# Characterization of recombinant *Saccharomyces cerevisiae* telomerase core enzyme purified from yeast

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Telomerase is a cellular reverse transcriptase that elongates the single-stranded chromosome ends and oligonucleotides *in vivo* and *in vitro*. In *Saccharomyces cerevisiae*, Est2p (telomerase catalytic subunit) and Tlc1 (telomerase RNA template subunit) constitute the telomerase core complex. We co-overexpressed GST (glutathione S-transferase)–Est2p and Tlc1 in *S. cerevisiae*, and reconstituted the telomerase activity. The GST–Est2p–Tlc1 complex was partially purified by ammonium sulphate fractionation and affinity chromatography on glutathione beads, and the partially purified telomerase did not contain the other two subunits of the telomerase holoenzyme, Est1p and Est3p. The purified recombinant GST–Est2p–Tlc1 telomerase core complex could specifically add nucleotides on to the single-stranded $TG_{1–3}$ primer in a processive manner, but could not translocate to synthesize more than one telomeric repeat. The purified telomerase core complex exhibited different activities when primers were paired with the Tlc1 template at different positions. The procedure of reconstitution and purification of telomerase core enzyme that we have developed now allows for further mechanistic studies of the functions of other subunits of the telomerase holoenzyme as well as other telomerase regulation proteins.

Key words: core enzyme, processivity, reconstitution, telomerase, telomere.

## INTRODUCTION

Telomeres are specialized nucleoprotein structures at the ends of eukaryotic chromosomes that protect chromosomes from degradation and end-to-end fusion. Telomeric DNA typically consists of simple repeated sequences in which the strand running 5′ to 3′ from the centre towards the end of the chromosome has clusters of G residues (reviewed in [1–3]). For example, in *Saccharomyces cerevisiae*, each end of every chromosome bears $\sim 300 \pm 75$ bp of $C_{1–3}A/TG_{1–3}$ repeated DNA. However, the telomeric repeat sequences cannot be maintained by the conventional DNA replication machinery because the RNA primer used to initiate DNA synthesis at the very end of the lagging strand has to be removed to leave a gap at the 5′ end of the daughter DNA strand. The chromosomal shortening problem is solved in most organisms (including yeast and humans) by telomerase, a telomere-specific reverse transcriptase (reviewed in [1,2]).

Telomerase is a ribonucleoprotein enzyme that catalyses the addition of telomeric DNA sequence to chromosome ends by using a small segment of the integral RNA component as template. In the budding yeast *S. cerevisiae*, at least five genes [1], *EST2*, *TLC1*, *EST1*, *EST3* and *CDC13*, are required *in vivo* for telomere maintenance [4,5], suggesting that the telomerase holoenzyme consists of multiple subunits. *EST2* encodes the 102-kDa catalytic subunit of telomerase with distinctive motifs that are common to reverse transcriptase, as well as telomerase-specific motifs [6,7], and *TLC1* encodes the 1301-nt RNA subunit which contains a template region (5′-$C^{468}$ACCACACCCACACACA$^{484}$-3′) [8]. Est2p and Tlc1 comprise the catalytic core of the enzyme, because Est2p and Tlc1 are essential and sufficient for elongation activity of oligonucleotide primers *in vitro* [9,10]. Cdc13p binds single-stranded telomeric DNA and appears to be a component of the telomere itself [11–13]. Est1p has been shown to bind to Tlc1 [14] and single-stranded G-rich telomeric DNA *in vitro* [15]. In addition, Cdc13 interacts with Est1p [16,17], so as to recruit telomerase to the telomere [18], and may convert inactive telomerase into an active form [19]. Est3p is a telomerase subunit [20], and may interact with the N-terminal domain of Est2p [21].

Telomerase activity from a wide range of organisms has been described. Among those, the telomerases from *S. cerevisiae*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Candida albicans* add one telomeric repeat per binding event *in vitro* [22–25]. However, the telomerase of *S. cerevisiae* has been reported to have the ability to add more than one repeat per binding event *in vivo* [26–28], indicating that telomerase is processive in terms of repeat addition *in vivo*. It has been shown that yeast telomerase that contains different protein subunits has different processivity [29]. The mechanisms causing the differences observed *in vivo* and *in vitro* remain to be elucidated.

The *in vitro* reconstitution system has been used widely to study the telomerase (mostly human telomerase) [30–33], and most of the reconstitution studies have used partially purified telomerase complex. In several studies, an immunoprecipitation approach was taken to affinity-purify telomerase in order to obtain reasonable amount of activity [7,34,35]. For the studies of *S. cerevisiae* telomerase, partially purified enzyme from wild-type cells [23], or from the cells in which Est2p is Protein-A-tagged [36,37], was analysed. However, the composition of the activity from preparations is not clear, and it has been difficult to carry out mechanistic studies with these enzymes. Reconstitution of the telomerase RNP (ribonucleoprotein) with purified components will be crucial to study the function of every single subunit. In the present study, we have overexpressed both the GST (glutathione S-transferase)-fused catalytic subunit Est2p and the RNA subunit Tlc1, and partially purified the telomerase activity, which contains the GST–Est2p–Tlc1 complex (i.e. comprising two subunits: the GST–Est2p fusion protein and Tlc1 RNA), but not endogenous Est1p and Est3p. The purified recombinant telomerase core

Abbreviations used: DTT, dithiothreitol; GST, glutathione S-transferase; RNP, ribonucleoprotein.

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enzyme was characterized to have the ability to perform one round of processive telomeric DNA synthesis. We propose that the other subunits, i.e. Est1p and Est3p, may be required for multiple rounds of telomeric DNA addition.

## MATERIALS AND METHODS

### Yeast strain

Yeast strain BCY123 (*MATa*, *Can1*, *ade2*, *trp1*, *Ura3-52*, *his3*, *leu2-3*, *112*, *pep4::his*$^+$, *prb1::leu2*$^+$, *bar1::HisG*$^+$, *lys2::pGAL1/10-GAL4*$^+$) was used to co-overexpress GST–Est2p and Tlc1 as the host strain.

### Construction of plasmid

The *EST2* gene was amplified by PCR using genomic DNA from *S. cerevisiae* strain BCY123 and primers 5′-CGGGATCCATGAAAATCTTATTCGAGTTC-3′ and 5′-ACGCGTCGACTTATCAGCATCATAAGCTG-3′, digested with BamHI and SalI, and then cloned into pUC19. The entire insert was sequenced, and all of the mutations revealed by comparison with the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) were replaced. The correct *EST2* was cut out from pUC19-*EST2* with BamHI and SalI, and was inserted into the pEG(KT) vector [38].

The *TLC1* gene was amplified by PCR using the genomic DNA from BCY123 and primers 5′-GCTCTAGAGAGAATAAAACTAGAGAGGAAGATAGG-3′ and 5′-GGGGTACCCTCGAGAGAAGAAGCCATTTGGTGG-3′, digested with XbaI and XhoI, and then inserted into the pUC19 vector. The entire insert was sequenced and all of the mutations revealed by comparison with the *Saccharomyces* Genome Database were replaced. The pUC19-*TLC1* plasmid was cut with HindIII, blunted with T4 DNA polymerase, and then cut with another enzyme EcoRI. The *TLC1* gene was inserted into the p424GAL1 vector [39] digested with EcoRI and SmaI.

### GST–Est2p and Tlc1 co-overexpression and affinity purification [40]

Yeast host strain BCY123 was co-transformed with plasmids pEG(KT)-*EST2* and p424GAL1-*TLC1* using the lithium acetate method. A single colony was inoculated into synthetic medium containing 2 % raffinose and was grown until reaching a $D_{660}$ of approx. 0.7. Then, 2 % galactose was added to induce GST–Est2p and Tlc1 expression for 12 h. Cells were harvested, resuspended in lysis buffer [50 mM Tris/HCl, pH 7.8, 150 mM NaCl, 2 mM EDTA, 0.4 mM PMSF, 10 mM DTT (dithiothreitol) and 40 units/ml RNasin (TaKaRa)] and passed through a cell disruptor twice. The soluble fraction of the total proteins was recovered with centrifugation at 27000 ***g***, and ammonium sulphate was added to 40 % saturation. The pellet of precipitate obtained by centrifugation was dissolved in PBS (with 3 % glycerol, 1 % Triton X-100, 10 mM DTT, 0.2 mM PMSF and 40 units/ml RNasin). The sample was cleaned up by centrifugation, and the GST-fused protein was purified further with glutathione–Sepharose 4B beads. After washing five times with PBS (with 3 % glycerol and 1 % Triton X-100), the fusion protein was eluted with an elution buffer (50 mM Tris/HCl, pH 7.8, 1 mM EDTA, 5 % glycerol, 0.01 % Triton X-100, 160 mM NaCl, 0.0005 % octanol, 20 mM glutathione and 5 mM DTT).

### Telomerase activity assay

The telomerase activity assay was modified from a method described by Forstemann and Lingner [35]. Telomerase reactions were carried out in 10 $\mu$l reaction mixtures with final concentrations of 20 mM Tris/HCl, pH 8.0, 25 mM NaCl, 1 mM DTT, 1 mM spermidine, 1 mM $MgCl_2$, 1 unit of RNasin, 50 $\mu$M dATP, dCTP and dGTP, 5 $\mu$M dTTP, 10 $\mu$Ci of [$\alpha$-$^{32}$P]dTTP (3000 mCi/mmol; Amersham Biosciences), 1 $\mu$M substrate and up to 50 % (v/v) of telomerase core complex sample. The reaction mixture was incubated at 30 °C for 45 min and was stopped by the addition of 400 $\mu$l of proteinase K buffer (100 mM Tris/HCl, pH 7.4, 150 mM NaCl, 12.5 mM EDTA, 1 % SDS and 400 ng/$\mu$l proteinase K). After digestion at 30 °C for 30 min, the samples were extracted with phenol/chloroform and precipitated with ethanol in the presence of 0.3 M sodium acetate and 30 $\mu$g of glycogen (Roche Molecular Biochemicals) as carrier. After one wash with 70 % ethanol, the pellets were dried and subsequently dissolved in 5 $\mu$l of formamide loading buffer. The reaction products were analysed on 14 % acrylamide–urea sequencing gels. After drying, gels were exposed on a PhosphorImager screen (Molecular Dynamics).

For processivity analysis, the radioactive signal for each band detected by PhosphorImager was quantified with ImageQuant software (Molecular Dynamics), and was normalized by dividing by the numbers of incorporated [$\alpha$-$^{32}$P]dTTP, i.e. from position +1 to +7, the radioactive signal was normalized by dividing by 1, 1, 2, 2, 2, 3 and 3 respectively (see Figure 2B). The processivity was determined according to the formula:

$$P_i = \left(\sum_{j=i}^{n} T_j\right) \Big/ T_i$$

($i = 1, 2, \ldots, 7; n = 7$), where $P_i$ means that the sum of radioactive signal for each band above position $+i$ (including position $+i$) is divided by the radioactive signal of position $+i$, where $T_i$ represents the radioactive signal for the primer $+i$ position; the $P_i$ value reflects the relative ability of the enzyme to pass through the position $i$.

### Northern blot analysis

Yeast total RNA was isolated using the hot acidic phenol method [41], separated by electrophoresis on agarose formaldehyde gel, transferred on to Hybond N+ membrane (Amersham Biosciences), cross-linked by UV and hybridized with *TLC1* probe (PCR fragment using primers 5′-ACCATCACCACACCCACA-3′ and 5′-AGACATAAAGTGACAGCGC-3′) in ExpressHyb hybridization solution (Clontech). After being exposed on a PhosphorImager screen, the membrane was stripped and rehybridized with an *ACT1* probe (PCR fragment using primers 5′-AGACACCAAGGTATCATGGTCG-3′ and 5′-AAACCAGCGTAAATTGGAACG-3′).

### Telomere length blot

DNA was prepared using a glass beads method and digested with XhoI. The resulting DNA fragments were separated by electrophoresis in 1 % agarose gel and transferred to HyBond N+ membranes, cross-linked by UV, and probed with $C_{1-3}A/TG_{1-3}$ telomeric probe in ExpressHyb hybridization solution. The membrane was exposed on a PhosphorImager screen.

### *In vitro* transcription of Tlc1 RNA

T7 RNA polymerase promoter sequence was cloned into the pUC19 vector by annealing the two oligonucleotides 5′-CGGGGTACCGTAATACGACTCACTATAGGGATCCCG-3′ and 5′-CGGGATCCCTATAGTGAGTCGTATTACGGTACCCCG-3′ and digesting with KpnI and BamHI, resulting in the pUC19-T7 plasmid. The *TLC1* fragment was then subcloned into the pUC19-T7 plasmid using BamHI and XbaI. The DNA template for *in vitro* transcription was created by linearizing the pUC19-T7-*TLC1* plasmid using XbaI, and the ends were blunted using T4

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DNA polymerase. Tlc1 *in vitro* transcription was carried out in a 20 $\mu$l reaction volume containing 1 $\mu$g of DNA template, 2.5 mM each of rATP, rCTP, rTTP and rGTP (Promega), 0.05 unit of pyrophosphatase (Sigma), 40 units of recombinant RNasin, 8 mM GMP (Sigma) and 700 units of T7 RNA polymerase (kindly provided by Professor En-Duo Wang's laboratory in our institute) in transcription buffer (40 mM Tris/HCl, pH 8.0, 5 mM DTT, 30 mM $MgCl_2$, 1 mM spermidine and 0.1 % Triton X-100) for 4 h at 30 °C. RNase-free DNase (1 unit) (Promega) was used to remove the DNA template at 37 °C for 30 min, followed by heat-inactivation at 70 °C for 10 min. The transcripts were then purified with RNeasy Minielute Cleanup Kit (Qiagen).

### Preparation of polyclonal antibodies against Est1p, Est2p and Est3p

*EST1* fragment (coding for amino acids 507–699), *EST2* fragments (coding for amino acids 1–270 and 739–884 respectively) or *EST3* was cloned into the pGEX-4T-3 plasmid. GST-fused truncated Est1p, Est2p or full-length Est3p was overexpressed in bacteria, purified and injected into rabbits to make antisera. The antisera were affinity-purified as reported previously [42].

## RESULTS

### GST–Est2p and/or Tlc1 overexpression in yeast

In order to reconstitute telomerase, we first tested the overexpression of GST–Est2p or Tlc1 in bacteria. The purification of GST–Est2p was not successful because of serious degradation (results not shown), and the overexpression of Tlc1 in bacteria was not detectable by Northern blot (results not shown) for unknown reasons. After failure with the *Escherichia coli* overexpression system, we attempted to overexpress GST–Est2p, or Tlc1, and co-overexpress GST–Est2p and Tlc1 in yeast strain BCY123 that is protease-deficient [40]. The overexpressed GST-fused Est2p was recognized by the anti-GST or anti-Est2p antibody (Figure 1A, lanes 3 and 6, and results not shown). In contrast, the endogenous Est2p was not detectable under the experimental conditions (Figure 1A, lane 1). The expression of Tlc1 was analysed by Northern blot. Both endogenous Tlc1 and overexpressed Tlc1 were detected, and the overexpressed Tlc1 seems longer than the endogenous Tlc1 (Figure 1B), presumably due to excess transcription from the p424-GAL1 vector [39].

Deletion of any one of *EST1*, *EST2*, *TLC1* and *EST3* genes causes progressive telomere shortening and cellular senescence, and overexpression of Tlc1 gives rise to shorter telomeres, but not senescence phenotypes [8], suggesting that components of telomerase complex have to keep a certain stoichiometric relation to function properly. Therefore the telomere length of the cells that overexpress GST–Est2p and/or Tlc1 was analysed by Southern blot. As shown in Figure 2(A), the overexpression of GST–Est2p, like that of Tlc1, leads to the shorter telomeres (Figure 2A, lanes 2 and 3) [8], while co-overexpression of GST–Est2p and Tlc1 did not result in telomere length change (Figure 2A, lane 4). It is likely that the overexpression of GST–Est2p or Tlc1 titrates out either Tlc1 or Est2p to reduce the total telomerase activity in the cell, and therefore to result in shorter telomeres [8]. Co-overexpression of both GST–Est2p and Tlc1 might reconstitute active telomerase core enzyme, and therefore produced no change in telomere length.

### Co-overexpression of GST–Est2p and Tlc1 was able to reconstitute telomerase activity

The telomerase activity of yeast cells was undetectable when crude extract was employed. In previous studies, a purification step, for example, DEAE ion-exchange chromatography, immunoprecipitation or glycerol gradient centrifugation, was usually undertaken to concentrate and/or partially purify the telomerase protein–RNA complex [9,10,23,35]. In the present study, we used ammonium sulphate precipitation to concentrate the proteins, as well as nucleic acid, in the total extract of the cells that overexpressed GST–Est2p, or Tlc1, or GST–Est2p and Tlc1. The assumed telomerase complex in the precipitates was resuspended, and subjected to telomerase activity assay as described in the Materials and methods section. Typical results are shown in Figure 2(B). The concentrated sample from the cells that overexpressed either GST-fused telomerase catalytic subunit Est2p or RNA subunit Tlc1 did not show any activities (Figure 2B, lanes 2 and 3). In contrast, the concentrated sample from the cells that co-overexpressed GST–Est2p and Tlc1 could elongate the oligonucleotide primers (Figure 2B, lane 4), indicating that co-overexpression of GST–Est2p and Tlc1 reconstitutes telomerase activity in yeast cells.

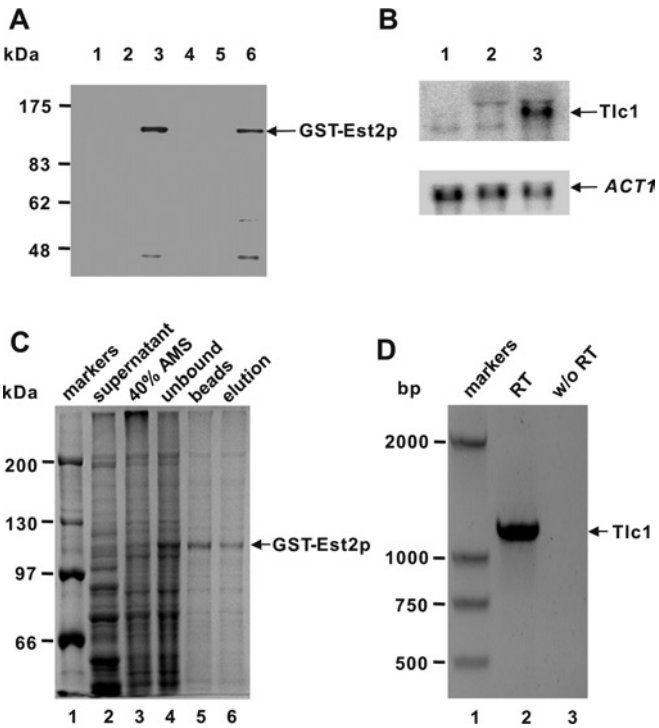


**Figure 1 Overexpression and purification of recombinant GST–Est2p and Tlc1 in the yeast expression system**

(**A**) Western blot analysis of GST–Est2p overexpression. Lane 1, BCY123 without transformed plasmids; lanes 2–6, BCY123 transformed with *EST2* (lanes 5 and 6), *TLC1* (lane 4) or co-transformed with *EST2* and *TLC1* (lanes 2 and 3) plasmids was grown with (lanes 3, 4 and 6) or without (lanes 2 and 5) galactose. Yeast total extract was resolved by SDS/7 % PAGE and detected by anti-GST antibody. Recombinant GST–Est2p (128 kDa) is indicated by the arrow. (**B**) Northern blot analysis of Tlc1 overexpression. Lane1, BCY123; lanes 2 and 3, BCY123 co-transformed with *EST2* and *TLC1* plasmids was grown with (lane 3), or without (lane 2) galactose. Total RNA was hybridized with *TLC1* probe, then stripped and rehybridized with an *ACT1* probe as loading control. Tlc1 is indicated by the arrow. (**C**) SDS/PAGE analysis of samples of the purification steps. Protein samples were resolved by SDS/7 % PAGE and detected by Coomassie Blue staining. Lane 1, molecular-mass markers; lane 2, soluble fraction of total extract; lane 3, 40 % ammonium sulphate (AMS) precipitate; lane 4, unbound fraction from glutathione–Sepharose 4B beads; lane 5, fraction of glutathione–Sepharose 4B beads before elution; lane 6, elution sample. (**D**) Tlc1 was co-purified with GST–Est2p analysed by reverse transcription–PCR. Lane 1, molecular-mass markers; lane 2, purified complex was reverse transcribed, and the resulting cDNA was PCR-amplified using *TLC1* primers 5′-CTAGAGAGGAAGATAGGTACCC-3′ and 5′-GCGATATACAAGTACAGTACGC-3′; lane 3, purified complex was directly PCR-amplified without reverse transcription. Sizes are indicated in kDa or bp as appropriate.

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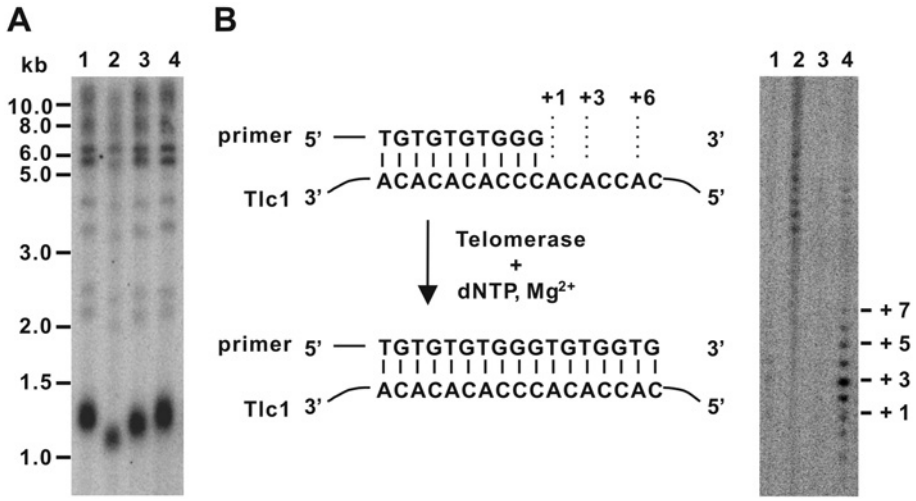


**Figure 2 Co-overexpression of GST–Est2p and Tlc1 was able to reconstitute telomerase activity**

Lanes 1, BCY123; lanes 2–4, BCY123 that overexpresses Tlc1 (lanes 2), GST–Est2p (lanes 3), or GST–Est2p and Tlc1 (lanes 4). (**A**) Telomere Southern blot analysis of yeast overexpressing GST–Est2p and/or Tlc1. Sizes are indicated in kb. (**B**) Telomerase activity assay of yeast extract precipitated with 40 % ammonium sulphate (see the Materials and methods section). The oligonucleotide DNA 5′-GTGTGTGTGGG-3′ was used as primer. Positions +1, +3, +5 and +7 are indicated.

### Purification of the GST–Est2p–Tlc1 complex

In several studies, an immunoprecipitation approach was taken to affinity-purify telomerase in order to obtain a reasonable amount of activity [7,34,35]; however, the composition of the preparations for the activity is not clear. To facilitate the mechanistic studies of yeast telomerase, we attempted to purify the overexpressed GST–Est2p–Tlc1 complex. The recombinant GST–Est2p–Tlc1 complex was partially purified by ammonium sulphate fractionation, and affinity chromatography on GST beads as described in the Materials and methods section. Western and Northern blot (or reverse transcription–PCR) were used to trace recombinant protein (GST–Est2p) and RNA (Tlc1) in both early and late stages of purification. The SDS/PAGE stained with Coomassie Blue indicated that the recombinant GST–Est2p was successfully retained on the GST beads (Figure 1C, lane 5), and the elution of GST–Est2p was also very efficient (Figure 1C, lane 6). Tlc1 was co-purified with GST–Est2p because the 1.3 kb DNA fragment was amplified with the primers specific for *TLC1* after the reverse transcription of the Tlc1 RNA in the eluate (Figure 1D, lane 2), while no fragment was detected in the PCR product when purified GST–Est2p–Tlc1 complex was amplified before reverse transcription (Figure 1D, lane 3), and neither were such bands observed when a purified GST–Est2p sample was amplified either before or after reverse transcription (results not shown). These results demonstrate that co-overexpressed GST–Est2p and Tlc1 allow them to form a complex, and the Tlc1 subunit has been co-purified with GST–Est2p.

From the Coomassie-Blue-stained gel (Figure 1C), it appeared that the recombinant GST–Est2p was the dominant band. However, there are many faint bands co-pulled-down by the GST-affinity column. Because Est1p and Est3p are considered to be the subunits of yeast telomerase, they might be co-pulled-down with the GST–Est2p–Tlc1 complex. To examine this possibility, we first performed a Western blot with anti-Est1p or anti-Est3p antibody which was affinity-purified with the antigen column (see the Materials and methods section), and neither Est1p nor Est3p was detected in the eluate of the GST-affinity column (results not shown). A second approach we used to analyse the co-purified proteins of Est2p–Tlc1 complex was MS. The peptides that were identified by MS matched Est2p, as well as structural components of ribosome, heat shock protein, mRNA-binding protein and translation factor, but not Est1p or Est3p (results not shown). These data indicated that the partially purified recombinant telomerase contains the Est2p–Tlc1 core enzyme, but not Est1p or Est3p.

### The recombinant GST–Est2p–Tlc1 complex can synthesize one round of telomeric repeat

To characterize the purified recombinant GST–Est2p–Tlc1 core enzyme, we first analysed its nucleotide addition activity [36], and compared it with that of total extract. The results are shown in Figure 3. The nucleotide addition activity of telomerase in the total extract was not processive, because fewer and fewer products were synthesized at positions +4, +5, +6 and +7 (Figure 2B, lane 4, and Figure 3A, lane 1). In contrast, the purified GST–Est2p–Tlc1 core complex was more processive, and it was able to add more nucleotides to the primers (Figure 3A, compare the signals at positions +5, +6 and +7 in lanes 1 and 2). To analyse how processive the enzyme was at certain points of the template, we calculated the processivity of the recombinant telomerase core enzyme with a formula described in the Materials and methods section. Figures 3(B) and 3(C) show the quantification of the processivity of the telomerase in total extract (Figure 3A, lane 1) and purified enzyme (Figure 3A, lane 2) respectively. The processivity of the enzyme at position +7 was defined as 1. The $P_i$ value reflects the relative ability of the enzyme to pass through the position $i$, and therefore the higher the $P_i$, the more processive the enzyme is. The telomerase in total extract was more processive at the +1 position than at other positions (Figure 3B). On the contrary, the purified telomerase exhibited higher processivity at +3 and +4 positions than at other positions, and lower processivity was shown at +5, +6 and +7 positions (Figure 3B). Although we normalized the activity assays by adding the enzyme with an equal amount of GST–Est2p, it was hard to quantitatively compare the processivity of the purified recombinant GST–Est2p–Tlc1 core enzyme with that of the enzyme in total extract. These

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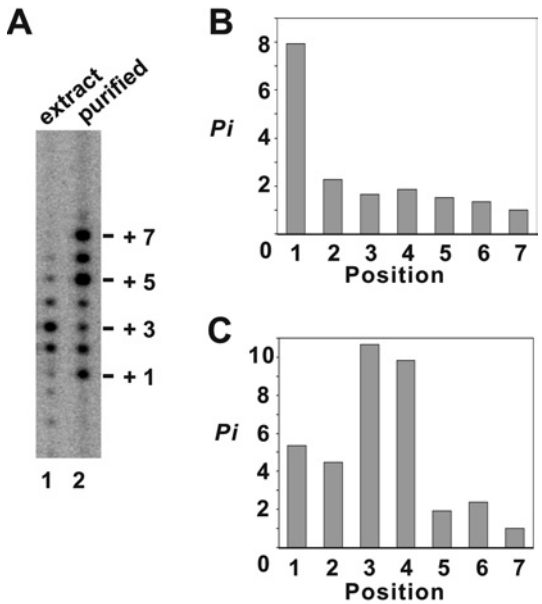


**Figure 3 Purified recombinant GST–Est2p–Tlc1 complex was more processive in terms of nucleotide addition than that of total extract**

(**A**) Telomerase activity assay for un-purified and purified recombinant GST–Est2p–Tlc1 core enzyme. Lane 1, total extract (40 % ammonium sulphate precipitate); lanes 2, purified telomerase core complex. Positions +1, +3, +5 and +7 are indicated. (**B**, **C**) Quantification of the processivity of nucleotide addition at different positions in lane 1 or 2 in (**A**) respectively (see the Materials and methods section).

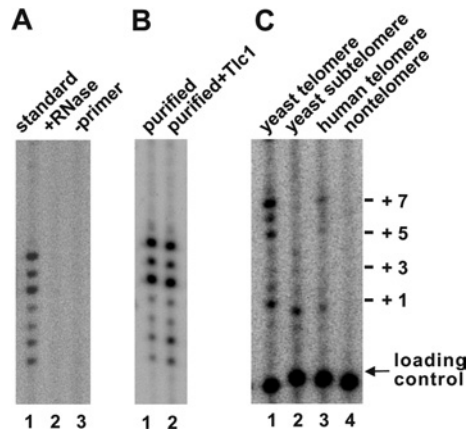


**Figure 4 Telomerase activity assays of purified core complex**

(**A**) The activity of the GST–Est2p–Tlc1 complex was dependent on Tlc1 RNA and primer. Lane 1, standard reaction as in Figure 3(A); lane 2, RNase A was added to pre-treat purified recombinant GST–Est2p–Tlc1 complex; lane 3, oligonucleotide primer was omitted. (**B**) The addition of *in vitro* transcribed Tlc1 RNA has little effect on the telomerase activity attributed to purified recombinant GST–Est2p–Tlc1 complex. Lane 1, purified telomerase core enzyme; lane 2, purified core enzyme with a roughly equal amount of *in vitro* transcribed Tlc1 RNA. (**C**) The recombinant GST–Est2p–Tlc1 complex had substrate preference for yeast telomeric DNA. Lane 1, primer with yeast telomere sequence; lane 2, primer with yeast subtelomere sequence; lane 3, primer with human telomere sequence; lane 4, primer with non-telomere sequence. As a loading control (indicated), $\alpha$-$^{32}$P-labelled 16-mer DNA (5′-GTGTGGTG-TGTGTGGG-3′) was co-precipitated with the products of telomerase. Positions +1, +3, +5 and +7 are also indicted.

results also indicated that the purified GST–Est2p–Tlc1 core enzyme fell off or halted on the primers less often at positions +1, +2, +3 and +4 than at positions +5, +6 and +7 [43], suggesting that processivity of nucleotide addition is an intrinsic feature of telomerase core enzyme. Moreover, a maximum seven nucleotide addition on to a primer by the purified GST–Est2p–Tlc1 core enzyme indicates that the *S. cerevisiae* telomerase core enzyme could add no more than one round of telomeric repeat at one time (Figure 3A, lane 2).

To confirm that the purified activity resulted from recombinant telomerase, not from other cellular polymerase(s), we examined its dependence on telomerase RNA, Tlc1, as well as telomeric primer. No activity was detected when Tlc1 was digested by RNase A (Figure 4A, lane 2), or when no primers (substrates) were added (Figure 4A, lane 3), demonstrating that both the Tlc1 and the primer were essential for its activity. Since the GST–Est2p–Tlc1 complex was affinity-purified with the GST column, it was possible that, in the partially purified telomerase, GST–Est2p could exist as either free protein or RNP with Tlc1. Therefore the *in vitro* transcribed Tlc1 was added to the partially purified telomerase, and the activity assay showed that the addition of extra Tlc1 did not increase the telomerase activity (Figure 4B), suggesting that co-expression of GST–Est2p and Tlc1 allows most, if not all, of the active GST–Est2p to form a complex with Tlc1. This result is consistent with the finding that overexpression of Tlc1 reverses the telomere shortening seen in the cells overexpressing GST–Est2p (Figure 2A).

### The recombinant GST–Est2p–Tlc1 complex has the substrate preference for single-stranded *S. cerevisiae* telomeric DNA

Although *Tetrahymena thermophila* can use non-telomeric DNAs as substrate primers [44], the yeast telomerase specifically elongates the telomeric DNAs [45]. Therefore we tested the recombinant enzyme with different DNA primers as substrates. As shown in Figure 4(C), the recombinant telomerase could efficiently add a telomeric repeat on to the primers with yeast telomeric DNA sequence (5′-GGGTGTGGTGTGTGTGGG-3′) (Figure 4C, lane 1), but not with the human telomeric DNA sequence $(TTAGGG)_3$ (Figure 4C, lane 3) or non-telomere DNA sequence (5′-ATTCGGCTACCTAGCCAT-3′) (Figure 4C, lane 4). The yeast subtelomeric DNA (5′-AGGGTAGTGTTAGGGTAG-3′) was not efficiently elongated by the recombinant Est2–Tlc1 complex, because only very faint bands were detected when yeast subtelomeric DNA was used as substrate (Figure 4C, lane 2). These data indicated that the recombinant GST–Est2p–Tlc1 core enzyme possessed substrate specificity.

### The recombinant GST–Est2p–Tlc1 complex exhibited different activities when primers were paired with Tlc1 template at different positions *in vitro*

The *S. cerevisiae* telomeric repeats are heterogeneous with the consensus sequences $(TG)_{1–4}G_{2–3}$ [46], and the heterogeneity of the repeats was thought to be due to different possible base pairing between telomere and template, as well as non-processive nucleotide addition [8]. Forstemann and Lingner [35] have demonstrated that the divergence of *S. cerevisiae* telomeric repeats is due to both abortive reverse transcription in the 3′ and 5′ regions of the template and the alignment of telomeres to multiple registers within the RNA template. We tested the telomerase activities with a set of primers that were designed to align with the template region of Tlc1 at different registration sites (Figure 5A). The recombinant GST–Est2p–Tlc1 complex could elongate primers Sc8–Sc15 efficiently (Figure 5B, lanes 8–15), but had little or no activity on primers Sc1–Sc7 (Figure 5B, lanes 1–7). To the primers Sc8, Sc9 and Sc10, the recombinant core enzyme could processively add TGTG, GTG and TG respectively,

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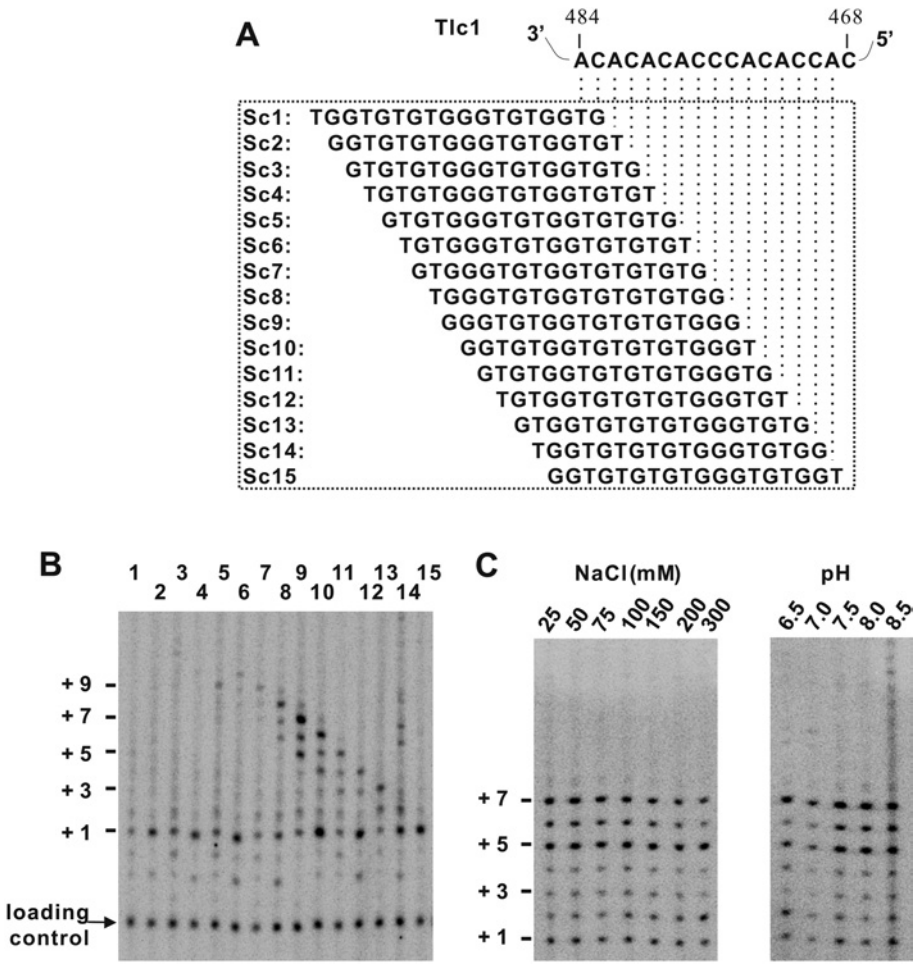


**Figure 5 Effects of primers, salt concentration and pH on telomerase activity**

(**A**) Primers aligned with different sites of Tlc1 template. (**B**) Recombinant telomerase exhibited different activities on primers with different 3′ ends *in vitro*. Numbers above the gel correspond to the primers shown in (**A**). The loading control (indicated) is as in Figure 4(C). Positions +1, +3, +5, +7 and +9 are indicated. (**C**) Telomerase activity was not sensitive to the salt concentrations (left-hand panel) and pH values (right-hand panel) tested. The oligonucleotide DNA (5′-GTGTGTGTGGG-3′) was used as a primer. Positions +1, +3, +5 and +7 are indicated.

corresponding to $A^{474}CAC^{471}$, $C^{473}AC^{471}$ and $A^{472}C^{471}$ in Tlc1, and was less processive at positions $C^{470}$, $A^{469}$ and $C^{468}$ (Figure 5B, lanes 8, 9 and 10). The 3′ end of primer Sc14 (5′-TGG-3′) appeared to be able to pair with $A^{478}CC^{476}$ in Tlc1, since telomerase could add approx. eight nucleotides on to the primer Sc14 (Figure 5B, lane 14), although these longer products were less dense. The wedge-shaped pattern that resulted from the different pairing of the primers (Sc6–Sc15) and template supported further the argument that telomerase core enzyme could only synthesize one round of telomeric repeat (Figure 5B).

Interestingly, the radioactive signals were detected at position +1 with all primers, suggesting that the purified recombinant core enzyme had mis-incorporated nucleotide dTTP instead of dGTP on to primers Sc2, Sc4, Sc6, Sc7, Sc8, Sc10, Sc12, Sc13 and Sc15 at position +1, because the source of radioactivity was [$\alpha$-$^{32}$P]dTTP. The nucleotide mis-incorporation might sterically inhibit a conformational change required for catalytic activity, and therefore cause a pause in the polymerization reaction [47]. We also analysed the recombinant core enzyme with different salt concentrations and pH; both the activity and the processivity of the recombinant telomerase were not sensitive to the changes in salt concentration and pH tested (Figure 5C).

## DISCUSSION

The Est2p–Tlc1 complex is believed to be yeast telomerase core enzyme, and sufficient to elongate the telomeric DNA tracts *in vitro*. The telomerase activity of the *S. cerevisiae* has previously been obtained with different approaches, e.g. DEAE–Sepharose fractionation [23], immunoprecipitation [7], affinity chromatography when Est2p is Protein-A-tagged [36,37] or glycerol gradient centrifugation [10]. However, the amount of activity fractionated and/or concentrated from endogenous telomerase by these approaches seemed relatively low. The activities reported previously are similar to the activity that we have seen in the total extract when GST–Est2p and Tlc1 were co-overexpressed. For example, the signals at the position +2 or +3 are stronger than that at other positions, i.e. +4, +5 and +6 (Figure 2B, lane 4, and Figure 3A, lane 1). One explanation for this low activity is that there is nuclease contamination co-fractionated with telomerase, and this argument was supported by the fact that the length of radiolabelled products is shorter than or equal to that of the input primer (faint signals at position −1 or −2 were detected; Figure 2B, lane 4). An alternative, but not exclusive, possibility is that the yeast telomerase, besides nucleotide addition

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activity, could have nucleolytic cleavage activity as seen with the *T. thermophila* telomerase [48,49]. In addition, the nature of the activity reported previously was not clear, and it might be difficult to carry out mechanistic studies with those enzymes. In the present paper, we have developed a procedure to purify recombinant GST–Est2p–Tlc1 telomerase core enzyme under mild conditions when it was co-overexpressed in yeast. The partially purified GST–Est2p–Tlc1 complex could processively elongate one round of telomeric repeat, but could not translocate to the second round after one cycle of copying the template. Since Est1p and Est3p were not detected in the partially purified recombinant telomerase activity, our data suggested that the core enzyme of yeast telomerase is essential and sufficient for the telomeric DNA elongation *in vitro*.

The activity of the purified GST–Est2p–Tlc1 core complex is higher than that of total extract, and this higher activity probably results from the increase in processivity. The purified core enzyme was more capable of adding five to seven nucleotides than unpurified enzyme when the primers were aligned with the 5′-$C^{471}$CCACACACA$^{484}$-3′ of Tlc1 (Figure 3A). This processive synthesis of yeast telomerase core enzyme was not observed in previous studies. Our results are consistent with the observation that the 5′-TGGGTGT-3′ telomeric sequence was prevalent *in vivo*, and support the finding that the reverse transcription in the central part of the template is non-abortive *in vivo* [35]. The recombinant telomerase core enzyme seemed to drop off or halt on the template, often when the polymerization reaction approached the position $C^{470}$, $A^{469}$ or $C^{468}$ in Tlc1 (Figures 3A and 5B), and the reason(s) for that was not clear. We also noticed that there were faint signals at positions +6 and +7 when the primer Sc14 was used (Figure 5B, lane 14). We speculated that the primer Sc14 was able to pair with the Tlc1 as primer Sc8 did (Figure 5B, lane 8), and resulted in the products that migrate at positions +6 and +7 (Figure 5B, lane 14). Both our *in vitro* data and other *in vivo* studies indicate that the processivity of nucleotide addition is an intrinsic feature of telomerase core enzyme. Interestingly, few radiolabelled products with sizes less than or equal to that of the input primer were detected when the purified recombinant telomerase core enzyme was analysed (Figure 3A). These results suggest that the purified telomerase core enzyme has little nucleolytic cleavage activity, at least under the assay conditions, and that the nuclease activity seen in the total extract has been eliminated after the purification.

The fact that the purified recombinant *S. cerevisiae* telomerase core enzyme could add no more than one round of telomeric repeat at one time (Figure 3A, lane 2, and Figure 5B) again raised a long-standing question of how the telomerase achieves the translocation after one round of telomeric DNA addition *in vivo* [26,27]. Our purified recombinant telomerase GST–Est2p–Tlc1 core enzyme could be highly useful in analysing whether the other subunits of telomerase holoenzyme, i.e. Est1p and Est3p, function as the long-hypothesized 'translocator'. It has been reported that, *in vivo*, yeast telomerase frequently allows telomeres to pair with the 3′ region of Tlc1 template and copy most of it [50]. But, *in vitro*, little or no activities were detected with the primers (Sc1–Sc5 in Figures 5A and 5B), which were annealed with the 3′ region of the template. Given that the orthologue of Est1p in *C. albicans* augments telomerase ability to extend some primers with different 3′ ends [22], the Est1p (and/or Est3p) in *S. cerevisiae* might help telomeric DNA to register at the 3′ region of the template. In addition, the activity assay with purified GST–Est2p–Tlc1 core enzyme could also be an initial step to perform mechanistic studies of the role of telomere binding proteins, such as Cdc13, Stn1 and Ku70/80, in telomere replication.

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