Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas

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Telomerase is a specialized type of reverse transcriptase which catalyzes the synthesis and extension of telomeric DNA (for review, see ref.1). This enzyme is highly active in most cancer cells, but is inactive in most somatic cells[2]. This striking observation led to the suggestion that telomerase might be important for the continued growth[3] or progression[4] of cancer cells. However, little is known about the molecular mechanism of telomerase activation in cancer cells. Human telomerase reverse transcriptase (hTRT) has recently been identified as a putative human telomerase catalytic subunit[5,6]. We transfected the gene encoding hTRT into telomerase-negative human normal fibroblast cells and demonstrated that expression of wild-type hTRT induces telomerase activity, whereas hTRT mutants containing mutations in regions conserved among other reverse transcriptases did not. Hepatocellular carcinoma (20 samples) and non-cancerous liver tissues (19 samples) were examined for telomerase activity and expression of hTRT, the human telomerase RNA component (hTR; encoded by *TERC*)[7] and the human telomerase-associated protein (hTLP1; encoded by *TEP1*)[8,9]. A significant correlation between hTRT expression and telomerase activity was observed. These results indicate that the hTRT protein is the catalytic subunit of human telomerase, and that it plays a key role in the activation of telomerase in cancer cells.

Telomerase is a ribonucleoprotein enzyme composed of a template RNA and several proteins. Recently, the genes encoding the catalytic subunits Ea_p123 and Est2p of the ciliate *Euplotes aediculatus* and the budding yeast *Saccharomyces cerevisiae* telomerases, respectively, have been cloned[10,11]. Interestingly, the amino acid sequences of Ea_p123 and Est2p contain motifs found in many reverse transcriptases (RTs). More recently, the human homologue, hTRT, has been described [5,6] and found to be expressed in cancer cells, but not in normal cells. Moreover, hTRT is activated when transformed human embryonic kidney cells and B lymphocytes overcome crisis and became telomerase-positive. These observations suggest hTRT may be the catalytic subunit of human telomerase, however, these studies did not address the functional and regulatory roles of hTRT, an issue addressed by the present study.

Six mutated cDNAs encoding hTRT proteins with single amino-acid substitutions were constructed by *in vitro* mutagenesis (Fig. 1). Mut 1, 4, 5 and 6 contain mutations at amino-acid residues that are invariably conserved in RTs, and Mut 2 and 3 have mutations in non-conserved regions. The wild-type and mutated *TRT* cDNAs were cloned into a CMV promoter/enhancer-based expression plasmid and transfected into human kidney 293 cells[12]. Polypeptides with an apparent molecular mass of 120 kD, as determined by SDS-PAGE, were specifically immunoprecipitated with the anti-hTRT polyclonal antibody (see Methods) from cells transfected with either the wild-type or one of the mutant *TRT* cDNAs, but not from mock-transfected cells (data not shown). The estimated molecular mass was close to that predicted from the amino-acid sequence (127 kD).

As shown in previous studies[5,6], we found that the three independently derived human mortal fibroblast cell lines TIG-3,

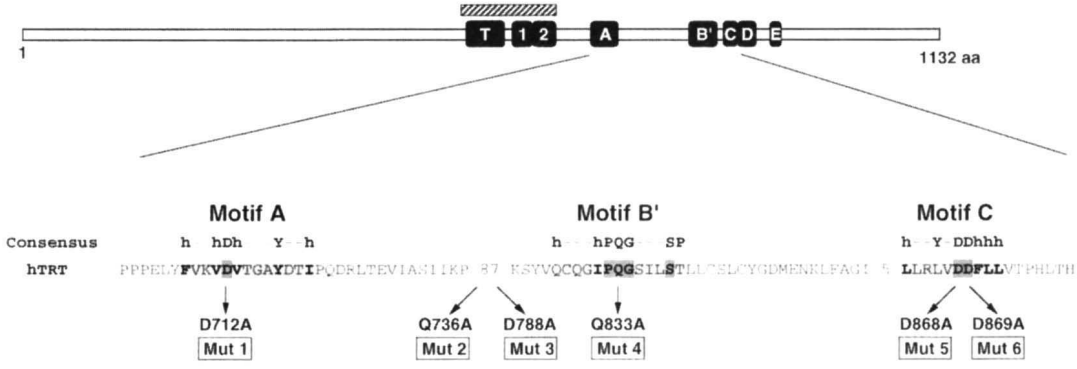

Fig. 1 Structure and RT motifs of the hTRT protein. Schematic diagram of the predicted hTRT amino acid sequence. The region of the recombinant protein used to raise the hTRT antibody is indicated by the hatched box. The motifs conserved within various reverse transcriptases are represented by boxes 1, 2, A, B′, C, D and E[20,21]. The motif found specifically within TRT proteins is represented by box T[6]. hTRT amino-acid sequences corresponding to these motifs are indicated below, together with the positions of the amino-acid substitutions present in hTRT mutants Mut 1–6. For example, D712A in Mut 1 designates the amino-acid change from D to A at amino acid 712.

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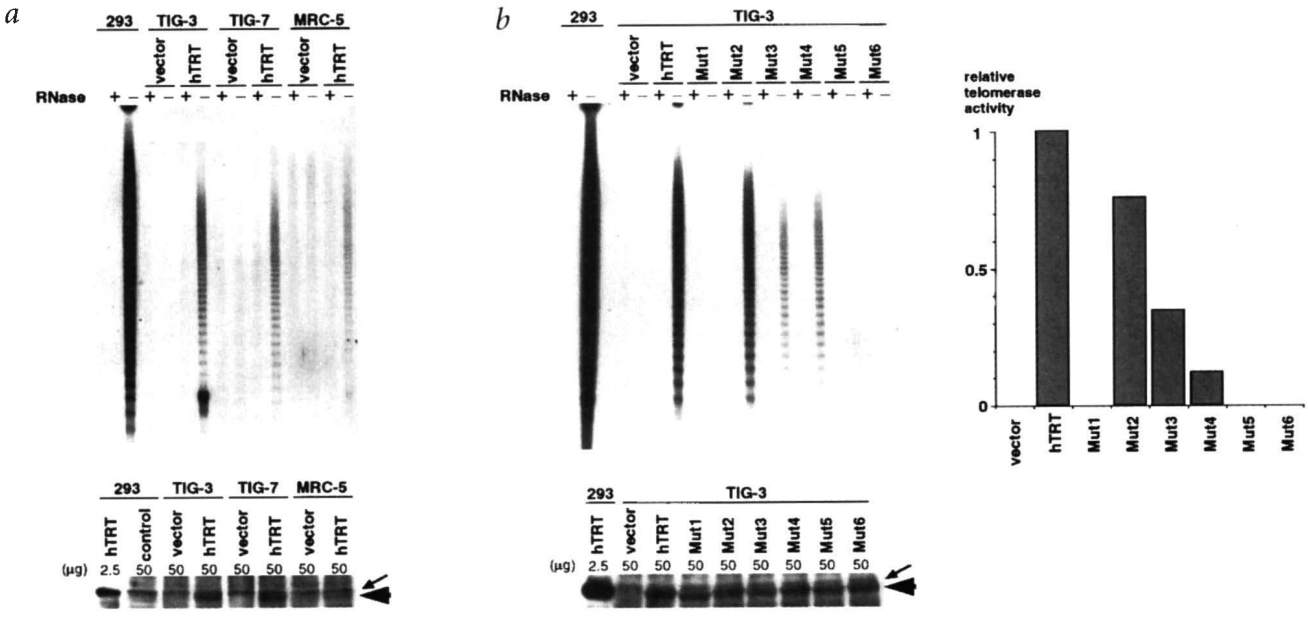

Fig. 2 Telomerase induction by hTRT in human normal fibroblasts. ***a***, Telomerase and hTRT protein induction in TIG-3, TIG-7 and MRC-5 cells by the *TRT* gene. TIG-3, TIG-7 and MRC-5 cells were transfected with the wild-type *TRT* cDNA or vector alone and examined for telomerase activity using the stretch-PCR assay (upper panel), with or without RNase pretreatment (+/-), and expression of the hTRT protein determined by western-blotting using the anti-hTRT antibody (lower panel). Telomerase activity in 293 cells and the hTRT protein in *TRT*-transfected 293 cells were assayed as controls. The amounts of extracts used for western-blotting are indicated. The arrowhead indicates the hTRT protein and the small arrow indicates an unrelated band cross-reacting with the antibody. ***b***, Levels of telomerase activity induced by expression of wild-type and mutant hTRT in TIG-3 cells. TIG-3 cells were transfected with wild-type *TRT* cDNA, with mutant cDNAs, Mut 1-6, or with the vector alone. Telomerase activity and the levels of hTRT protein (left panel) were determined. Telomerase activity was normalized to hTRT protein levels. The value obtained for wild-type *TRT* was defined as one unit and the relative values obtained for mutant hTRTs are plotted (right panel). No telomerase activity was detected in the mock-transfected cells. Similar results were obtained in three independent experiments.

TIG-7 and MRC-5 (refs 13,14) do not express hTRT, whereas they do express hTR[7] and hTLP1[8,9] (data not shown). We addressed the question of whether the absence of telomerase activity in these cells was due to the lack of hTRT expression. These three fibroblast cells were transfected with the plasmid encoding wild-type hTRT. S100 extracts were prepared after 48 h and telomerase activity was measured using the quantitative stretch PCR assay[15]. Significant levels of telomerase activity were observed in hTRT-transfected cells, but not in mock-transfected cells (Fig. 2*a*). As the plasmid was transiently transfected, the amount of expressed hTRT protein was affected by a number of factors, including transfection efficiencies (20–30%) and the rate of hTRT protein production. The levels of hTRT protein were analysed by western-blotting using the same S100 extracts. No hTRT was detected in the mock-transfected cells, but similar levels of hTRT were detected in all *TRT*-transfected cells (Fig. 2*a*). Therefore, we concluded that the hTRT protein is the only missing factor required for telomerase activity in these normal fibroblast cells, and that the expression of hTRT alone can induce telomerase activity.

To analyse the function of hTRT, we determined the effects of amino-acid substitutions on the induction of telomerase activity. Wild-type and mutant hTRT-encoding plasmids (Mut 1-6) were transfected into TIG-3 cells and telomerase activity and hTRT expression quantitated (Fig. 2*b*). All mutations reduced telomerase activity. Mut 2 induced a moderate level of telomerase activity, and Mut 3 and Mut 4 induced slight levels. Mut 2 and Mut 3 contain mutations in non-conserved regions. Mut 4 contains a mutation in a conserved region, but the corresponding yeast mutant does not show any phenotypic change[10]. In contrast, no telomerase activity was detected on transfection with Mut 1, 5 and 6, which harbour mutations in the region encoding the catalytic centre of RT[16], consistent with the observations for the corresponding yeast mutants. Given that hTRT and yeast Est2p share remarkably conserved sequences of their RT regions, together with our observation that an authentic RT motif is essential for telomerase activity induced by expression of hTRT in TIG-3 cells, we concluded that the hTRT protein is the catalytic subunit of human telomerase.

These studies suggested that hTRT expression might be the primary mechanism regulating telomerase activity in cells. To test this hypothesis, 20 cancerous and 19 non-cancerous liver tissues were examined for telomerase activity using the TRAP assay[2] (Fig. 3*a*). Telomerase activity was negligible in the non-cancerous tissues, but levels of telomerase activity ranging from undetectable to very high were found in the cancerous tissues, as reported previously[17,18]. Expression of *TRT*, *TERC* and *TEP1* was determined by RT-PCR (Fig. 3*a*). Telomerase activity was found to correlate with *TRT* expression, but not with *TERC* or *TEP1* expression. In a parallel assay using the gastric cancer cell line HSC39 as a positive control, semi-quantification was possible when telomerase activity was in a range greater than that contained in 100 HSC39 cells, and when hTRT expression was in a range greater than that found in 0.01 μg of HSC39 RNA (Fig. 3*a*, lane B). When TRAP and RT-PCR signals below this range were tentatively designated as 'negative', all 19 non-cancerous tissues except one case (sample 9), and six of the 20 cancerous tissues were negative for both telomerase activity and hTRT expression. It was found, therefore, that hTRT is not expressed in most non-cancerous and some cancerous liver tissues. Telomerase activities and the levels of hTRT expression in the remaining one non-cancerous and fourteen cancerous tissues were quantified and plotted (Fig. 3*b*). The relative titres of telomerase and hTRT expression showed good correlation in most cases where these values were relatively high. However, in some cases, telomerase activity and hTRT expression levels

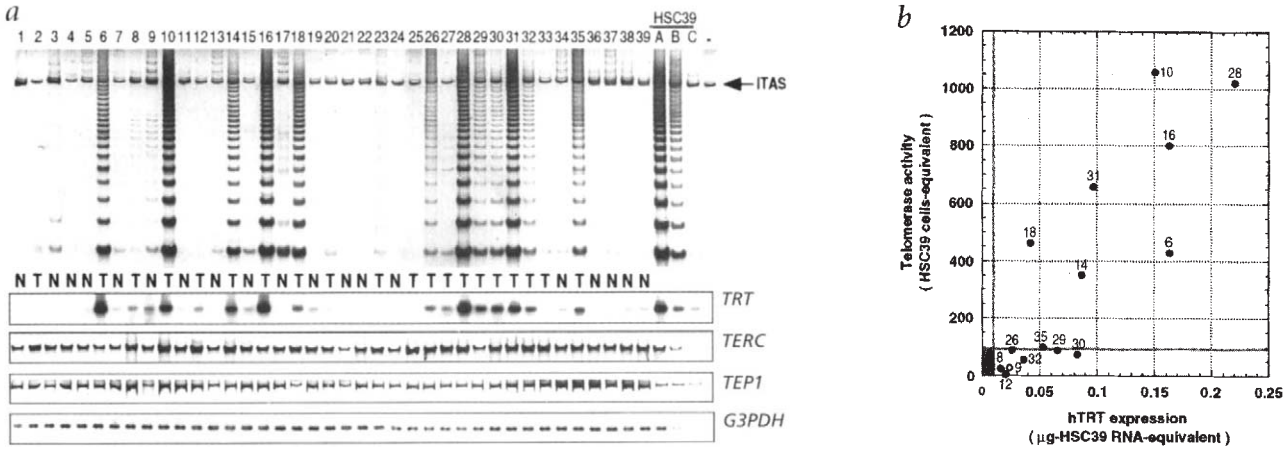

Fig. 3 Telomerase and hTRT expression in liver tissues. ***a***, Telomerase activity and hTRT, hTR and TLP1 expression in human liver tissues. Human hepatocellular carcinomas (T) and non-cancerous (N) tissues were assayed for telomerase activity by the TRAP method (upper panel) and for the expression of *TRT, TERC* and *TEP1* (encoding hTRT, hTR, hTLP1, respectively) and *G3PDH* (control) by RT-PCR (lower panel). The 19 non-cancerous hepatic tissues included samples from normal liver and from patients with chronic or acute hepatitis, liver cirrhosis and focal nodular hyperplasia nodules. The 20 cancerous tissues used were all hepatocellular carcinoma. The numbers at the top of the upper panel represent the sample number. The human gastric cancer cell line HSC39 was used as a positive control; A, 1000 cells for TRAP and 0.1 μg RNA for RT-PCR; B, 100 cells for TRAP and 0.05 μg RNA for RT-PCR; and C, 10 cells for and 0.001 μg RNA for RT-PCR. Buffer containing no sample extract was assayed as a negative control (–). ITAS : internal telomerase assay standard. ***b***, Relative telomerase activities and hTRT expression levels in positive cases. The TRAP signals and the hTRT RT-PCR signals were quantitated as described in Methods. These values were quantitatively correlated with the amounts of the input protein and with the RNA for HSC39 lanes A and B, but not C. Therefore, TRAP and hTRT RT-PCR signals lower than those of HSC39 (lane B) were designated as 'negative'. Telomerase activity is represented as the number of HSC39 cells containing an equivalent activity (ordinate), and hTRT expression level is represented as the amount of HSC39 RNA producing an equivalent hTRT RT-PCR signal (abscissa). Telomerase titers below the 100 HSC39 cells-equivalent and hTRT expression levels below the 0.01 μg HSC39 RNA-equivalent are considered to be 'negative'. The gray area indicates that both TRAP and hTRT RT-PCR signals were in these ranges. Eighteen of nineteen non-cancerous tissues and six of the twenty cancerous tissues are classified in this group (both TRAP and hTRT 'negative'). The remaining one non-cancerous (sample 9; indicated by an open circle) and fourteen cancerous tissues (samples 6, 8, 10, 12, 14, 16, 18, 26, 28, 29, 30, 31, 32 and 35; indicated by closed circles) are positive for either TRAP or hTRT. Telomerase titres of samples 8, 9, 12, 26, 29, 30 and 32 are below the quantitative range and thus should be considered as 'negative'.

showed no correlation. For example, samples 8, 9, 12, 26, 29, 30, 32 and 35 showed significant levels of hTRT expression, but negligible telomerase titres. On the other hand, sample 18 showed a relatively higher level of telomerase activity compared with the hTRT expression level. Factors other than hTRT expression might have a role in regulating telomerase activity in these cases. Future studies are necessary to clarify this possibility.

In conclusion, we have demonstrated that hTRT expression is the primary determinant regulating telomerase activity in human mortal fibroblasts and, possibly, in cancers. It will be of great interest to investigate the mechanisms leading to constitutive hTRT expression in cancer cells.

Note in proof: The gene symbol *TRT* is provisional and the allocation of an official gene symbol is currently under consideration by the HUGO nomenclature committee.

Methods

Cloning of cDNA encoding hTRT. We have independently isolated a number of *TRT* cDNA clones from a cDNA library constructed from the human cell line HeLa, using the human EST clone g1924194 (GenBank accession number: AA281296) as a probe. The composite nucleotide sequence and the predicted amino-acid sequence were essentially identical to those already reported[5,6]. As described in these reports, two types of cDNA, with or without a 182-bp insertion, were cloned with comparative frequencies. The longer cDNA containing the insert was predicted to produce a 1132-amino-acid-polypeptide starting from a putative initiation methionine, whereas the shorter form without the insert was predicted to produce a truncated 807-amino-acid-polypeptide due to a shift in the open reading frame. It is likely that the putative initiation codon is actually used as the translation start site, because there is an in-frame stop codon upstream to this codon in the mouse cDNA (Hatakeyama *et al.*, unpublished). At present, we have no evidence suggesting that the relative abundance of these two forms has any correlation with the relative titres of telomerase activity; the ratio of the amounts of these two forms did not differ significantly among samples in Fig. 3*b*. We therefore focused on the longer form of cDNA producing the longer polypeptide containing all RT motifs.

Anti-hTRT antibody. A PCR fragment encoding amino acids 545–662 of hTRT was subcloned into the expression vector pGEXH12 (a MCS-modified version derived from the pGEX series [Pharmacia]) and introduced into the *E. coli* strain XL1-Blue MRF'. The recombinant protein was purified with Mini-Prep Cell (Bio-Rad), concentrated using a Centricon 10 (Amicon), mixed at a 10:11 ratio with Freund's adjuvant, and injected into New Zealand white rabbits in 7 x 100 μg doses. Affinity-purified anti-hTRT polyclonal antibody was used in this study. The immune sera were precipitated in 3M ammonium sulfate and subsequently dissolved in TBS (20 mM Tris-HCl (pH7.4), 150 mM NaCl). It was then subjected to mock-affinity chromatography with crosslinking to extracts obtained from bacteria transformed with expression plasmid without insert. The flow-through fraction was collected, and subjected to positive-affinity chromatography with crosslinking to the purified recombinant hTRT protein. The bound antibody was eluted using elution buffer (0.1 M glycine-HCl; pH 2.5). After neutralization with 1 M Tris-base, the purified anti-hTRT antibody was used for immunoprecipitation and western-blotting analyses.

Transient expression of hTRT in human cells. The wild-type and mutant full-length cDNAs encoding hTRT were subcloned into the mammalian expression vector pcDNA3 (Invitrogen). The resultant plasmids were transfected into cells at 60–70% confluence in 6-well dishes by calcium phosphate precipitation or Lipofectamine (GIBCO BRL). The cells were incubated for 48 h before harvesting. The transfection efficiency was measured using the control plasmid pGreen Lantern-1 (GIBCO BRL). S100 cell extracts were prepared as described [15]. Telomerase activity was assayed by the stretch-PCR assay using protein extract (10 μg) as described[12]. The amplified products were analysed on a 7 M urea, 7% polyacrylamide sequencing gel and quantified using a phosphorimager (Fuji Bas 2000, Fuji). Protein extracts (50 μg) were fractionated in 6% SDS-PAGE gels and electroblotted onto membranes. Blots were incubated with the anti-hTRT antibody, followed by an

anti-rabbit IgG horseradish peroxidase antibody (Amersham). The ECL Plus kit (Amersham) was used to detect the antigen/antibody complex and chemiluminescent signals were detected using X-ray film. The film was scanned and analysed using the NIH Image software.

Telomerase activity and expression of hTRT, hTR and TLP1 in liver tissues. Hepatocellular carcinoma tissues and non-cancerous hepatic tissues, obtained by surgical operation or needle biopsy from tumor-bearing or non-bearing patients, were rapidly frozen and stored at -80 °C until use. of Frozen tissue (approximately 20 mg) was powdered in liquid nitrogen and divided into two samples, one for the telomerase assay and the other for RT-PCR, allowing the telomerase assay and RT-PCR to be carried out with identical samples. Telomerase activity was assayed by the TRAP method[2] with modifications[19] using extract of tissue protein (0.2 μg). The PCR products were analysed by electrophoresis on a 12% polyacrylamide gel, stained with SYBR Green I and visualized. The TRAP signals were shown to be sensitive to RNase pretreatment of the tissue extract. RNA was isolated using the RNeasy mini kit (Qiagen) and treated with DNase. RT-PCR was performed with total RNA (0.1 μg) using the GeneAmp EZ r*Tth* RNA PCR kit (Perkin-Elmer). The thermal cycles were: 94 °C for 30 s and 62 °C for 45 s (65°C for 60 s for hTR) for 25 cycles for *TERC*, *G3PDH*, and 30 cycles for *TRT* and *TEP1*. The amplified products were fractionated on a 6% non-denaturing polyacrylamide gel (hTR, TLP1) or on a 2% agarose gel (hTRT, G3PDH). Gels were stained with SYBR Green I and analysed using a fluorescence imaging analyser. To minimize experimental deviation, all samples were assayed simultaneously for a particular set of primers. The primer sets used were: for *TRT*, TRT/U1426 (5′–CCTCTGTGCTGGGCCTGGACGATA–3′) and TRT/L253 (5′–ACGGCTGGAGGTCTGTCAAGGTAG–3′); for *TEP1*, TLP1/U4792 (5′–CTTGGAATTGGGTCTGGTCTCTCG–3′) and TLP1/L5102 (5′–CACAGCAGTAGGGGATGAGGAAAC–3′); for hTR, hTR-F (5′–CCTAACTGAGAAGGGCGTAGGC–3′), *TERC* (5′–CTAGAATGAACGGTGGAAGGCG–3′); and for *G3PDH*, G3PDH-F (5′–ACCACAGTCCATGCCATCAC–3′) and G3PDH-R (5′–TCCACCACCCTGTTGCTGTA–3′). The TRAP signals and the *TRT* RT-PCR products were quantified using a fluorescence image analyser (FUJI FLA-2000), normalized to the ITAS signal and the *G3PDH* RT-PCR signal, respectively. The telomerase titres were inferred from the normalized RT-PCR signals using a dose response curve obtained with serially diluted HSC39 samples. Telomerase titre was represented as the number of HSC39 cells containing an equivalent activity, and *TRT* expression level was represented as HSC39 RNA producing an equivalent *TRT* RT-PCR signal.

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