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TLP1: A Gene Encoding a Protein Component of Mammalian Telomerase Is a Novel Member of WD Repeats Family

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Summary

We have cloned and characterized the rat *telomerase protein component 1* gene (*TLP1*), which is related to the gene for Tetrahymena p80. The cDNA encodes a 2629 amino acid sequence and produces the TLP1 proteins p240 and p230. The anti-TLP1 antibody specifically immunoprecipitated the telomerase activity. Moreover, p240 and p230 were copurified with telomerase activity in a series of extensive purification experiments. These results strongly suggest that the TLP1 proteins are components of, or are closely associated with, the rat telomerase. A pulse-chase experiment showed that p240 is modified to p230 in vivo. p230 was the dominant form in telomerase-positive cells, suggesting that modification of the TLP1 protein may regulate telomerase activity in vivo.

Introduction

In the early 1970s, it was suggested that linear genomic DNA loses terminal sequences each time it replicates, because DNA polymerases initiate de novo DNA synthesis in a unidirectional orientation from an RNA primer (Watson, 1972; Olovnikov, 1973; Levy et al., 1992; Lingner et al., 1995). The actual demonstration of this end replication problem in vertebrates became possible with the identification of the human telomeric DNA sequences, which were found to be TTAGGG repeats (Moyzis et al., 1988). Molecular studies using TTAGGG probes then indicated that normal human somatic cells typically lose terminal DNA sequences at a rate of 50–200 bp per population doubling (Harley et al., 1990; Hastie et al., 1990). In sharp contrast to somatic cells, germ cells do not show any telomere reduction and maintain a long tract of repetitive telomere DNA, in spite of their extensive proliferation (Hastie et al., 1990). Many types of cancer cells also do not show this loss of telomeric DNA. These cells continue to proliferate without apparent telomere shortening (Counter et al., 1992).

It was presumed that some mechanism overcomes the end replication problem in cells that maintain their telomeric lengths. Telomerase is now known to play a major role in telomeric maintenance. This enzyme specifically adds telomeric repetitive DNA to the chromosome 3′ ends (Greider and Blackburn, 1985; for review, see Blackburn, 1992). Telomerase was originally isolated from the macronucleus of the ciliate Tetrahymena. Elegant studies indicated that telomerase is a multiple-component enzyme, with both RNA and protein components essential for its activity (Greider and Blackburn, 1987). The 159 nt RNA component serves as a template for synthesizing the Tetrahymena telomeric sequence (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990). Thus, telomerase is a specialized type of reverse transcriptase, containing an RNA template as an intrinsic component of the enzyme. Recently, the genes encoding p80 and p95, two proteinaceous components that copurify with Tetrahymena telomerase, have been isolated (Collins et al., 1995). p95 has a limited homology with the consensus sequences of the catalytic regions of the RNA and DNA polymerases. p80 was shown to bind to the RNA component. More recently, two proteins of 120 and 43 kDa were identified as the telomerase components derived from another ciliate, Euplotes (Lingner and Cech, 1996).

Telomerase activity has been detected in a wide variety of eukaryotic species, including yeasts, ciliates, Xenopus, mouse, and human (Morin, 1989; Mantell and Greider, 1994; Cohn and Blackburn, 1995; Lin and Zakian, 1995; Lue and Wang, 1995; Prowse and Greider, 1995). In mammalian cells, however, there is much less telomerase activity than in the Tetrahymena macronuclei. Accordingly, only after the development of a sensitive PCR-based assay did studies on mammalian telomerase become practical (Kim et al., 1994). High levels of telomerase activity were found in cancer and germ cells, but only weak activity or no activity was generally observed in normal somatic cells (for review, see Harley and Villeponteau, 1995).

Telomerase RNA subunits have been identified and sequenced in several organisms. In spite of the wide prevalence of telomerases among the eukaryotic kingdoms, it was surprising that these RNA components have very different lengths and sequences (Lingner et al., 1994; Singer and Gottschling, 1994; Blasco et al., 1995; Feng et al., 1995; McEachern and Blackburn, 1995). Recent studies examining the expression of human RNA component (hTR) showed no correlation between the hTR expression level and telomerase activity (Avilion et al., 1996). Thus, telomerase activity must be regulated by other mechanisms, presumably by protein components.

We know very little about the biochemical and structural aspects of mammalian telomerases, because of the very low abundance of these enzymes. Attempts to isolate the mammalian genes encoding telomerase protein components by cross-hybridization using the Tetrahymena p80 or p95 sequences have been unsuccessful.

Here, we report the isolation of a rat cDNA that encodes an amino acid sequence related to Tetrahymena p80. We provide evidence that this cDNA encodes a

protein that is one component of, or is closely associated with, the rat telomerase, and thus we have named the gene *telomerase protein component 1* (*TLP1*). Surprisingly, the TLP1 proteins p240 and p230 are much larger than p80 and contain functional domains not found in p80. One domain consists of WD-40 repeats, a motif known to mediate protein–protein interactions.

Results

One major difficulty in the biochemical study of the mammalian telomerase was the lack of a sensitive and quantitative method for analyzing activity. We have developed a new PCR-based detection method called the "stretch PCR assay" for this purpose (Tatematsu et al., 1996). Using this method, we have succeeded in extensively purifying mouse, rat, and human telomerases (unpublished data; see Figure 4). We observed 2 or 3 major protein molecules of about 55–60 kDa and a few minor species of more than 200 kDa in these highly purified telomerase samples. However, we were unable to obtain amino acid sequence information from these purified materials, owing to the scarcity of the proteins.

cDNA Cloning of a Rat Gene Related to p80

As a result of a database search for DNA sequences homologous to the component genes for Tetrahymena telomerase p80 or p95, we identified a rat EST (expression sequence tag) clone showing low homology with p80. This cDNA clone, H33937 (GenBank ID), was originally cloned from a rat pheochromocytoma cell line PC12 cDNA library as a part of the EST database project. However, it initially seemed unlikely that this clone encoded the rat p80 homolog for two reasons. First, the open reading frame showing homology with the Tetrahymena p80 protein was supposedly the antisense sequence of the cDNA clone. Second, there was an in-frame stop codon in the DNA region homologous to the gene for p80. We constructed an oligonucleotide primer, PC5, from the reported DNA sequence of H33937 and performed a vectorette PCR experiment (Figure 1A). Vectorette PCR utilizes one specific primer and one adaptor primer ligated to restricted DNA ends (Riley et al., 1990). Since only one specific primer is needed for this type of PCR, it is possible to clone a DNA fragment including a contiguous region of unknown DNA sequence. Random-primed cDNA was synthesized from the mRNA derived from a rat adenovirus-transformed 3Y1 fibroblast cell line. Vectorette PCR using the PC5 primer and an adaptor primer produced a PCR fragment clone V5 from the cDNA. DNA sequencing of V5 identified a 1181 bp DNA sequence. A 168 bp sequence starting from the PC5 primer of V5 was found to be identical to H33937. However, the DNA sequence of the rest of V5 was completely different from H33937. A tctctcctAG sequence that was a good match with the consensus splicing acceptor site was found at the point where homology between H33937 and V5 diverged (Figure 1A). These results were most simply explained by assuming that V5 and H33937 were both derived from transcripts of a common rat gene, and that the reported DNA sequence of H33937 was antisense and included an intron.

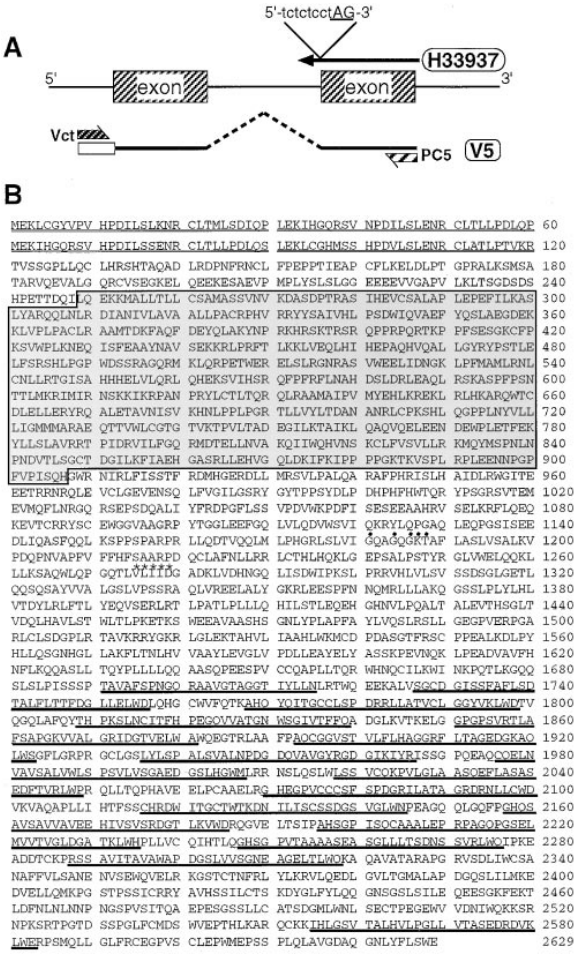

Figure 1. Cloning of the Rat cDNA Related to Tetrahymena p80

(A) The rat EST clone H33937 and the PCR clone V5 are schematically represented. The reported sequence of H33937 in the EST database is antisense to the open reading frame encoding the rat p80-homologous amino acid sequence and contains an intron sequence. The sequence of the splice acceptor site of the intron is shown at the top of the diagram, and the in-frame stop codon found in H33937 is underlined. A vectorette PCR experiment using PC5 and a vectorette primer (Vct) produced the fragment V5. V5 did not contain the intron or the stop codon and showed greater homology with the Tetrahymena p80 sequence than the original H33937 EST clone. At present, we do not know the detailed exon-intron structures of the gene, and the boxes shown as exons are drawn here for clarity. The relative lengths and positions of the clones and the exons are not to scale.

(B) Deduced amino acid sequence of the rat TLP1 protein. The 4 tandem repeats of the well-conserved 30 amino acid sequences are underlined at the N-terminus. The region homologous with the Tetrahymena p80 protein is indicated by a shaded box. The Walker's A- and B-box sequences, which have been found in many nucleotide binding proteins, are shown by dots and asterisks, respectively, indicating the conserved amino acids (Walker et al., 1982). The 16 WD-40 motifs at the C-terminus are underlined.

Importantly, the conceptually translated amino acid sequence from the V5 sequence showed greater homology with the Tetrahymena p80 protein (p, the probability that the homology is coincidental, $= 9.1 \times 10^{-7}$) than the original H33937 sequence ($p = 0.0017$).

We constructed a cDNA library from a rat SV40-transformed subline of 3Y1 fibroblasts (SV-3Y1-C66), in which

we had previously detected potent telomerase activity. V5 was used as a probe to screen 1×10^6 independent clones, and 3 positive clones were identified. Further screening of the library using DNA probes constructed from the ends of the longest cDNA clone identified a total of seven overlapping cDNA clones. The 5′ end of the cDNA clone was obtained by a 5′ RACE (rapid amplification of cDNA end) experiment. DNA sequencing was performed and a composite cDNA sequence of 8193 nt (from the 5′ end of the 5′ RACE clone to the 3′ end of the unique sequence, just upstream of the long poly[A] tract) was determined. Two observations suggested that the cDNA sequence was not chimeric. First, restriction mapping of the seven clones indicated that every region of the composite sequence was colinear in at least two different cDNA clones. Second, a 5′-terminal probe (744–1955 nt) and a 3′-terminal probe (6066–7424 nt) identified the same 9 kb mRNA in a Northern blotting experiment using total RNA from rat SV-3Y1-C66 cells (data not shown). As described below, this cDNA encodes one protein associated with the rat telomerase. Hereafter, we will refer to this gene as *rat telomerase protein component 1* (*rTLP1*).

Structural Analysis of rTLP1 Protein

Figure 1B shows the 2629 amino acid sequence conceptually translated from the determined *rTLP1* cDNA sequence. The size of the predicted rTLP1 protein was calculated to be 292 kDa. The most 5′-terminal ATG codon of the cDNA was tentatively assigned as the initiation codon. The DNA sequence around this codon (GCTATGG) matches well with the consensus DNA sequence for the eukaryotic initiation codon. The exact assignment of the initiation codon, however, awaits amino acid sequencing of the N-terminus of the protein product.

Several interesting features were observed in the deduced amino acid sequence of the rTLP1 protein (Figure 2A). First, amino acids 1–120 had 4 tandem repeats of a well-conserved 30 amino acid sequence (Figure 2B). No sequence with significant homology to this repeat motif was identified in the GenBank database. The amino acid sequence of the TLP1 protein can be grossly divided into three domains. The region comprising amino acids 200–908 shows significant homology with the total sequence of the Tetrahymena telomerase p80 component ($p = 3.3 \times 10^{-52}$; Figures 2A and 2C). A potential metal-binding motif, CxxC(27)CxxC, has been described in p80 (Collins et al., 1995), but no similar sequence was found in the rTLP1 sequence. Following the p80-homologous region, a canonical ATP/GTP binding sequence is present in the middle region of the protein (Walker et al., 1982) (Figure 2A). In the C-terminal third of the protein, a characteristic spacing of tryptophan (W) and aspartic acid (D) was noted (Figures 2A and 2D). These W-D sequences, separated by about 40 amino acids, are known as WD-40 repeats (Neer et al., 1994). WD-40 repeats were first described for the β subunit of the heterotrimeric G protein complex (Fong et al., 1986) and have a role in protein–protein interactions. A consensus sequence for WD-40 repeats has been deduced. An alignment of the rTLP1 amino acid sequence with the consensus sequence indicated the presence of at least 16 repeats in the C-terminus of the rTLP1 protein. The number of these repeats in rTLP1 is surprising, because in most WD repeats proteins, the number of repeats has been reported to be between 5 and 10 (Neer et al., 1994). The most C-terminal region of the protein is also rich in tryptophan. This region may contain remnants of additional WD-40 repeats or may represent a totally unrelated structure.

rTLP1 Protein Is Associated with the Rat Telomerase

To examine the role of the rTLP1 protein in telomerase activity, we first raised rabbit antisera against recombinant proteins representing the p80-homologous region. To confirm that the purified antibody recognizes the rTLP1 protein, an immunoprecipitation experiment was performed (Figure 3). A mammalian expression plasmid that produced the full-length rTLP1 protein fused with a FLAG tag (DYKDDDDK) at its C-terminus was transfected into COS7 cells. Cells transiently expressing the recombinant proteins were cultured in the presence of [^{35}S]methionine and cysteine. Total cell lysates were prepared and immunoprecipitated by the anti-FLAG tag antibody (Figure 3, lanes 1 and 2) or the affinity-purified anti-rTLP1 antibody (Figure 3, lanes 3 and 4). Both antibodies specifically recognized a protein with an apparent molecular mass of 240 kDa in the cDNA-transfected COS7 cells (Figure 3, lanes 2 and 4), but not in the mock-transfected cells (Figure 3, lanes 1 and 3). Any other protein recognized by the anti-FLAG or anti-rTLP1 antibody was present in both the mock- and cDNA-transfected cells and was not recognized simulataneously by both antibodies, indicating that the reactions were nonspecific. This experiment confirmed that the antibody we had raised actually recognized the rTLP1 protein. The molecular mass of the rTLP1 protein calculated from its migration in SDS-PAGE (240 kDa) was significantly less than the calculated value (292 kDa). At present, we do not know if this difference resulted from anomalous migration of the protein in the gel or from translational initiation at a site other than that which we had tentatively assigned.

To determine whether the rTLP1 protein is involved in telomerase activity, we examined fractions obtained during the purification of rat AH66F cell telomerase for the presence of the rTLP1 protein. We have extensively purified rat, mouse, and human telomerases using a combination of heparin-Sepharose, anion-exchange, hydroxyapatite, and metal-chelate chromatography, and glycerol-gradient centrifugation (unpublished data). The fractions obtained by these different separation techniques were examined for the presence of the rTLP1 protein by Western blotting and were assayed for telomerase activity by stretch PCR (Tatematsu et al., 1996). Any PCR-based detection method is weak in quantitative ability, because it is difficult to control the efficiency of exponential amplification reactions. The stretch PCR assay was developed to overcome this weakness as much as possible by careful control studies (Tatematsu et al., 1996). We found that the assay is highly specific and quantitative, and we were able to determine not only the presence or absence of telomerase activity but also the relative titers of the activity in the purified

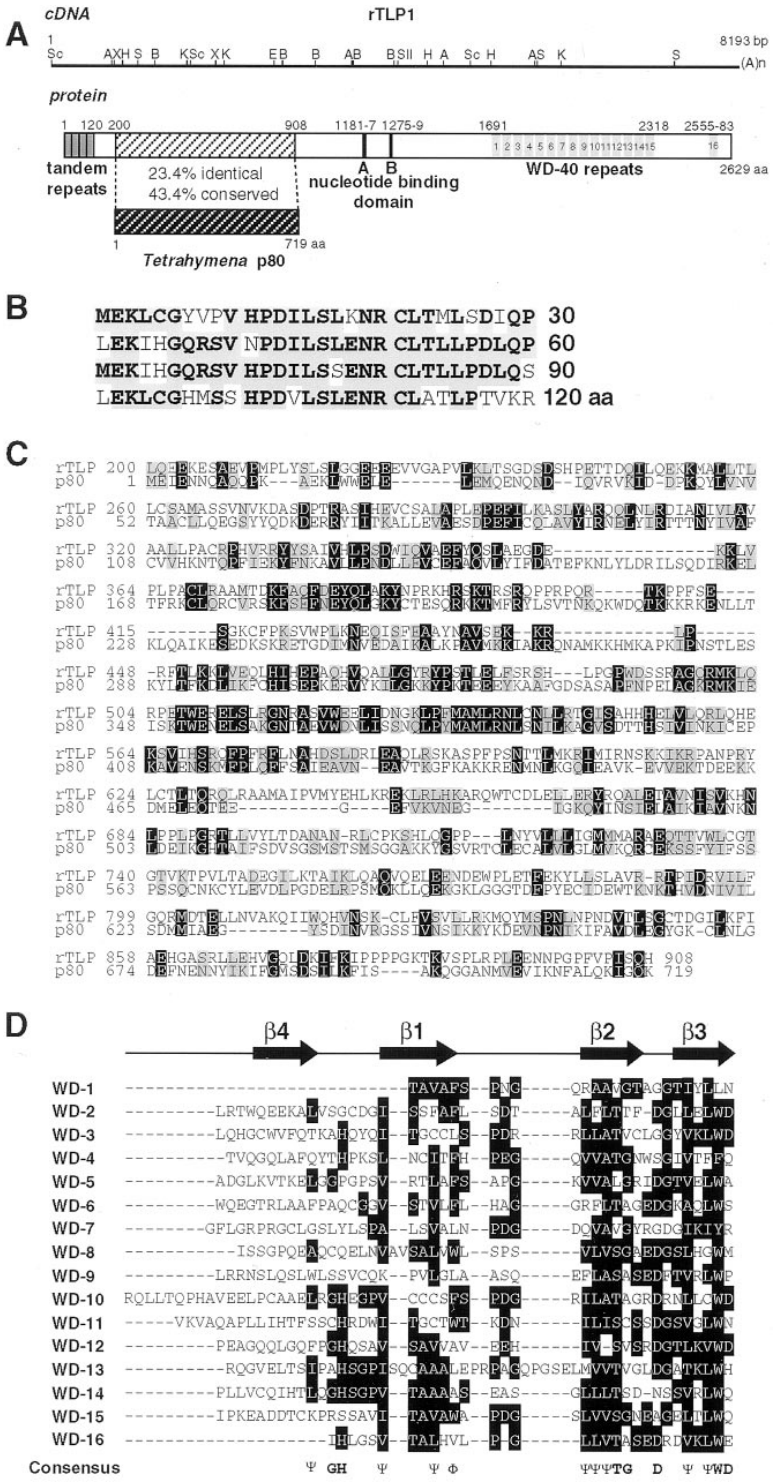

Figure 2. Domain Structures of the rTLP1 Protein

(A) The overall structure of the deduced rat TLP1 amino acid sequence. The amino acid sequence 200–908 shows homology with the Tetrahymena p80 protein. The alignment of rTLP1 and p80 is indicated in (C). This region corresponds to almost the total length of p80. The Walker's A- and B-box consensus sequences for nucleotide binding proteins lie at amino acids 1181–1187 and 1275–1279, respectively. The 16 repeats of the WD-40 sequence are depicted by boxes. Restriction enzymes used are as follows: A, ApaI; B, BamHI; E, EcoRI; H, HindIII; K, KpnI; Sc, SacI; SII, SacII; S, SmaI; and X, XhoI.

(B) Putative four 30-amino acid repeats at the N-terminus of the rTLP1 protein.

(C) The rTLP1 protein and the Tetrahymena p80 amino acid sequences were aligned by the ClustalW 1.6 program. Identical amino acids and conserved amino acids are shown by heavy and light shades, respectively. The potential metal-binding motif, CxxC(27)CxxC, reported to be present in p80 by a previous study (Collins et al., 1995), is found at the residues 537–570 of p80 amino acid sequence. No corresponding metal-binding motif was found in the TLP1 amino acid sequence.

(D) The 16 WD-40 repeats shown in Figure 2A are aligned along with the reported consensus sequence (Neer et al., 1994). (ψ) and (Φ) indicate any hydrophobic and aromatic amino acid, respectively, and (T) designates Thr or Ser. The positions of the putative four β-strand sheets, which were deduced from the WD-40 repeats of the β subunit of the heterotrimeric G protein complex whose crystal structure was recently resolved (Wall et al., 1995; Sondek et al., 1996), are indicated above.

fractions. In all cases, the rTLP1 protein was detected in the telomerase-active fractions at concentrations corresponding to the relative telomerase activity. Figure 4 shows the rTLP1 proteins and the telomerase activity of the fractions obtained in the final step of purification by glycerol-gradient centrifugation. Three kinds of rTLP1 proteins, having apparent molecular masses of 240, 230, and 55 kDa, were detected in the telomerase-active fractions (For a better separation between p240 and p230, see Figure 7). The 55 kDa protein was found to be a degradative product derived from the 240 and 230 kDa proteins (hereafter designated as p240 and p230, respectively) by the heat treatment of the samples in the presence of reducing agents for SDS–PAGE analyses. The relative concentrations of these proteins showed a good correlation with the telomerase titers obtained for each fraction. The protein that migrated slightly slower than the 120 kDa marker protein in fractions 1–5 was

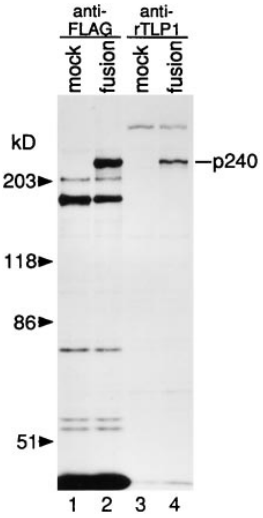

Figure 3. Immunoprecipitation Analysis of the Transiently Expressed rTLP1 Proteins

COS7 cells were transfected either by the expression plasmid vector that produced the full-length rTLP1 protein fused with the anti-FLAG at its C-terminus (lanes 2 and 4) or by the plasmid without the insert cDNA (lanes 1 and 3). These cells were metabolically labeled with [^{35}S]methionine and cysteine. Total cell lysates were prepared and immunoprecipitated either by the anti-FLAG antibody (lanes 1 and 2) or the affinity-purified anti-rTLP1 antibody (lanes 3 and 4). The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. The rTLP1 protein p240 was specifically detected in the cDNA-transfected cells by both the anti-FLAG antibody and anti-rTLP1 antibody (indicated as p240).

Figure 5. Immunoprecipitation of the Rat Telomerase Activity by the Anti-rTLP1 Antibody

An S100 extract was prepared from rat AH66F cells. It was immunoprecipitated either by the preimmune IgG (lanes 2 and 3) or the anti-rTLP1 antibody (lanes 4 and 5). The immune complexes were absorbed by Protein A–Sepharose. The supernatants (lanes 2 and 4) and the absorbed fractions (lanes 3 and 5), along with the original S100 extract, were assayed for telomerase activity. Owing to limited sample volumes, only one-fifth of each supernatant sample was used in the assays. Therefore, a direct quantitative comparison is not possible between lanes 1, 3, and 5 and lanes 2 and 4. In order to show the weak activity detected in lane 3, an autoradiograph after a long exposure is shown here.

thought to be unrelated to the TLP1 protein, because another anti-rTLP1 antibody did not recognize this protein. In summary, the rTLP1 proteins p240 and p230 were shown to be consistently copurified with telomerase activity in a series of extensive purification experiments. Three marker proteins, thyroglobulin (19.3 S, 669 kDa), catalase (11.3 S, 232 kDa), and aldolase (7.6 S, 158 kDa), were centrifuged in an identical glycerol gradient. The sedimentation coefficient of the rat telomerase was calculated to be 44 S from the migration rates of these marker proteins. Assuming that telomerase has a partial specific volume and a friction coefficient similar to these marker proteins, the molecular mass of the enzyme complex is estimated to be about 1500 kDa.

To obtain further evidence that the rTLP1 protein is a component of telomerase, we next examined the ability of the antibody to immunoprecipitate rat telomerase activity (Figure 5). An S100 extract derived from the rat

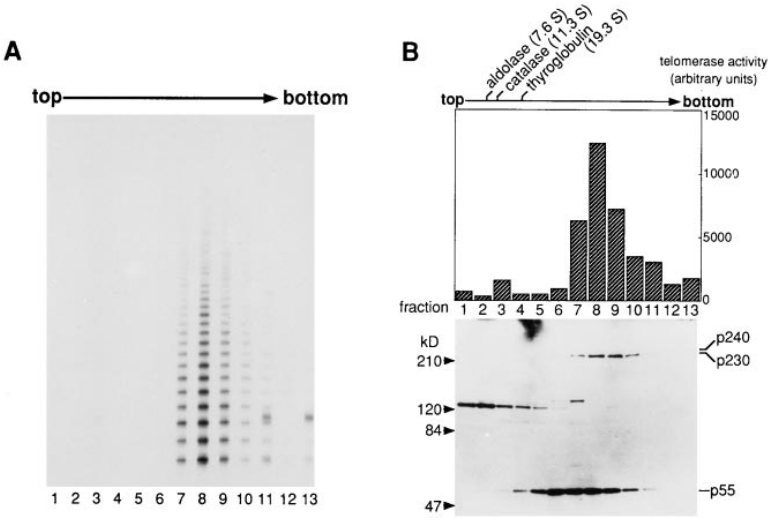

Figure 4. Detection of the rTLP1 Proteins p240 and p230 in the Rat Telomerase Samples Purified by Glycerol-Gradient Centrifugation

The rat telomerase purified by heparin-Sepharose, anion-exchange, hydroxyapatite, and metal-chelate chromatography was further purified by glycerol-gradient centrifugation. (A) The telomerase activity of each fraction was assayed using the stretch PCR assay. (B) The quantitated telomerase activity is depicted in the top panel, and the rTLP1 proteins p240 and p230 detected by Western blotting are shown in the bottom panel for each purified fraction. The sedimentation positions of the standard proteins are shown at the top. The positions of p240, p230, and p55 are indicated on the right.

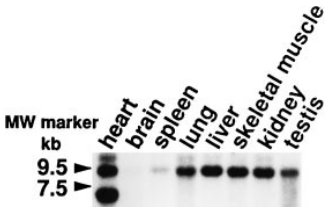

Figure 6. Northern Blotting Analysis of Mouse Tissues

A membrane blotted with 2 μg of each poly(A)$^+$ RNA derived from the mouse tissues indicated above (Clontech) was hybridized with the rat *TLP1* probe. Similar levels of the β-actin transcript were detected in all lanes when hybridized with the actin probe (data not shown), indicating that similar levels of mRNA from the different tissues were loaded. The positions of the 9.5 and 7.5 kb RNA molecular weight markers are indicated on the left.

hepatoma cell line AH66F showed strong telomerase activity (Figure 5, lane 1). When the extract was treated with the preimmune IgG, no significant telomerase activity was detected in the fraction immunoprecipitated by Protein A Sepharose (Figure 5, lane 3), and most activity remained in the unbound fraction (Figure 5, lane 2). In contrast, when the extract was immunoprecipitated by the anti-rTLP1 antibody, a significant level of telomerase activity was detected in the immunoprecipitated fraction (Figure 5, lane 5), although considerable activity still remained in the unbound fraction (Figure 5, lane 4). Quantitative analysis indicated that the antibody immunoprecipitated about 15%–20% of the total activity present in the S100 sample. A similar result was obtained with the highly purified rat telomerase samples shown in Figure 3B (data not shown). In these experiments, we were unable to immunoprecipitate all the activity; significant levels of activity always remained in the unbound fractions. Western blotting analysis, however, identified the rTLP1 proteins present in these unbound fractions. Therefore, the failure to absorb all the activity in these immunoprecipitation experiments did not necessarily indicate the presence of a subset of telomerase that did not contain rTLP1 proteins. Taken together, these results indicate that the rTLP1 products p240 and p230 are component of, or are closely associated with, the rat telomerase.

Ubiquitous Expression of *TLP1* Gene in Mouse Tissues

We examined the expression of the *TLP1* gene by a Northern blotting analysis in mouse (Mus musculus) tissues, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (Figure 6). In all tissues except the brain, a 9 kb mRNA was easily detected in 2 μg of poly(A)$^+$ RNA. A relatively weak level of expression in brain tissues was also found in human brain tissue when the human *TLP1* gene was used as a probe (unpublished data). Interestingly, another small 5 kb *TLP1* mRNA was found to be specific in heart tissue. We have not yet elucidated the structural difference between this small transcript and the widely observed 9 kb transcript. It has been reported that no telomerase activity is detected in the mouse kidney or brain (Prowse and Greider, 1995). The presence of the *TLP1* transcript in kidney observed in this study suggests that telomerase activity is not regulated by the expression of the *TLP1* gene.

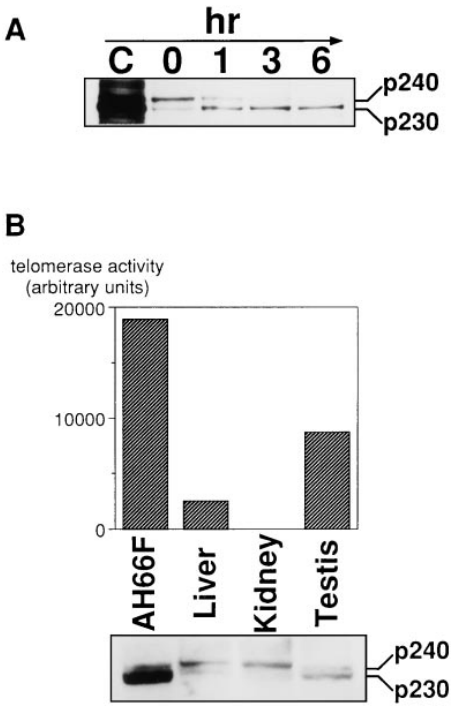

Figure 7. Posttranslational Modification of rTLP1 and the Telomerase Activity

(A) AH66F cells were labeled by [^{35}S]methionine and cysteine for 30 min (pulse) and chased for 0, 1, 3, or 6 hr in complete medium supplemented with 2.5 mM methionine. The labeled extracts were immunoprecipitated using the anti-rTLP1 antibody. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. An extract obtained from cells labeled for 6 hr without chase was also analyzed (lane C, control). The positions of p240 and p230 are indicated. p240 was modified to p230 within a 3 hr chase after pulse labeling.

(B) S100 extracts were prepared from AH66F cells and rat liver, kidney, and testis. Extracts derived from the same wet weights of cells were analyzed quantitatively for telomerase activity by the stretch PCR assay (upper panel) and for p240 and p230 by Western blotting (lower panel). The positions of p240 and p230 are indicated.

Modification of rTLP1 p240 to p230 May Regulate the Telomerase Activity

To analyze the relationship between p240 and p230, we performed a pulse–chase experiment. AH66F cells were pulse labeled with [^{35}S]methionine for 30 min, followed by the addition of excess cold methionine. Cells were further cultured for chase times of 0, 1, 3, and 6 hr, and the total cell lysates were then prepared for SDS–PAGE and autoradiography (Figure 7A). Immediately after the pulse labeling (0 hr chase), it was observed that the level of p240 was greater than p230. However, as the chase time was extended from 1 to 6 hr, the p230 fraction gradually increased and the p240 fraction decreased. At 3 and 6 hr chase times, the p230 fraction was greater than p240 and similar to that observed with stably labeled cells (Figure 7A, lane C, control), suggesting that the change from p240 to p230 is completed within 3 hr of translation. This experiment clearly indicated that

p230 is a mature form derived from p240. At present, we do not know whether posttranslational modification or cleavage of the protein is responsible for this change. COS7 cells transfected by the *rTLP1* cDNA and transiently over-expressing the rat TLP1 protein dominantly produced p240 and a relatively small amount of p230 (Figure 3). This result suggested that the COS7 cells were not competent to modify the large amount of p240 produced by the expression vector.

Since our results suggested that the telomerase activity is not regulated by *TLP1* expression, it was interesting to examine whether modification of the rTLP1 proteins is related to activity. Rat liver, kidney, and testis tissues were assayed for telomerase activity using the stretch PCR assay and examined for p240 and p230 abundance by Western blotting analysis (Figure 7B). To avoid possible contamination of the genomic DNA and telomerase inhibitors, the cell extract was partially purified on a heparin Sepharose column for the quantitative analysis of telomerase activity. In the liver and testis extracts, about 20% and 45% levels of activity were detected, respectively, as compared to that of the control AH66F cells extract . In contrast, only a negligible level of activity was found in the kidney extract. The TLP1 proteins were detected in all tissues. This result confirmed the hypothesis that the expression of the *TLP1* gene does not control telomerase activity. However, the relative abundance of p240 and p230 differed depending on the tissues tested. In the telomerase-negative kidney, p240 was the predominant form, with only a relatively small amount of p230 present. In contrast, p230 was more abundant in the telomerase-positive testis. The liver, which had a moderate level of telomerase activity, displayed an intermediate pattern, with similar levels of p240 and p230. Finally, a very high level of p230 was present in the AH66F cells. These results suggested that the modified p230 is associated with the active telomerase, whereas the p240 precursor is associated with an inactive form.

Discussion

We successfully identified a mammalian homolog by a BLAST search of the EST database using the Tetrahymena p80 sequence. This study illustrates the usefulness of the informatics of the genome project. Several lessons were learned here. First, the presumed orientation of the ORF reported with each EST clone is not necessarily correct. Second, the EST clone may have intron sequences, disrupting the continuity of the anticipated ORFs. Apparent "junk" candidates should not be discarded in a database search simply because of these reasons. Finally, a systematic strategy is needed to extract significant clones from among the numerous candidates. The method employed here, namely extending the sequence information of a region of interest by vectorette PCR to see if the homology would significantly increase over the extended region, was shown to be effective for this purpose. Coupled with this strategy, a database search may identify a sequence of very weak but significant homology (Figure 2C) that conventional experiments cannot detect. However, we would like to emphasize that the final verification of the identified clones requires biochemical evaluation. In our case, the newly developed stretch PCR assay was a key to this process.

TLP1 Protein as a Component of Telomerase

All the results obtained in this study strongly suggested that the TLP1 protein, a mammalian homolog of Tetrahymena p80, is a protein component of telomerase. First, the TLP1 proteins p240 and p230 were copurified with telomerase activity in different purification steps by different methods of separation. Second, immunoprecipitation experiments indicated that the TLP1 protein is closely associated with the active telomerase. Third, modification of the TLP1 protein appeared to correlate with the telomerase activity. Finally, we found that the TLP1 proteins were among the major proteins present in the highly purified telomerase preparation (unpublished data). However, the possibility that the TLP1 protein is closely associated with, but not essential for, telomerase activity is still present. The answer to this question will only be obtained by a reconsititution experiment using all the components, which is not possible at present.

It is not known how many protein components comprise the catalytically active mammalian telomerase complex. However, a glycerol-gradient centrifugation experiment suggested that the molecular mass of telomerase is more than 1000 kDa. Although the rat telomerase RNA component is not known, the mouse RNA (mTR) is about 450 nt, and the calculated molecular mass is about 150 kDa (Blasco et al., 1995). Thus, it is likely that another protein component(s), in addition to the TLP1 protein, is necessary for the active telomerase. One such protein may be a mammalian homolog of Tetrahymena p95.

Possible Functions of the TLP1 Protein

The finding that *TLP1* encodes a large 290 kDa protein containing a nucleotide binding domain and WD repeats was unexpected. It was reported that Tetrahymena telomerase p80 and p95 bind to each other by protein–protein interactions, even in the absence of the RNA component (Collins et al., 1995). The TLP1 protein contains a region of length equivalent to p80 in its amino-terminal one-third. This region may play all the roles performed by Tetrahymena p80, including the interaction with the putative p95 homolog. If this is the case, the nucleotide binding domain and WD repeats found in the TLP1 protein might perform serve functions specific to mammalian telomerases. Alternatively, there might be a Tetrahymena protein that has not yet been identified and that plays similar roles to those performed by these additional mammalian domains.

Two possibilities can be inferred about the functions of the nucleotide binding and WD repeats domains. First, it is possible that these domains are involved in the catalytic activity of the enzyme. These putative functions may include melting the substrate single-stranded telomeric DNA and regulation of the telomerase processivity. The second possibility is that these domains do not directly participate in telomerase activity. The telomere is a complex composed of the telomeric DNA and many

proteins, and has a number of divergent functions. In yeast and ciliates, nonnucleosomal domains were identified in the telomeres using structural, functional, and biochemical approaches (Gottschling and Cech, 1984; Wright et al., 1992). In human cells, micrococcal nuclease digestion experiments have indicated the presence of an unusual chromatin structure at the very ends of the telomere (Tommerup et al., 1994). Telomere binding proteins (TBPs) have been identified in several speicies (Fang and Cech, 1995). In mammals, a protein named TRF was shown to be specifically localized at the telomeres (Chong et al., 1995). These proteins are presumably members of the large telomeric domain called the telosome (Wright and Zakian, 1995). Several lines of evidence have suggested that TBPs play a role in regulating the telomerase activity in vivo (Runge and Zakian, 1989; Krauskopf and Blackburn, 1996; for review, see Greider, 1996). Therefore, it is possible that telomerase has either direct or indirect interactions with TBPs. The WD repeats in the TLP1 protein might play a role as an interface between the telomerase and other protein members of the telosome. In this respect, it is of interest that the TLP1 protein is constitutively expressed in most tissues. The inactive TLP1 p240 may function as a "platform protein" mediating the assembly of the various proteins, including telomerase and TBPs, at the telosome. When p240 is activated to p230, the composition of and interactions among the members of the telosome may change, leading to the activation of the telomerase. This type of regulatory role in a signaling pathway has been described for the β subunit of the heterotrimeric G proteins, which is the prototype of the WD repeats proteins (Neer, 1995).

Experimental Procedures

Cells

Adenovirus- and SV-40-transformed rat 3Y1 fibroblast cell lines (Ad-3Y1 and SV-3Y1) and African green monkey kidney COS7 cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum. The rat hepatoma cell line AH66F was grown in RPMI1640 medium supplemented with 10% fetal bovine serum.

Cloning of *rTLP1* cDNA

An oligonucleotide primer PC5 (5′-CATACCTGGTAGAACTCGG CTA-3′) was constructed according to the reported sequence of H33937. Vectorette PCR was performed as described (Riley et al., 1994) using adaptor primers VCT-A (5′-AAGGAGAGGACGCTGT CTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG-3′), VCT-B (5′-CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGT CCTCTCCTT), and VCT-G (5′-CGGTACCGAATCGTAACCGTTCG TACGAGAATCGCT). The template cDNA for the vectorette PCR was synthesized from 1 μg of Ad-3Y1 poly(A)$^+$ RNA. The first strand cDNA was synthesized using SuperScript reverse transcriptase (GIBCO-BRL) and random primer (hexadeoxyribonucleotide mixture; TaKaRa). After the synthesis of the second strand cDNA, an aliquot of double-stranded cDNA (5 μl of total 160 μl reaction volume) was digested with PvuII. These blunt-ended cDNA fragments were then ligated with 3 pmol of annealed bubble oligonucleotides, which consisted of the two oligonucleotides VCT-A and VCT-B. Using an aliquot of bubble-ligated cDNA (1 μl of total 50 μl ligation volume) as the template, vectorette PCR was carried out in a 50 μl reaction volume containing 0.4 μM of H33937-specific primer (PC5), 0.4 μM of VCT-G primer, 0.2 mM dNTPs, 0.5% polyoxyethylene ether W-1 (Sigma), 1× PCR buffer, and 2.5 U of Taq polymerase (Boehringer-Mannheim). The thermal cycle was 1 min at 93°C, 1 min at 65°C, and 2 min at 72°C for 35 cycles, followed by 10 min at 72°C.

A rat SV-3Y1 cell cDNA library was constructed using the ZAP-cDNA synthesis kit (Stratagene). The cDNA with an EcoRI adaptor at both ends was ligated to predigested λ ZAP II vector. The 1.2 kb PCR fragment clone, V5, was used as a probe for screening 1×10^6 independent clones of the library. Three positive clones (rλTLP1–3) were detected by autoradiography and isolated. Restriction analysis and DNA sequencing of the three positive clones revealed that they were all colinear and derived from the same transcript, and the 3′ ends of these plasmids did not contain poly(A)$^+$ tracts. Further screening of another group of 1×10^6 clones using the 3′-end fragment of rλTLP3, the longest cDNA clone, identified 10 positive clones. Restriction analysis and DNA sequencing of 6 of these clones revealed that 3 clones (rλTLP01, -09, and -13) had the same 3′-end sequence followed by different lengths of poly(A)$^+$ tract, indicating the 3′ end of the cDNA.

The 5′ end of the cDNA clone was obtained by a 5′ RACE experiment according to the the manufacturer's directions (Clontech). The four oligonucleotides used were NcEX3 (5′-GATCCATGGTCAG CTAATTATGGCCTTTGGAGCTTCATCCGTT- 3′), PC5 (see above), RACE-ANC (5′-CACGAATTCACTATCGATTCTGGAACCTTCAAGG-NH_2-3′), and RACE-PRM (5′-TAAGCTTCCTCTGAAGGTTCCAGAAT CGATAG-3′).

Antibody

A PCR fragment encoding amino acids 241–490 of the *rTLP1* was subcloned into pET-32a (Novagen), which is designed to produce a thioredoxin fusion protein that contains a 6-histidine tag. The plasmid was introduced into the E. coli strain BL21 (DE3). After disrupting the bacterial cells by sonication, the recombinant protein was isolated as inclusion bodies and treated with 8M-UTS buffer (20 mM Tris–HCl [pH 7.5], 0.5 M NaCl, 8 M urea). The solubilized recombinant protein was then loaded on an Ni^{2+}-bound HiTrap Chelating column (Pharmacia). The column was washed with 6M-UTP8 buffer (6 M urea, 0.1 M NaH_2PO_4, 10 mM Tris–base, pH adjusted to 8.0 with NaOH), and the protein was eluted by a pH gradient to 6M-UTP3 buffer (6 M urea, 0.1 M NaH_2PO_4, 10 mM Tris–base, pH adjusted to 3.0 with HCl). The eluted material was mixed in a 1:1 ratio with Freund's adjuvant and injected into New Zealand white rabbits in 3×100 μg doses.

The affinity column used for purification of the anti-TLP1 antibody from immune sera was prepared as described below. A restriction fragment (HindIII–SacI site) encoding the 217–481 amino acid sequence of rTLP1 was inserted into pGEX (Pharmacia) and introduced into the E. coli strain XL1-Blue MRF′. The bacterial extracts were prepared in lysis buffer (20 mM Tris-HCl [pH 7.5], 300 mM NaCl, 20 mM EDTA, 10% w/v sarcosyl) by disrupting cells by sonication and clarifying the supernatant by centrifugation at $12,000 \times g$ for 5 min. The supernatant was then loaded onto a Glutathione Sepharose 4B column and eluted with GST elution buffer (75 mM HEPES [pH 8.0], 75 mM NaCl, 5 mM DTT, 0.01% Triton X-100, 20 mM reduced glutathione). The eluted recombinant protein was concentrated and crosslinked to NHS-activated HiTrap, as recommended by the supplier (Pharmacia). The immune sera was loaded onto the affinity column, and 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-TLP1 antibody was used for Western blotting and immunoprecipitation. For control experiments, preimmune IgG was purified using a HiTrap protein G column according to the manufacturer's directions (Pharmacia).

Transient Expression of rTLP1 Protein in COS Cells

The full-length *rTLP1* cDNA was introduced into the mammalian expression vector pcDNA3-FLAG, which had a FLAG epitope sequence at the 3′ end of the MCS of original vector pcDNA3 (Invitrogen). The resultant plasmid, pcRTLP1, produced the full-length TLP1 protein with a FLAG tag (DYKDDDK) at the C-terminus. Transfection of COS7 cells was performed by calcium phosphate precipitation. Five hours after transfection, the medium was replaced with prewarmed culture medium, and the cells were incubated for another 48 hr before harvesting.

Immunoprecipitation

For metabolic labeling with [^{35}S]methionine, semiconfluent cells in 10 cm dishes were washed once with prewarmed labeling media (methionine-, cysteine-free Dulbecco's modified Eagle's medium [GIBCO-BRL] supplemented with 10% dialyzed fetal bovine serum [JRH Biosciences]). [^{35}S]methionine (Tran ^{35}S-label, ICN) was added to 1 ml of labeling medium (approximately 250 μCi/ml), and incubation was continued for 6 hr before lysis. The cells were washed with cold phosphate-buffered saline, scraped into 1 ml of RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatinA), and further incubated on ice for 30 min. The nuclei were removed by centrifugation at 19,000 $\times$ g for 5 min, and the lysate was precleared by incubation with 10 μg of preimmune IgG and 25 μl of Protein A–Sepharose (Pharmacia) for 1 hr, followed by a 1 min centrifugation at 5000 $\times$ g. Antibody was added to the clarified lysates, which were then incubated on ice for 4 hr. The 10 μl of Protein A-Sepharose was added to the lysate and incubated on a rotating shaker for 30 min at 4°C. The immunoprecipitates were washed three times with RIPA buffer, resuspended in SDS sample buffer, and separated on SDS-PAGE.

Western Blot

Whole-cell lysates of tissues or partially purified samples were resolved on 6% SDS-PAGE (14 cm $\times$ 14 cm). Gels were transferred to PVDF membrane (Immobilon, Millipore) at 0.5 A for 2 hr. After blotting, the membranes were blocked in Blotto (5% w/v nonfat dry milk, 0.02% sodium azide in PBS) for 2 hr at room temperature, incubated with anti-TLP1 antibody (2 μg/ml in PBS containing 3% w/v BSA) for 1 hr, and washed 3 times for 10 min with TBST (20 mM Tris–HCl [pH 7.5], 140 mM NaCl, 0.05% Tween 20). The secondary antibody (1/1000 dilution of horseradish peroxydase-linked donkey anti-rabbit immunogloblin, Amersham) was incubated with the membranes for 30 min, and specific proteins were detected using ECL (Amersham).

Telomerase Activity Measurement

Cytoplasmic S100 extracts of rat cell lines and tissues were prepared as described (Tatematsu et al., 1996). Telomerase activity was assayed by using the stretch PCR assay as described (Tatematsu et al., 1996). The amplified products were analyzed on a 7 M urea, 7% polyacrilamide sequencing gel, and the telomerase activity was measured using a phosphorimager (Fuji Bas 2000, Fuji).

Partial Purification of Rat Telomerase

An S100 extract derived from 3×10^9 cells of the rat hepatoma cell line AH66F was applied to heparin-Sepharose CL-6B (Pharmacia) equilibrated in TMG buffer (10 mM Tris-acetate [pH 8.0], 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol) containing 50 mM KCl, and telomerase was eluted by a gradient to 1 M KCl in TMG buffer. The fractions with peak activity were pooled and then loaded onto a hydroxyapatite chromatography column (Bio-Rad) equilibrated in TMG buffer containing 50 mM KCl. The column was washed in 5 mM KP buffer (5 mM potassium phosphate, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol), and the telomerase was eluted by a gradient to 0.5 M potassium phosphate in KP buffer. The active fractions were loaded onto an anion-exchange chromatography column (Resource Q, Pharmacia) equilibrated with 50 mM KCl in TMG buffer (without DTT), and the telomerase was eluted by a gradient to 1 M KCl in TMG buffer (without DTT). The fractions with peak activity were pooled and then loaded onto an immobilized metal ion (Zn^{2+}) affinity chromatography column (HiTrap Chelating, Pharmacia) equilibrated with 0.5 M KCl and 1 mM imidazole in TMG buffer (without DTT). The telomerase was eluted in a step increase to 50 mM imidazole. The eluent was then layered on 15%–40% glycerol gradient in 20 mM Tris–acetate (pH 8.0), 300 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT, and centrifuged in an SW28 rotor (Beckman) at 25,000 rpm for 24 hr at 2°C.

Northern Blot

Using a restriction fragment of rλTLP2 (XhoI, 744–1955 bp) as a probe, expression of *TLP1* mRNA in mouse tissues was examined. A multiple tissue Northern blot was purchased from Clontech.

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GenBank Accession Number

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