes. RT motifs are extended in some cases to include other adjacent invariant or conserved amino acids. The sequence of the expressed sequence tag AA281296 is underlined.

Since the identified expressed sequence tag provided only a fragment of the putative human telomerase open reading frame, we screened a human Jurkat T-cell lymphoma and a human Nalm-6 pre-B cell leukemia cDNA library with a probe derived from the expressed sequence tag and retrieved a total of seven cDNA clones. The resulting sequence was extended further in the 5' direction by rapid amplification of cDNA ends (RACE) on

human testis cDNA and on an independently generated human testis cDNA library.

Assembly of the cDNA clones and RACE products, together with the clone containing the expressed sequence tag, resulted in a contiguous sequence spanning 4030 bp. Conceptual translation of this 4 kbp sequence reveals an open reading frame of 1132 amino acids that is predicted to encode a 127 kDa protein (Figure 1).

Although we have not identified an in-frame upstream stop codon in the sequence, the first methionine identified by RACE (Figure 1) is a candidate for the translation start site, as the sequence of this putative translation initiation site, CCCGCGAUGC, is similar to the consensus GCC(A/G)CCAUGG characteristic of translation initiation sites (Kozak, 1986).

The predicted 127 kDa protein shares extensive sequence similarity with the entire sequences of the Euplotes and yeast telomerase subunits (Figure 1) and extends beyond the amino and carboxyl termini of these proteins. A BLAST search reveals that the probabilities of these similarities occurring by chance are  $1.3 \times 10^{-18}$  and  $3 \times 10^{-13}$ , respectively. By way of comparison, the probability of similarity between the yeast and Euplotes telomerases in a protein BLAST search is  $6.9 \times 10^{-6}$ . We have named the human gene *hEST2* (human *EST2* homolog) to reflect its clear relationship with the yeast gene, the first of these genes to be described. *EST2* was named because of the phenotype of ever shortening telomeres caused by its mutant alleles (Lendvay et al., 1996) and was later demonstrated to encode the yeast telomerase catalytic subunit (Counter et al., 1997; Lingner et al., 1997).

Like the yeast and ciliate telomerase proteins, *hEST2* is a member of the reverse transcriptase (RT) family of enzymes (Figures 1 and 2). Seven conserved sequence motifs, which define the polymerase domains of these enzymes, are shared among the otherwise highly divergent RT family (Poch et al., 1989; Xiong and Eickbush, 1990). *p123* and *Est2p* share six of these motifs with, most prominently, the *a2-Sc* enzyme, an RT that is encoded within the second intron of the yeast *COX1* gene (Kennell et al., 1993). These six motifs, including the invariant aspartic acid residues known to be required for telomerase enzymatic function (Counter et al., 1997; Lingner et al., 1997), are found at the appropriate positions of the predicted sequence of *hEST2* (Figures 1 and 2). Thus, the proposed human telomerase catalytic subunit, like its yeast and ciliate counterparts, belongs to the RT superfamily of enzymes.

Although *hEST2* shares some sequence similarity with RTs, it is not a conventional RT. Rather, it is far more closely related to the telomerase catalytic subunits of yeast and ciliates than to other RTs. Whereas the BLAST probability of sequence similarity between *hEST2* and the telomerase subunits of the single-cell eukaryotes arising by chance is  $10^{-13}$ – $10^{-18}$ , the chance probability of sequence similarity with the next most closely related RT, *a2-Sc*, is 0.12. Beyond the motifs that define the polymerase domains of these various enzymes, *hEST2* shows no sequence similarity with RTs. In contrast, in its domains that lie N-terminal to the polymerase domain, BLAST searches identify clear relatedness between *hEST2* and both *p123* and *Est2p*, the chance occurrence of these similarities being  $1.6 \times 10^{-9}$  and  $1.8 \times 10^{-4}$ , respectively. We note that many of the sequence identities in the N termini of *hEST2*, *p123*, and *Est2p* reside in a region just before motif 1 (Figure 1, boxed region). This sequence is not found in RTs, nor is it apparent in other proteins, suggesting the presence of motifs that may be unique to telomerases. Identification of the catalytic subunits of yet other telomerases will be required to validate this possibility.

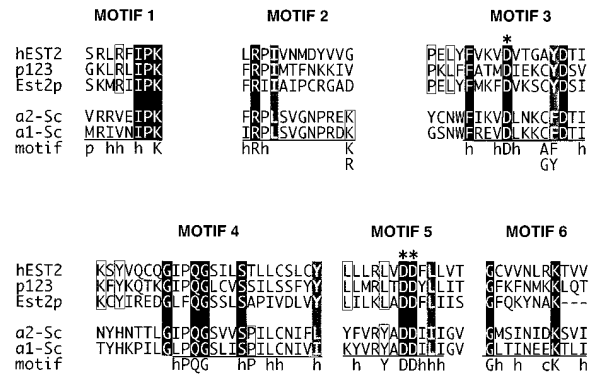


Figure 2. Alignment of RT Motifs 1–6 of Telomerase Subunits *hEST2*, *p123*, and *Est2p* with *S. cerevisiae* Group II Intron-Encoded RTs *a2-Sc* and *a1-Sc*

The consensus sequence of each RT motif is shown ([h], hydrophobic residues; [p], small polar residues; [c], charged residues). Amino acids that are invariant among telomerases and the RT consensus are in closed boxes, while those that are invariant among telomerases and similar to the RT consensus are in shaded boxes. Open boxes identify highly conserved residues unique to either telomerases or to nontelomerase RTs. Asterisks denote amino acids essential for polymerase catalytic function.

Even within the *hEST2* domains that share sequence similarity with RTs, it is clear that *hEST2* is more closely related to the already described telomerases than it is to nontelomerase RTs. For example, the sequence similarity of the region encompassing the RT motifs between *hEST2* and the catalytic subunits of yeast and Euplotes has a probability of chance occurrence of  $5.7 \times 10^{-6}$  and  $1.9 \times 10^{-5}$ , respectively, compared to 0.0056 for *a2-Sc*, the next most closely related nontelomerase RT. Within the RT motifs are several amino acids that are invariant among the telomerases but divergent between telomerases and nontelomerase RTs, or alternatively, nearly invariant among nontelomerase RTs but divergent between these RTs and telomerases (Figure 2).

In summary, *hEST2*, *Est2p*, and *p123* form a clearly defined subgroup within the RT family. For these reasons, we conclude that *hEST2* is a human homolog, and very likely an ortholog, of the microbial enzymes described to date.

We mention in passing that our sequencing of a number of cDNA clones has revealed two distinct forms of *hEST2* transcripts. Four independent cDNA clones, isolated from three independently generated libraries deriving from distinct cell types, lack an identical 182 bp segment within the open reading frame (data not shown). The absence of this segment leads to a shift in reading frame that introduces a premature termination codon. Both forms of the *hEST2* transcript were detected by RT-PCR in a variety of human cell types (data not shown). Although we lack any information on the intron-exon boundaries of *hEST2*, the simplest interpretation of these data is that the sequence difference between the two groups of cDNAs reflects the existence of two alternatively spliced mRNAs of the *hEST2* gene. The physiological consequences of the expression of the potential nonfunctional *hEST2* transcript are obscure at present.

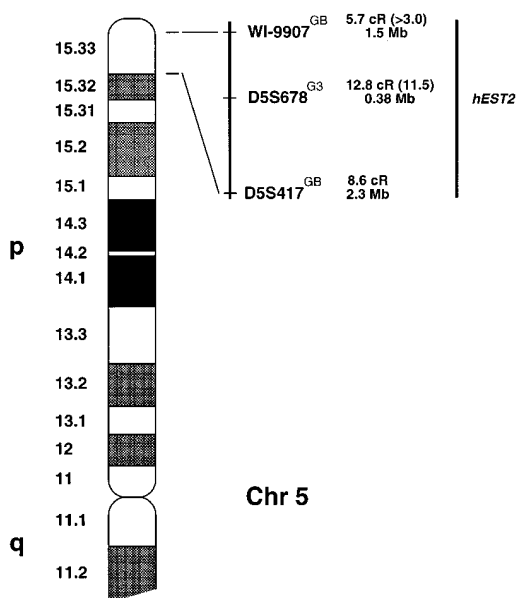


Figure 3. Chromosomal Localization of *hEST2*

Ideogram of human chromosome 5p showing linkage of *hEST2* to sequence-tagged site (STS) markers WI-9907 and D5S417 determined by radiation hybrid mapping employing the Genebridge 4 RH panel (GB) (1 cR = 270 kb). The higher resolution Stanford G3 RH panel (G3) placed *hEST2* close to marker D5S678 (1 cR = 30 kb). The calculated distance between *hEST2* and these STS markers is in centiRays (cR) with LOD scores in parentheses.

#### Chromosomal Localization of *hEST2*

The existing *hEST2* cDNA was used as a probe in Southern blot analyses of human genomic DNA. These reveal *hEST2* to be a single-copy gene with an estimated size of 40 kb (data not shown). All of the genomic sequences reactive with the cDNA probe appear to be components of this ~40 kb locus, suggesting that there are no other closely related genes in the human genome.

*hEST2* was localized to a specific chromosomal region by analyzing two independent panels of hamster-human radiation hybrid (RH) cells with two markers spanning different regions of *hEST2*. Initial mapping using the Genebridge 4 RH panel placed both *hEST2* markers between sequence-tagged sites (STS) WI-9907 and D5S417 (Figure 3). Independent confirmation of this localization was then obtained by mapping carried out with a second panel, the Stanford G3 RH panel. This second mapping placed both *hEST2* markers next to STS marker AFMA139YA9 (GDB locus D5S678), which itself is localized between the above-mentioned markers WI-9907 and D5S417. These markers are present at the telomeric end of chromosome 5p (Hudson et al., 1995).

Mapping of the *hEST2* locus was further refined by localizing it to subband 5p15.33, since the STS markers D5S678 and D5S417 have been assigned to this band on the Genethon YAC contig map (Chumakov et al., 1995). This localization is consistent with fluorescence in situ hybridization (FISH) analysis of YAC 767E1. This YAC maps further away from the telomere than D5S678 and has been assigned to chromosome 5p15.33 by FISH (Chumakov et al., 1995).

We note in passing that chromosome 5p is one of the

most common targets for amplification in non-small cell lung cancers, being amplified in ~70% of tumors (Balsara et al., 1997; Petersen et al., 1997). The effects of this amplification on *hEST2* expression levels are unknown.

#### Expression of *hEST2* Transcripts

Much of our work has been motivated by the question of how telomerase becomes activated during tumor progression. As discussed above, this activation has been associated with the immortalization of tumor cells. A variety of mechanisms might be invoked to explain such activation, among which is the induction of the expression of one or more telomerase subunits. Since the telomerase holoenzyme is presumed to exist as a multisubunit ribonucleoprotein complex, the levels of any one of the subunits, including those of the catalytic subunit described here, might be rate-limiting in determining enzyme activity. Alternatively, the components of the telomerase holoenzyme might be expressed constitutively and subject to various types of posttranslational modification that would govern their activity. As mentioned earlier, transcript levels of the telomerase RNA subunit and of the *TP1/TLP1* gene do not necessarily correlate with telomerase activity (Feng et al., 1995; Avilion et al., 1996; Blasco et al., 1996; Harrington et al., 1997; Nakayama et al., 1997), implying that at least one other mechanism is responsible for regulating human telomerase.

To address one of the remaining possible regulatory mechanisms, we analyzed the expression levels of *hEST2* mRNA in various cell types, using both RNA Northern hybridizations and RNase protection assays to do so. RNA blots prepared from a panel of normal human tissues and human cancer cell lines were probed with cDNA fragments deriving from two independent, nonoverlapping regions of the *hEST2* gene. This probing revealed two major RNA species migrating near the 4.4 kb and the 9.5 kb markers, as well as a minor species of ~6 kb (Figure 4). Each of these RNA species was recognized by both probes, confirming that each represents an *hEST2* mRNA.

*hEST2* message was detectable in several normal tissues including thymus, testis, and intestine. Of these, testis (Kim et al., 1994; Wright et al., 1996) and intestine (Hiyama et al., 1996) are known to be telomerase-positive, while the telomerase status of the thymus has not been reported. In marked contrast, the *hEST2* transcript was undetectable in our assays in most other normal human tissues, including heart, brain, placenta, liver, skeletal muscle, and prostate (Figure 4), all of which have been reported to be telomerase-negative (Kim et al., 1994; Wright et al., 1996; Shay and Bacchetti, 1997). The absence of detectable *hEST2* message in ovary may seem paradoxical, as ovary is a germline tissue and germline tissues have been reported to harbor significant levels of telomerase. However, oocyte division is completed by birth, and while fetal ovary is telomerase-positive (Kim et al., 1994; Wright et al., 1996), both adult ovarian epithelium and mature oocytes are telomerase-negative (Counter et al., 1994b; Wright et al., 1996). It is also possible that the levels of *hEST2* are below the

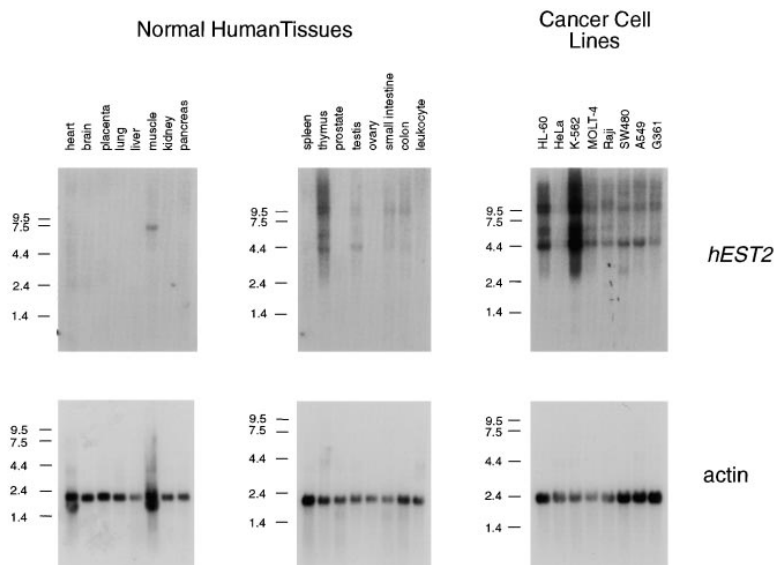


Figure 4. Expression of *hEST2* in Normal Human Tissues and Cancer Cell Lines

Duplicate RNA blots were probed with an *hEST2* probe (top panels) or with a  $\beta$ -actin probe as internal control (bottom panels). The 4.4 kb, 6 kb, and 9.5 kb transcripts are *hEST2*-specific.

threshold of detection. We note that the 7 kb band seen in muscle tissue appears to be an artifact, as it was not observed with an independent *hEST2* probe (data not shown).

In contrast to its absence in the majority of normal tissues, *hEST2* mRNA was strongly expressed in a variety of cancer cell lines, most strikingly in the leukemic cell lines HL-60 and K-562 (Figure 4). It is unclear why only very low levels of *hEST2* message were observed in HeLa cells on this particular Northern blot (Figure 4, rightmost panel, lane 2); reanalysis of independently prepared HeLa cell RNA both by Northern blot and by RNase protection demonstrated that *hEST2* mRNA is present in HeLa cells at high levels comparable to those seen in K-562 cells (data not shown; Figure 6 below). The two major and one minor *hEST2* transcripts appear to be expressed in the same relative proportions in all cell types that yielded detectable *hEST2* mRNA.

#### *hEST2* Expression in Primary Human Cancers

The expression of *hEST2* mRNA in cancer cell lines suggested that *hEST2* transcript levels might be elevated in primary tumors as well. To test this, we extracted RNA from a variety of tumor samples as well as from normal control tissues and analyzed this RNA for the presence of *hEST2* mRNA using an RNase protection assay. In total, 11 of 11 tumor samples examined showed detectable levels of *hEST2* message (Figure 5). *hEST2* RNA was undetectable in normal breast and ovarian tissue but was expressed at significant levels in 2 of 2 breast tumors as well as 2 of 2 breast tumor-derived cell lines, and in 4 of 4 ovarian tumors.

As expected from Northern hybridization analysis of *hEST2* RNA (Figure 4) and the known pattern of telomerase catalytic activity in human tissues (Kim et al., 1994; Hiyama et al., 1996), the *hEST2* transcript was detected at high levels by the RNase protection assay in testis and at moderate levels in colon. Similarly, using the RNase protection assay, four colon tumor samples and a testicular tumor sample were found to express

detectable levels of *hEST2* RNA (Figure 5); two of the colon tumors showed significantly elevated levels as well. These data suggest that the *hEST2* message is expressed in a very high percentage of tumors and is specifically induced in tumors that arise from telomerase-negative tissues.

#### Up-Regulation of *hEST2* mRNA Is Associated with Telomerase Activation and Cellular Immortalization

The strong expression of the *hEST2* message observed in several tumors and cancer cell lines suggested that the levels of this transcript might be correlated with the amount of telomerase enzyme activity in these various cell types. To investigate this possibility, we analyzed telomerase activity and *hEST2* RNA level in a panel of nonimmortalized and immortalized cell lines. Using the TRAP telomerase assay (Kim et al., 1994), two mortal fibroblast strains, WI-38 and IMR-90, were found to lack detectable telomerase activity, in contrast to three immortal cell lines, HeLa, 293, and K-562, in which telomerase activity was readily detectable (Figure 6A). The telomerase-negative cells also lacked detectable *hEST2* message as gauged by an RNase protection assay, while the immortal telomerase-positive cell lines expressed significant levels of *hEST2* RNA (Figure 6A). These results indicate that *hEST2* message levels correlate closely with telomerase activity.

The association between *hEST2* RNA expression and telomerase activity, both being present in immortal transformed cells but absent in mortal normal cells, suggests that the induction of *hEST2* expression might underlie the activation of telomerase that occurs during cellular immortalization. As a direct test of this hypothesis, *hEST2* transcript levels were analyzed by RNase protection and were compared to the levels of hTR and telomerase activity in precrisis cells prior to the up-regulation of telomerase and in postcrisis telomerase-positive immortal cells from two different transformed human cell populations: Epstein-Barr virus-transformed

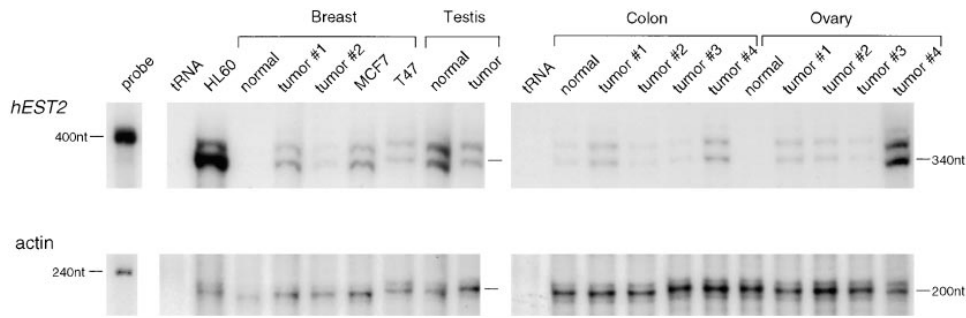


Figure 5. *hEST2* Expression in Primary Human Tumors

RNase protection assays are shown for *hEST2* and  $\beta$ -actin controls. Sizes of the full-length and protected bands are indicated. Shown are the HL-60 leukemia cell line (control), normal breast tissue, two primary breast tumors, the MCF7 and T47D breast cancer cell lines, and normal and primary tumor tissues from the testis, colon, and ovary. The doublet seen protected by the *hEST2* probe is invariant and may be a result of probe secondary structure.

B lymphocytes (B4 cells) and SV40 T antigen-transformed embryonic kidney cells (HA1 cells). As previously described (Counter et al., 1992, 1994b; Avilion et al., 1996), little or no telomerase activity was detected in cells prior to crisis when telomere length decreases, but abundant levels were detected in postcrisis cells that maintain telomere length (Figure 6B). In contrast, as

previously reported (Avilion et al., 1996), the levels of *hTR* remained essentially constant throughout the immortalization process. The levels of *hEST2* RNA, on the other hand, parallel telomerase activity during cell immortalization. *hEST2* RNA was undetectable in the precrisis cells but was clearly present in the postcrisis, telomerase-positive cells (Figure 6B). These results

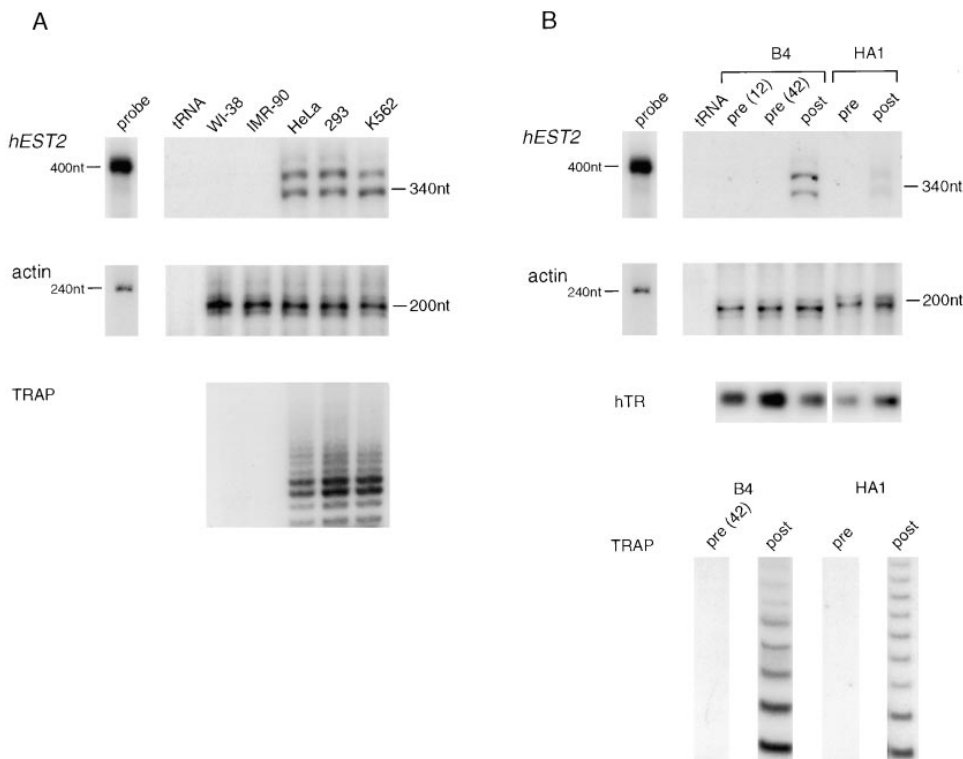


Figure 6. Correlation in Cultured Cells of *hEST2* RNA with Telomerase Activity and Immortalization Status

(A) Comparison of normal and immortalized cells. *hEST2* and  $\beta$ -actin control RNA levels were determined by RNase protection analysis using 40  $\mu$ g of total RNA from each cell type. Sizes of the full-length probes and protected fragments are indicated. Telomerase was assayed with the TRAP protocol on 100 ng of cell lysate (bottom panel).

(B) Comparison of EBV-transformed B cells (B4) and SV40 T antigen-transformed kidney cells (HA1) precrisis and postcrisis. Cell line passage numbers are indicated in parentheses. The top two panels show RNase protection of *hEST2* and  $\beta$ -actin as a control. The third panel shows Northern blot analysis of the *hTR* transcript. The bottom panel shows TRAP telomerase assays on 1  $\mu$ g of cell lysate.

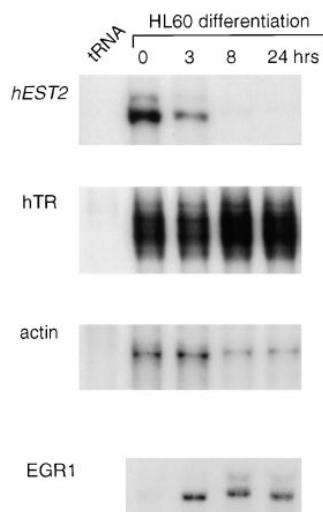


Figure 7. Changes in *hEST2* RNA Expression during Induced Differentiation of HL-60 Cells

Differentiation of the human promyelocytic leukemia cell line HL-60 was induced with all-*trans* retinoic acid as described in Experimental Procedures, and cells were collected for analysis at the indicated times. Total cellular RNA (20  $\mu$ g) was analyzed for each time point. The first three panels show RNase protection analysis of the *hEST2*,  $\beta$ -actin control, and *hTR* transcripts, respectively. Analysis of the human  $\beta$ -actin transcript was performed in the same reaction tube as that for *hEST2*. The fourth panel shows Northern blot analysis of the early growth response gene *EGR-1*, whose expression is rapidly induced during HL-60 differentiation (Nguyen et al., 1993); this is used as a control to verify cell differentiation. RNA quantity and integrity were assessed by ethidium bromide staining (data not shown).

strongly suggest that the induction of *hEST2* message may be a rate-limiting step for the activation of telomerase during immortalization.

#### Down-Regulation of *hEST2* Expression upon Cellular Differentiation

Since the up-regulation of *hEST2* RNA is associated with the activation of telomerase during cell immortalization, we wondered whether conditions that repress telomerase activity in cultured cells might similarly operate by shutting down expression of the *hEST2* mRNA in these cells. Human HL-60 promyelocytic leukemia cells can be induced to differentiate to mature granulocytes by treatment with retinoic acid. During this process, telomerase activity has been shown to decline over a period of 2–5 days (Sharma et al., 1995; Bestilny et al., 1996; Savoysky et al., 1996; Xu et al., 1996).

As demonstrated in Figure 7, induction of HL-60 differentiation leads to the disappearance of the *hEST2* mRNA within 24 hours, foreshadowing the loss of telomerase activity that declines more slowly. Once again, this contrasts with the behavior of the human telomerase RNA subunit, whose expression during induced differentiation remains constant (Figure 7) as previously reported (Bestilny et al., 1996). We note that the levels of the *hEST2* mRNA decline precipitously within the first 3 hours after induced differentiation. This rapid down-modulation is compatible with a short half-life for the *hEST2* message and suggests that the levels of this

RNA species are under tight control in these cells. The contrasting delay in the decline of telomerase activity is consistent with the reported  $\sim$ 24 hour half-life of the enzymatic activity after cycloheximide treatment (Holt et al., 1996).

Taken together, our findings suggest the possibility that modulation of *hEST2* RNA expression levels is an important mechanism used in a variety of developmental contexts to determine the amount of telomerase activity present in specific cell lineages. Indeed, the levels of the catalytic subunit of telomerase may represent a rate-limiting determinant of enzyme activity in many types of cells. Moreover, up-regulation of *hEST2* message may be an important mechanism through which telomerase becomes activated during both cellular immortalization and the progression of malignant tumors.

#### Discussion

*hEST2*, the putative human telomerase subunit described here, shares extensive sequence similarities with the catalytic subunits of the yeast and ciliate telomerase enzymes. The amino acid sequence conservation between these three enzymes is extensive and is scattered throughout their reading frames. In addition, we have been unable to detect related genes in the human genome by Southern blotting analysis (not shown). Together, these data lead us to conclude that the gene described here is very likely to specify the catalytic subunit of the human telomerase holoenzyme. Formal proof will depend upon experiments analogous to those performed with the yeast *EST2* gene (Counter et al., 1997; Lingner et al., 1997). Thus, such experiments will need to demonstrate that mice lacking the mouse *mEST2* homolog also lack telomerase activity; that the *hEST2* protein is physically associated with a ribonucleoprotein complex that exhibits telomerase activity; and that alteration of critical residues in the domain of *hEST2* that is homologous to RTs inactivates its catalytic function.

While all three telomerases described to date give indications of being distant homologs of a variety of RTs, they also share several unique, telomerase-specific sequence motifs. Because yeast, ciliates, and mammals represent highly diverged branches of the phylogenetic tree, this suggests that the catalytic subunit of the telomerase was developed early in eukaryotic evolution, being present possibly in the cell that became ancestral to all contemporary eukaryotes. Further back, it appears that this enzyme shares ancestry with the precursors of the RTs specified by a variety of transposons and viruses.

The repression of *hEST2* mRNA in telomerase-negative cells and tissues and its up-regulation that is associated here with a number of human tumor cell lines and primary human tumors suggest one mechanism by which telomerase activity might be modulated. During development, the expression of the *hEST2* mRNA may be repressed in many postembryonic cell lineages, depriving cells in these lineages of the telomerase catalytic subunit. This in turn may underlie the observed progressive telomeric shortening associated with aging in the cells in many of these lineages.



In contrast, the reappearance of telomerase enzyme activity when transformed cells escape from crisis or when tumors progress toward malignancy may in many cases be explained mechanistically by the derepression of *hEST2* mRNA expression. As reported here, in cell cultures, this derepression occurs in both transformed embryonic kidney cells and lymphocytes when they emerge from crisis and begin to exhibit telomerase activity. Our work does not address whether the enhanced *hEST2* RNA expression is achieved at the transcriptional or posttranscriptional level.

Although we have found a general correlation between *hEST2* mRNA levels and assayable telomerase activity, the present experiments do not demonstrate that these two manifestations of *hEST2* gene expression are always present in some constant, predictable ratio. This leaves open the possibility that other mechanisms besides the presently observed modulation of *hEST2* mRNA levels may intervene to modulate telomerase activity.

Perhaps most important, the identification of *hEST2* as the gene specifying the putative telomerase catalytic subunit may provide an entree into understanding one of the essential steps in human tumor pathogenesis—that leading to cell immortalization. Enzymatic assays have demonstrated that telomerase is often activated at a relatively late step in tumor progression (Harley et al., 1994). This step may occur when evolving, premalignant cell clones have surmounted the senescence barrier and subsequently, having exhausted their telomeric ends, encounter the successive barrier of crisis. At this stage, the activation of telomerase may enable cells to breach these barriers to further clonal expansion, thereby conferring great selective advantage on the rare cell that has acquired the ability to resurrect the long-repressed telomerase activity. Indeed, activation of telomerase may represent an essential step in tumor progression.

Such dependence on telomerase activity would suggest that this enzyme represents an attractive target of drugs designed to interfere with malignant cell proliferation. The finding that *hEST2* message is up-regulated in human tumors and in immortalized cells lends further credence to this idea. Furthermore, the identification of *hEST2* as the candidate telomerase catalytic subunit may provide a biochemical reagent for identifying such drugs.

#### Experimental Procedures

##### Cloning of *hEST2*

The expressed sequence tag database (dbEST) was searched for sequences related to the yeast protein Est2p (Lendvay et al., 1996) and the Euplotes protein p123 (Lingner et al., 1997), using the program TBLASTN. This resulted in the identification of a homologous expressed sequence tag, GenBank accession number AA281296, which derived from Soares NbHTGBC cDNA clone 712562. DNA from clone plasmid 712562 was PCR-amplified with primers HT-1 (5'-AAGTTCCTGCACTGGCTGATGAG-3') and HT-5 (5'-TCGTAGTTGAGCAGCTGAACAG-3'). The resulting 377 bp fragment was used to probe  $\lambda$  ZAP phage cDNA libraries derived from the human Jurkat T-cell lymphoma (Stratagene, La Jolla, CA) and human Nalm-6 pre-B cell leukemia cell lines (Weissbach et al., 1994). A total of seven independent clones were isolated from these two libraries. Three of these cDNA clones together with plasmid 712562 were sequenced completely in both directions. The remaining clones were sequenced in specific regions. Reprobing of the Jurkat library with

the 5'-most 500 bp region identified from the *hEST2* cDNA clones yielded one new clone containing an insert that overlapped with already determined sequences.

Rapid amplification of cDNA ends (RACE) was performed by PCR amplifying testis Marathon-ready cDNA (Clontech, Palo Alto, CA) with flanking primer AP-1 (Clontech) and *hEST2* primer R0096 (5'-CAAGAAACCCACGGTCACTCGGTCCACGCG-3'), and then reamplifying with flanking primer AP-2 and *hEST2* primer R0098 (5'-CAGCTCCTTCAGGCAGGACACTGCGGG-3'). The product was subcloned and sequenced in both directions. RACE was also performed on aliquoted pools of plasmid DNA from a human testis cDNA library (Reduced Complexity cDNA Analysis, Q. L. and Fang Chen, unpublished data) with primers HT-21 (5'-CAGGTGACACCACACAGAAA-3') or HT-22 (5'-TTCCAAGCAGCTCCAGAAA-3') and a vector primer, reamplified with the vector primer and *hEST2* primers R0098 or R0097 (5'-CCTTCGGGGTCCACTAGCGTGTGGCGG), then purified and sequenced.

DNA sequencing reactions were performed with the AmpliTaq FS Prism ready reaction cycle sequencing kit (Perkin-Elmer/ABI) and electrophoresed on a 373 A Stretch ABI DNA sequencer. The resulting sequences were assembled into a contig using the program Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI). The 1132 amino acid ORF identified in this contig was aligned with p123 and Est2p using the Pattern-Induced Multi-Sequence Alignment Program version 1.4 (R. F. Smith, Baylor College of Medicine, and T. F. Smith, Boston University), with minor modifications to the final alignment. BLASTP searches were performed against the nonredundant protein databases.

##### Radiation Hybrid Mapping

Genomic DNA (25 ng) from hybrid clones of the Genebridge 4 and Stanford G3 radiation hybrid (RH) mapping panels (Research Genetics, Inc. Huntsville, AL) was PCR-amplified with the primer pair M1 (forward-5'-CACAGCCAGGCCGAGAGCAGA-3' and reverse-5'-AGG CCTCAGCCGGGACACTCAG-3'), yielding a 170 bp fragment in the 3'-untranslated region, and with primer pair M2 (forward-5'-GAAGA AAACATTCTGTGCGTG-3' and reverse-5'-GCCCTTGCCCCCAGC GACAT-3'), generating a 180 bp fragment crossing a putative intron-exon boundary near the *hEST2* stop codon. PCR was carried out for 35 cycles of 94°C for 0.5 min, 69°C for M1 or 65°C for M2 for 0.5 min, and 72°C for 1.5 min. The results of the PCR screening were analyzed using the statistical program RHMAP (Hudson et al., 1995; Stewart et al., 1997). STS markers were linked to both a physical chromosomal map and to YACs physically mapped by FISH (Cohen et al., 1993; Chumakov et al., 1995; Hudson et al., 1995).

##### RNA Isolation from Cell Lines and Primary Tissues

Cell lines were obtained from the American Type Culture Collection and grown under standard conditions. For HL-60 differentiation assays, cells were pelleted and resuspended in normal growth medium plus all-*trans* retinoic acid (Sigma) at a final concentration of 1  $\mu$ M.

All primary normal and tumor tissues were obtained from the Massachusetts General Hospital tumor bank. Tissues were processed in a tissue homogenizer. RNA samples were prepared in the RNA Stat-60 solution (Tel-Test "B," Friendswood, Texas) according to the manufacturer's protocol.

##### Northern Hybridization

Duplicate filters containing poly A(+)-selected mRNAs from various human tissues and cell lines (Multiple Tissue Northern Blots, Clontech, Palo Alto, CA), were incubated according to the manufacturer's instructions with two independent *hEST2* probes. One probe was derived by PCR-amplifying plasmid 712562 with primers HT-1 and HT-5 to generate the 377 bp fragment described above; the second, more 3' probe was generated from the 1064 bp Stul fragment of the same plasmid. The duplicate blots were then rehybridized with a  $\beta$ -actin probe. Northern blots for detection of *hTR* were performed with total RNA and an *hTR*-specific probe (Feng et al., 1995).

##### RNAse Protection Analysis

Radiolabelled RNA probes for RNAse protection analysis were synthesized using [ $\alpha$ -<sup>32</sup>P]UTP, T7 RNA polymerase, and the MAXIScript

kit (Ambion, Austin, TX). DNA templates for probe synthesis were created as follows. For the *hEST2* probe, the insert from plasmid clone 712562 was subcloned into pUHD  $10^{-3}$  (a gift from M. Gossen), and template DNA was synthesized by PCR using a forward plasmid-specific primer and a reverse primer containing 18 bp of *hEST2* complementary sequence (5'-TCTCTGCGGAGTTCTG) and the T7 promoter sequence. For the  $\beta$ -actin probe, template DNA was synthesized by PCR on a human  $\beta$ -actin cDNA insert (Clontech). The *hEST2* and the  $\beta$ -actin control probes were hybridized in the same reaction tube. The *hTR* probe was synthesized directly from the linearized pGRN83 plasmid (Feng et al., 1995).

RNAse protection analysis was performed using the HybSpeed RPA kit (Ambion) according to the manufacturer's protocol. Briefly, sample RNA and radiolabeled RNA probes were coprecipitated in ethanol, resuspended in hybridization buffer, hybridized at 68°C, then digested with RNases A and T1. Samples were then reprecipitated and analyzed on a 6% denaturing polyacrylamide gel.

#### Telomerase Assays

Telomerase repeat amplification protocol (TRAP) assays were performed as described (Kim et al., 1994), in some cases using the TRAPEze telomerase detection kit (Oncor, Gaithersburg, MD).

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#### GenBank Accession Number

The accession number for the cDNA sequence of *hEST2* reported in this paper is AF018167.

#### Note Added in Proof

As a clarification, we note that the initial TBLASTN search that identified the *hEST2* expressed sequence tag was performed with the Euplotes p123 protein (Lingner et al., 1997). Comparison with the yeast *Est2p* sequence (Lendvay et al., 1996) was used to confirm the significance of this homology.