Reconstitution of human telomerase activity *in vitro*

Tara L. Beattie*, Wen Zhou†, Murray O. Robinson† and Lea Harrington*

Telomerase is a ribonucleoprotein enzyme complex that adds single-stranded telomere DNA to chromosome ends [1]. The RNA component of telomerase contains the template for telomeric DNA addition and is essential for activity [1,2]. Telomerase proteins have been identified in ciliates, yeast and mammals [3–12]. In *Saccharomyces cerevisiae*, the Est2 protein is homologous to the 123 kDa reverse transcriptase subunit of *Euplotes* telomerase, and is essential for telomerase activity [8]. In humans, telomerase activity is associated with the telomerase RNA *hTR* [13], the telomerase RNA-binding protein TP1/TLP1 [5,12] and the TP2 protein encoded by the human *EST2* homolog [12] (also known as *TRT1*, *hEST2* or *TCS1* [9–11]). The minimal complex sufficient for activity is, however, unknown. We have reconstituted human telomerase activity in reticulocyte lysates and find that only exogenous *hTR* and TP2 are required for telomerase activity *in vitro*. Recognition of telomerase RNA by TP2 was species specific, and nucleotides 10–159 of *hTR* were sufficient for telomerase activity. Telomerase activity immunoprecipitated from the reticulocyte lysate contained *hTR* and recombinant TP2. Substitution of conserved amino acid residues in the reverse transcriptase domain of TP2 completely abolished telomerase activity. We suggest that TP2 and *hTR* might represent the minimal catalytic core of human telomerase.

Addresses: *Ontario Cancer Institute-Amgen Institute, Department of Medical Biophysics, University of Toronto, 620 University Avenue, Toronto, Ontario M5G 2C1, Canada. †Amgen, Inc. 1840 DeHavilland Drive, Thousand Oaks, California 91320, USA.

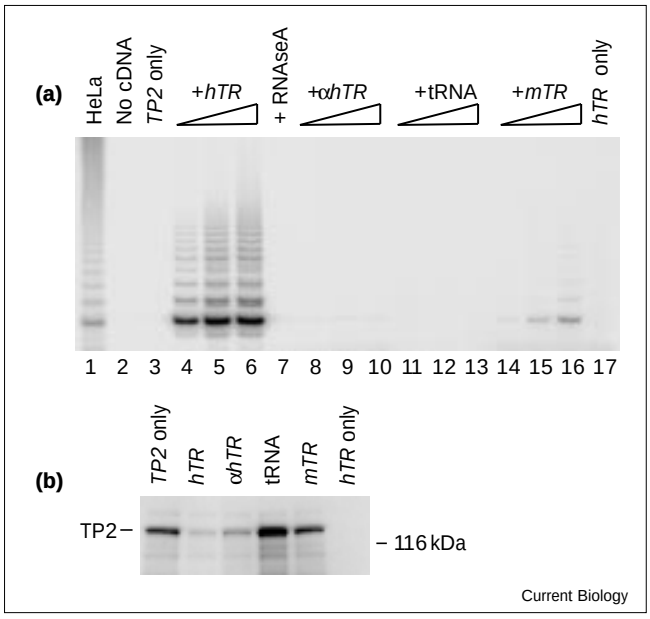
Correspondence: Lea Harrington
E-mail: leah@amgen.com

Received: 11 November 1997
Revised: 4 December 1997
Accepted: 5 December 1997

Published: 19 January 1998

Current Biology 1998, 8:177–180
http://biomednet.com/elecref/0960982200800177

Figure 1



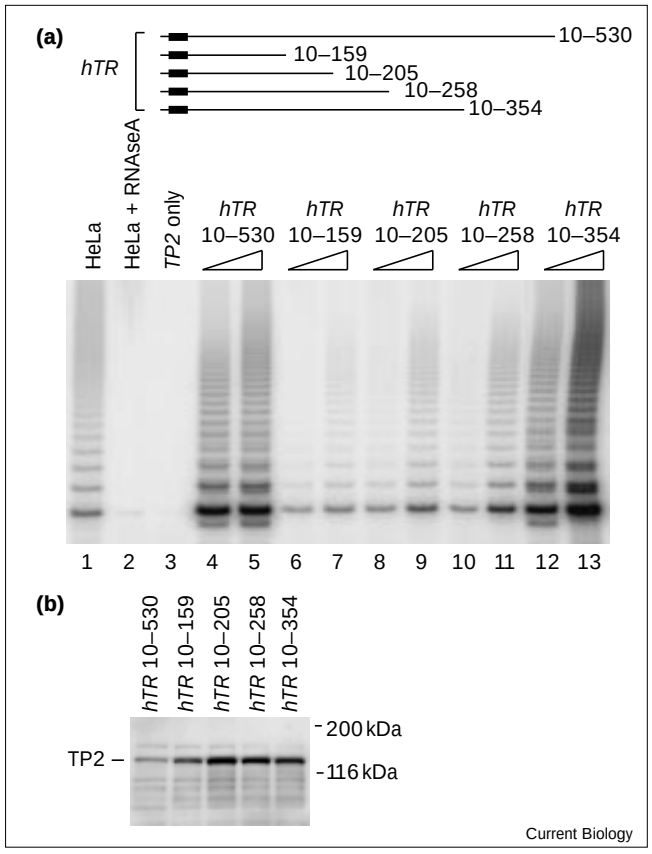
Human TP2 protein and telomerase RNA reconstitute telomerase activity in reticulocyte lysates. (**a**) Results of telomerase assays. Lane 1, 5 μg HeLa cell lysate; lane 2, no template cDNA added; lane 3, *TP2* cDNA only (0.1 μg translated in a 10 μl reticulocyte reaction); lanes 4–16, *TP2* cDNA in the presence of increasing concentrations of the indicated RNA species (0.001 μg, 0.01 μg, or 0.1 μg RNA per μl lysate): lanes 4–6, *hTR* RNA; lane 7, the same reaction in lane 6, preincubated with ribonuclease A (RNAse A) prior to the telomerase assay; lanes 8–10, antisense human telomerase RNA, *αhTR*; lanes 11–13, transfer RNA, tRNA; lanes 14–16, full-length sense mouse telomerase RNA, *mTR*; lane 17, 0.1 μg *hTR* RNA only. (**b**) Each of the *in vitro* translation mixtures (approximately 2 μl) in (a) containing 0.001 μg/μl RNA was resolved by SDS–PAGE and analyzed by western blotting with 0.2 μg/ml anti-TP2 antiserum [12]. The position of a 116 kDa protein marker is indicated at the right, and that of the FLAG–TP2 fusion protein is indicated at the left (TP2). The 520-nucleotide *hTR* (nucleotides 10–530) [13] was purified from a genomic *hTR* PCR product containing a T7 promoter, the antisense *hTR* using a T7 primer to the 3′ end of *hTR*, and the *mTR* from a previously described plasmid [5]. Full-length *TP2* cDNA containing sequences encoding the eight amino-acid FLAG epitope [12] was transcribed and translated using a Promega reticulocyte lysate (TNT) extract according to the manufacturer's instructions. RNA preparation and telomerase assays (TRAP-EZE, Oncor Inc. [24]) were carried out at the Ontario Cancer Institute.

Results and discussion

Reconstitution of telomerase activity using *hTR* RNA and recombinant TP2 protein

The reconstitution of ciliate and human telomerases has been characterized using recombinant telomerase RNA in the presence of partially purified, micrococcal-nuclease-treated lysates [1,14], but the minimal protein composition of active human telomerase remains unknown. Recently, we have functionally characterized TP2 as the catalytic subunit of human telomerase in human cells [12], and sought to determine whether recombinant TP2 could reconstitute human telomerase activity *in vitro*. We found that rabbit reticulocyte lysates did not contain endogenous telomerase activity (Figure 1a, lane 2), but telomerase activity could be detected when purified *hTR* RNA (nucleotides 10–530 of the *hTR* gene [13]) was

Figure 2



Deletion analysis of *hTR* in the reconstitution assay. (a) A schematic representation is shown of the *hTR* truncations that were tested for the ability to reconstitute telomerase activity in the presence of full-length TP2 protein (the boxed region indicates the telomere template). The numbers refer to the positions of nucleotides within *hTR* [13]. Lanes 1,2, 5 μg HeLa cell lysate in the absence and presence of RNAse A; lane 3, *TP2* cDNA only; lanes 4–13, *TP2* in the presence of the indicated truncated *hTR* RNA species (0.01 μg or 0.1 μg RNA per μl lysate). (b) Each of the *in vitro* translation mixtures (approximately 2 μl) in (a) containing 0.1 μg/μl of the indicated RNA was analyzed by western blotting using 0.2 μg/ml anti-TP2 antiserum. The position of the TP2 protein is indicated at the left, and the protein markers, in kDa, at the right. The truncated *hTR* RNAs were generated by digestion of a 520-nucleotide *hTR* PCR product containing a 5′ T7 promoter sequence with the following restriction enzymes: nucleotides 10–159, *Xba*I; nucleotides 10–205, *Sma*I, nucleotides 10–258, *Sac*II; nucleotides 10–354, *Stu*I.

added at the onset of transcription and translation of a full-length *TP2* cDNA [12] (Figure 1a, lanes 4–6). Telomerase activity was not detected in the presence of either *hTR* RNA or TP2 protein alone (Figure 1a, lanes 3,17). TP2 protein in the presence of antisense *hTR* (*αhTR*), transfer RNA (tRNA) or mouse telomerase RNA (*mTR*) [15] did not reconstitute telomerase activity (Figure 1a, lanes 8–16). These results suggest a species-specific interaction between TP2 and *hTR*. In the micrococcal-nuclease reconstitution of partially purified human telomerase described by Autexier *et al.* [14], activity was also reconstituted with *hTR* but not *mTR*. Western analysis of the lysates using an anti-TP2 antibody showed lower TP2 protein levels in the *hTR* sample compared with the samples containing inactive RNAs (Figure 1b), suggesting that the lack of telomerase activity in the latter samples was not due to lower TP2 protein levels. To rule out the possibility that high TP2 protein levels might inhibit telomerase activity, each purified RNA was mixed with a constant amount of TP2 that had been translated in a separate reaction, and the same results were obtained (data not shown). Therefore, TP2 can reconstitute telomerase activity in reticulocyte lysates and the reconstituted enzyme shows the same species-specific interaction with telomerase RNA as that seen with the native human enzyme [14].

Nucleotides 10–159 of *hTR* are sufficient for telomerase activity *in vitro*

In micrococcal-nuclease-treated human telomerase extracts, nucleotides 44–204 of *hTR* are sufficient to reconstitute telomerase activity, but nucleotides 44–170 are not [14]. We compared the activity of a number of *hTR* RNAs, truncated from the 3′ end and lacking the first nine nucleotides, in the reticulocyte telomerase reconstitution assay (Figure 2a). All truncated RNAs, the shortest of which consisted of nucleotides 10–159, had detectable telomerase activity, although the shorter *hTR* RNAs showed reduced activity compared with an *hTR* RNA consisting of nucleotides 10–354 (Figure 2a). The levels of TP2 protein in the presence of the truncated RNAs were higher than in the presence of a 520-nucleotide *hTR* (nucleotides 10–530; Figure 2b); higher TP2 levels did not inhibit the reconstitution, however, because the activity with the *hTR* RNA consisting of nucleotides 10–354 was similar to that with the 520-nucleotide *hTR*. Therefore, nucleotides 10–159 of *hTR* are sufficient for telomerase activity in this assay. Our ability to detect activity with a shorter RNA in the reticulocyte lysate assay than in the micrococcal-nuclease reconstitution assay may reflect an increased reconstitution efficiency or the increased sensitivity of the PCR-based telomerase assay [14]. Therefore, the reconstituted telomerase enzyme requires a similar region of the *hTR* RNA as does the native human telomerase.

hTR and TP2 are the only limiting and necessary components for activity *in vitro*

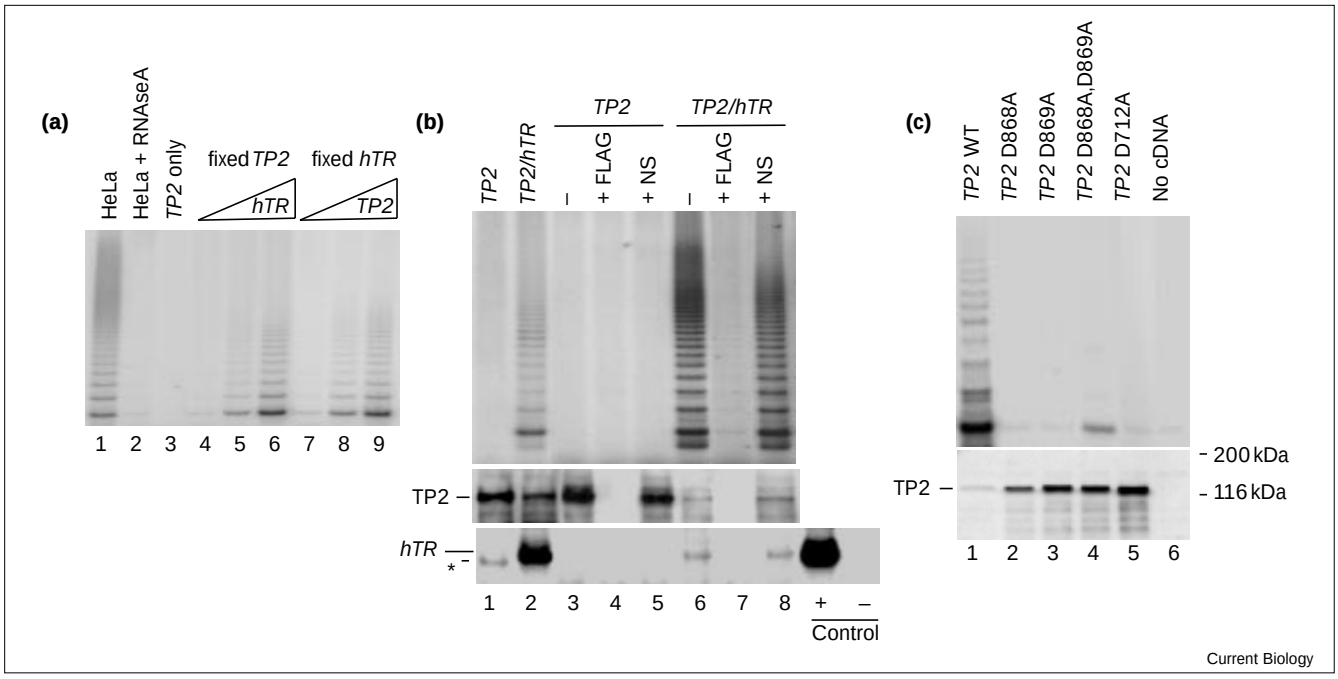
To examine whether *hTR* and TP2 were the only limiting components required for activity, we titrated the levels of *hTR* and TP2 in the reconstitution system (Figure 3a). Increasing the amount of telomerase RNA while maintaining a fixed concentration of *TP2* cDNA caused a parallel increase in telomerase activity (Figure 3a, lanes 4–6). Similarly, at fixed *hTR* concentrations, increasing the amount of *TP2* cDNA template in the reaction also resulted in an

increase in telomerase activity (Figure 3a, lanes 7–9). Therefore, both TP2 and *hTR* are required and are the limiting components for telomerase activity.

To confirm that the reconstituted activity contained recombinant TP2 and purified *hTR*, the recombinant TP2 protein, which contained a FLAG epitope tag, was immunoprecipitated from the lysate using an anti-FLAG antibody. Although FLAG–TP2 was efficiently immunoprecipitated from the lysates (again, the levels of TP2 protein were lower in the presence of *hTR*), only the immunoprecipitates containing FLAG–TP2 and *hTR* had telomerase activity (Figure 3b, lanes 6,8). Prior incubation of the antibody with a FLAG peptide, which had the effect of preventing FLAG–TP2 immunoprecipitation, did not result in immunoprecipitation of telomerase activity (Figure 3b, lane 7), whereas a non-specific peptide had no effect (Figure 3b, lane 8). Reverse transcription and PCR analysis of the immunoprecipitated material showed that *hTR* was associated with FLAG–TP2 and telomerase activity (Figure 3b, lanes 6,8). Thus, the telomerase activity observed resides in a complex that contains recombinant human TP2 and *hTR*. Although we cannot exclude the possibility that other rabbit proteins in the lysate contribute to the activity, we examined the lysates and immunoprecipitates using polyclonal antibodies against the telomerase-associated protein TP1/TLP1 (encoded by the *TEP1* gene [5,6]), and we did not detect a cross-reactive species.

To determine whether the reverse transcriptase domain of TP2 was essential for telomerase activity *in vitro*, we

Figure 3



TP2 and *hTR* are limiting and necessary for telomerase activity *in vitro*. (a) Results of telomerase assays. Lanes 1,2, 5 μg HeLa cell lysate in the absence and presence of RNAse A; lane 3, *TP2* cDNA only (0.1 μg cDNA in a 10 μl transcription–translation reaction); lanes 4–6, 0.02 μg full-length *TP2* cDNA (in 50 μl reticulocyte lysate) translated in the presence of 0.001 μg, 0.005 μg or 0.01 μg *hTR* RNA per μl lysate; lanes 7–9, increasing amounts of *TP2* cDNA (0.01 μg, 0.02 μg, or 0.05 μg in 50 μl reticulocyte lysate) translated in the presence of 0.002 μg/μl *hTR* in the lysate. (b) The reticulocyte lysate (approximately 10 μl) containing TP2 only (0.5 μg, lane 1) or TP2 and *hTR* (0.5 μg *TP2* cDNA and 0.5 μg *hTR* in a 50 μl transcription–translation reaction, lane 2) was immunoprecipitated using 10 μl agarose beads containing anti-FLAG antibody (Kodak), washed, and then analyzed for telomerase activity (upper panel), or subjected to western blot analysis using 0.2 μg/ml anti-TP2 antiserum (middle panel) or reverse transcription (RT)-PCR analysis using *hTR*-specific primers (lower panel). The immunoprecipitations were carried out with no competing peptide (lanes 3,6), a specific peptide (FLAG, lanes 4,7), or a non-specific peptide derived from TP1 [5] (NS, lanes 5,8). In the lower panel, as controls, the RT-PCR was carried out in the absence (–) or presence (+) of *hTR* RNA. The slightly smaller species detected by RT-PCR in lanes 1 and 2 (indicated by asterisk) is not specific for the *hTR* primers. (c) Effect of TP2 point mutations on telomerase activity. Lane 1, wild-type *TP2* cDNA (0.5 μg in a 50 μl reticulocyte lysate reaction containing 0.1 μg/μl *hTR* RNA); lanes 2–5, *TP2* cDNAs carrying the indicated point mutation (0.5 μg in a 50 μl reticulocyte lysate reaction containing 0.1 μg/μl *hTR*); lane 6, no cDNA template added. In the lower panel, each of the *in vitro* translation mixtures (approximately 2 μl) in (a) was analyzed by western blotting using 0.2 μg/ml anti-TP2 antiserum. The positions of protein markers are shown at the right and the position of full-length TP2 is indicated at the left. *TP2* cDNAs carrying point mutations were transcribed and translated as described for wild-type, FLAG-tagged TP2 in the legend to Figure 1.

introduced point mutations in sequences encoding conserved aspartate residues within the reverse transcriptase domain of TP2 [12]. Mutant TP2 proteins were produced which carried the single amino acid substitutions D868A, D869A and D712A, and the double substitution D868A,D869A (using the single-letter amino-acid code). We showed previously that each of these point mutations severely decrease telomerase activity associated with transfected, recombinant TP2 in human cell lysates [12]. Reticulocyte lysates supplied with wild-type TP2 and *hTR* contained telomerase activity (Figure 3c, lane 1), however the point mutated forms of TP2 could not reconstitute activity (Figure 3c, lanes 2–5). Although the levels of mutant TP2 proteins were higher than the level of wild-type TP2 (Figure 3c, lower panel), similar results were also obtained when the proteins were first translated and equal amounts mixed with purified *hTR*. The fact that telomerase activity is completely abolished only when mutant TP2 protein is present shows that a functional reverse transcriptase domain within TP2 is essential for catalytic activity. In yeast, the introduction of these analagous mutations in the reverse transcriptase domain of the TP2 homolog Est2 completely abolishes telomerase activity in crude cell lysates [8,16].

In yeast, the telomerase RNA *TLC1* and the Est1, Est2, Est3, and Est4/Cdc13 proteins are each essential for maintenance of telomere length *in vivo* [17–19], but only Est2 and *TLC1* are essential for telomerase activity in cell lysates [8,20,21]. Despite the known association of telomerase activity with other protein subunits *in vivo*, such as TP1/TLP1 [5,6], human telomerase might contain only two essential components — TP2 and *hTR*. Further purification of the reconstituted enzyme will be required to rule out the contribution of other reticulocyte proteins to telomerase activity. The reticulocyte-lysate telomerase-reconstitution system described here will be useful in further elucidating the role of TP2 and other associated proteins in telomerase function, such as elongation, processivity, telomerase RNA binding and telomeric primer recognition.

Recently, the reconstitution of human telomerase activity in reticulocyte lysates and in human cell lines with recombinant TRT1 was reported by Weinrich *et al.* [22] and Nakayama *et al.* [23]. The gene symbol TP2 is provisional and the allocation of an official gene symbol is currently under consideration by the Human Genome Organization nomenclature committee.

Acknowledgements

We thank V. Mar, T. McPhail and D. Yeung for reagents, and M. Tyers and N. Oleski for helpful comments. T.L.B. is a Research Fellow of the National Cancer Institute of Canada supported with funds provided by the Terry Fox Run. This work was supported in part by a grant from the Medical Research Council of Canada to L.H.

References

1. Greider CW: Telomere length regulation. *Annu Rev Biochem* 1996, 65:337-365.
2. Blasco MA, Lee H-W, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW: Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* 1997, 91:25-34.
3. Harrington LA, Hull C, Crittenden J, Greider CW: Gel shift and UV crosslinking analysis of ***Tetrahymena*** telomerase. *J Biol Chem* 1995, 270:8893-8901.
4. Collins K, Kobayashi R, Greider CW: Purification of ***Tetrahymena*** telomerase and cloning of genes encoding two protein components of the enzyme. *Cell* 1995, 81:677-686.
5. Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass MB, *et al.*: A mammalian telomerase-associated protein. *Science* 1997, 275:973-977.
6. Nakayama J, Saito M, Nakamura H, Matsuura A, Ishikawa F: TLP1: a gene encoding a protein component of mammalian telomerase is a novel member of WD repeats family. *Cell* 1997, 88:875-884.
7. Lingner J, Cech TR: Purification of telomerase from ***Euplotes aediculatus***: requirement of a primer 3′ overhang. *Proc Natl Acad Sci USA* 1996, 93:10712-10717.
8. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR: Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* 1997, 276:561-567.
9. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, *et al.*: Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997, 277:955-959.
10. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, *et al.*: hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* 1997, 90:785-795.
11. Kilian A, Bowtell DDL, Abud HE, Hime GR, Venter DJ, Keese PK, *et al.*: Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet* 1997, 6:2011-2019.
12. Harrington L, Zhou W, McPhail T, Oulton R, Yeung D, Mar V, *et al.*: Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev* 1997, 23:3109-3115.
13. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu C-P, *et al.*: The RNA component of human telomerase. *Science* 1995, 269:1236-1241.
14. Autexier A, Pruzan R, Funk WR, Greider CW: Reconstitution of human telomerase activity and identification of a minimal functional region of the human telomerase RNA. *EMBO J* 1996, 15:5928-5935.
15. Blasco MA, Funk W, Villeponteau B, Greider CW: Functional characterization and developmental regulation of mouse telomerase RNA. *Science* 1995, 269:1267-1270.
16. Counter CM, Meyerson M, Eaton EN, Weinberg RA: The catalytic subunit of yeast telomerase. *Proc Natl Acad Sci USA* 1997, 94:9202-9207.
17. Singer M, Gottschling DE: ***TLC1***: template RNA component of ***Saccharomyces cerevisiae*** telomerase. *Science* 1994, 266:404-408.
18. Lundblad V, Szostak JW: A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell* 1989, 57:633-643.
19. Lendvay TS, Morris DK, Sah J, Balasubramanian B, Lundblad V: Senescence mutants of ***Saccharomyces cerevisiae*** with a defect in telomere replication identify three additional EST genes. *Genetics* 1996, 144:1399-1412.
20. Cohn M, Blackburn EH: Telomerase in yeast. *Science* 1995, 269:396-400.
21. Lingner J, Cech TR, Hughes TR, Lundblad V: Three ever shorter telomere (***EST***) genes are dispensable for ***in vitro*** yeast telomerase activity. *Proc Natl Acad Sci USA* 1997, 94: 11190-11195.
22. Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, *et al.*: Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat Genet* 1997, 17:498-502.
23. Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura H, *et al.*: Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet* 18:65-68.
24. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, *et al.*: Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994, 266:2011-2015.