Published online 4 January 2010

Nucleic Acids Research, 2010, Vol. 38, No. 7 ***2279–2290***
doi:10.1093/nar/gkp1173

Investigating the role of the Est3 protein in yeast telomere replication

Jaesung Lee[1], Edward K. Mandell[1], Timsi Rao[2], Deborah S. Wuttke[2] and Victoria Lundblad[1,*]

[1]The Salk Institute for Biological Studies, La Jolla, CA 92037 and [2]Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0125, USA

Received September 14, 2009; Revised November 25, 2009; Accepted December 1, 2009

ABSTRACT

The Est3 subunit of yeast telomerase, which adopts a predicted OB-fold, is essential for telomere replication. To assess the possible contributions that Est3 might make to enzyme catalysis, we compared telomerase activity from wild type and *est3-Δ* strains of *Saccharomyces castellii*, which revealed that loss of the Est3 subunit results in a 2- to 3-fold decline in nucleotide addition. This effect was not primer-specific, based on assessment of a panel of primers that spanned the template of the *S. castellii* telomerase RNA. Furthermore, using nuclear magnetic resonance chemical shift perturbation, no chemical shift change was observed at any site in the protein upon addition of single-stranded DNA, arguing against a role for Est3 in recognition of telomeric substrates by telomerase. Addition of exogenous Est3 protein, including mutant Est3 proteins that are severely impaired for telomere replication *in vivo*, fully restored activity in *est3-Δ* telomerase reactions. Thus, Est3 performs an *in vivo* regulatory function in telomere replication, which is distinct from any potential contribution that Est3 might make to telomerase activity.

INTRODUCTION

In telomerase-expressing cells, telomerase is carefully controlled by both negative and positive regulation (1). These regulatory mechanisms ensure that the shortest telomeres are preferentially elongated by telomerase (2,3) and, conversely, that telomerase is repressed at over-elongated termini (4–6), thereby maintaining telomere length homeostasis. Regulation can occur at multiple levels, including controlling access to the site of catalysis (the protruding G-rich 3′ overhang present at chromosome termini) and the extent of nucleotide addition per catalytic event (7,8), as well as assembly and transport of the telomerase holoenzyme complex (9,10).

At least a subset of these regulatory steps are performed by subunits of the telomerase holoenzyme, and, thus, a full appreciation of telomerase regulation will benefit from a mechanistic understanding of biochemical properties of each telomerase subunit. However, a complete picture of the telomerase complex in various experimental systems is still emerging. In the budding yeast *Saccharomyces cerevisiae*, telomerase is composed of three Est (*e*ver *s*horter *t*elomere) proteins in association with the telomerase RNA, referred to as TLC1 (11–13). A secondary structure model for TLC1, constructed on the basis of both phylogenetic and *in vivo* analysis, reveals a central core with flexible arms protruding from this core (14,15). Est2 binds to the central core, which contains the template region of TLC1. Est1 also binds the telomerase RNA, through a bulged stem–loop structure located at the end of one of the flexible arms (16). Consistent with distinct binding sites on TLC1 for Est1 and Est2, these two subunits do not interact in the absence of TLC1 (17), arguing against a stable interaction between these two proteins. In contrast, Est3 is dependent on the Est2 subunit for complex association (11; this work), suggesting that Est2 and Est3 bind each other directly. The overall architectural features displayed by the *S. cerevisiae* enzyme are shared with the *Schizosaccharomyces pombe* telomerase RNP (18–20), although an Est3 subunit of fission yeast telomerase has not been identified. This may be a consequence of the pronounced sequence divergence exhibited by telomerase subunits among the *Ascomycota* phylum, which is particularly notable for Est3; so far, homologs of Est3 have only been identified in the subphylum *Saccharomycotina* (20; Mandell, E.K., unpublished data).

The individual functions of the components of the *S. cerevisiae* telomerase have been intensively studied

*To whom correspondence should be addressed. Tel: +1 858 453 4100 (Ext. 1913); Fax: +1 858 457 4765; Email: lundblad@salk.edu
Present address:
Jaesung Lee, University of California at San Diego, La Jolla, CA 92093-0651, USA

over the past decade. Two subunits form the catalytic core of the enzyme: TLC1 provides the template that dictates the sequence of telomeric repeats synthesized by the reverse transcriptase domain of Est2 (12,13). Est1 and Est3, in contrast, are dispensable for enzyme catalysis (21,22). Instead, the primary role of the Est1 protein is to recruit telomerase to its site of action, through an interaction with the telomere-bound Cdc13 protein (8,23–25). The contribution that Est3 makes to telomere replication has remained elusive, although recent work has demonstrated that Est3 consists of a predicted oligo-saccharide/oligo-nucleotide binding (OB)-fold which is structurally similar to an OB-fold present in the mammalian TPP1 protein (26,27).

The conclusion that the Est1 and Est3 telomerase subunits are not essential for enzymatic activity has been supported by studies in multiple fungal organisms. Telomerase is capable of elongating telomeric primers in partially fractionated extracts prepared from *est1-Δ* strains of *S. cerevisiae*, *Candida albicans* and *S. pombe*, as well as *est3-Δ* derivatives of *S. cerevisiae*, *Kluyveromyces lactis* and *C. albicans* (18,19,21,22,28–30). However, telomerase activity from each of these fungal species exhibits an atypical property, when compared to the behavior of the telomerase enzyme from most other species. As was first demonstrated in *Tetrahymena*, a characteristic feature of telomerase is the ability to elongate a primer to the end of the RNA template, followed by translocation of the primer on the template to allow another round of elongation (31). Repeated rounds of elongation and translocation can allow synthesis of >500 nt before dissociation (32), giving rise to a periodicity in the pattern of the reaction products due to pausing that occurs at the translocation step. In contrast, telomerase extracts from *S. cerevisiae*, *K. lactis* and even the more phylogenetically distant *S. pombe* appear to be capable of synthesizing only up to ~10 nt *in vitro*, with no evidence for translocation (19,21,30).

This does not appear to be an intrinsic property of fungal species, however, because telomerase from *Saccharomyces castellii* is capable of elongating a telomeric primer through multiple rounds of translocation (21). Since this provides a more sensitive assay for assessing the potential effects of regulatory factors on telomerase activity, this current study re-addresses the role of Est3 in telomerase activity. Comparison of enzyme activity prepared from *EST3* and *est3-Δ* strains of *S. castellii* demonstrated that loss of the Est3 telomerase subunit resulted in a 2- to 3-fold reduction in nucleotide addition, in a primer-independent manner. Activity could be restored by adding *S. castellii* Est3 protein, which was affinity purified from *Escherichia coli,* to *est3-Δ* telomerase reactions. This add-back assay was used to demonstrate that Est3 proteins bearing mutations in an invariant arginine residue could fully restore enzyme activity, even though these same mutations confer a profound telomere replication defect *in vivo*. This demonstrates that Est3 performs a regulatory activity, which is unlinked to any contribution to enzymatic activity.

MATERIALS AND METHODS

Yeast strains

Construction of an est3-Δ derivative of S. castellii. The *S. castellii* strain Y258, a generous gift by Jure Piskur (33), was modified by transformation with a *ura3-Δ::NATMX6* cassette, to create a Ura$^-$derivative. In parallel, the *S. castellii EST3* gene was isolated by functional complementation of an *S. cerevisiae est3-Δ rad52-Δ* strain, using a genomic library constructed by partial Sau3A digestion of *S. castellii* genomic DNA cloned in BamHI-digested YEP351 (34). A plasmid containing an ~7-kb genomic insert which included the *S. castellii EST3* gene was recovered and used to construct a *est3-Δ::KANMX6* derivative with the KAN cassette replacing aa 19–50 of the Est3 ORF (pVL2617). This disruption cassette was used to construct YVL2733, which also contained pVL2722 (YCplac33 containing the *S. castellii EST3* gene). Isolates of YVL2733 which had lost the covering *EST3* plasmid were generated either by isolating single colonies on plates containing 5-FOA, or by passive loss by screening colonies grown on rich media (YPAD) for loss of the *URA3* plasmid. In either protocol, Ura$^-$isolates were propagated for a minimum number of generations prior to preparation of extracts for telomerase assays, to minimize possible complications when preparing extracts from senescing strains.

For co-immunoprecipitation experiments, two isogenic protease-deficient strains (YVL3187 and YVL3188) were constructed with $(myc)_{13}$::*TRP1* tags at the C-terminus of Est1 and Est2, respectively, and with *tlc1-Δ148-440* integrated in place of the wild-type *TLC1* gene. Both strains contained pVL2076, which expressed Est3-$(Flag)_3$ and either pVL4061 (*URA3 CEN tlc1-47*) or pVL4062 (*URA3 CEN tlc1-59*), respectively. The strains used for Figure 6C were constructed similarly, with genomic myc-tagged versions of Est1 or Est2, and containing pVL2076 and pVL4126 (*URA3 CEN tlc1-Δ535-707*).

Telomerase assays

Extracts were minimally fractionated, using a previously published protocol (21). Briefly, cell pellets were lysed under liquid nitrogen by mechanical disruption, with a mortar and pestle, extracts were clarified by two successive 15-min spins at 20 000 *g*, loaded onto a DEAE column which was washed 2 × with 0.5 M Na acetate and eluted with 0.7 M Na acetate; eluates were desalted and concentrated on a Microcon-30 column. The levels of TLC1 RNA in eluates prepared from wild type and *est3-Δ* strains was monitored by northern analysis in more than a half dozen experiments, and always found to be equivalent (Figure 4A, Supplementary Figure S3). Freshly prepared eluates were assayed for telomerase activity in 40 µl reactions incubated at 30°C for 30 min, in 50 mM Tris pH 8.0, 100 mM K glutamate, 1 mM spermidine, 2.5 mM $MgCl_2$, 1 mM DDT, 1 µM oligo, 0.25 µM [α-32P]-dGTP (3000 Ci/mol), 5 µM dGTP, 50 µM dCTP and 50 µM dTTP. Oligomers, which were polyacrylamide gel electrophoresis (PAGE)-purified, were obtained from Integrated DNA Technologies;

the sequences of the 16-mer oligomers used in this study are primer 1, $(GTCTGGGT)_2$; primer 2, $(GTGTCTGG)_2$; primer 3,$(GGTGTCTG)_2$; primer 4, $(GGGTGTCT)_2$; primer 5, $(TGTCTGGG)_2$; primer 6, $(TGGGTGTC)_2$; and primer 7, $(CTGGGTGT)_2$. For chain termination assays, dTTP was replaced with 50 μM ddTTP. Completed reactions were treated with 0.5 mg/ml proteinase K + 0.5% SDS at 55°C for 30 min, 500 cpm of a [γ-^{32}P]-labeled oligomer was added as a loading control, and the mixture was extracted with phenol/chloroform; reactions were run on 10% acrylamide plus 7M urea sequencing gels.

For add-back assays, a frame-shift corrected version of the *S. castellii* Est3 protein was cloned in pRSET, with a C-terminal Flag epitope (pVL3057). Protein was expressed and affinity purified in BL21(DE3) *E. coli*, as described previously (35). In brief, cultures were grown to OD_{600} ~0.6 and induced with 0.5 mM isopropyl-β-thio galactopyranoside (IPTG) for 3.5 h at 26°C. Pelleted cells were resuspended in lysis buffer, lysed by sonication and cleared by two successive spins at 20 000 *g* for 10 min. Clarified extracts were incubated with anti-Flag M2 beads (Sigma) for 2–3 h at 4°C, and bound protein was eluted from washed beads with 0.5 μg/ml Flag peptide (Sigma). Protein concentration was assessed on Coomassie-stained 15% sodium dodecyl sulfate (SDS)-PAGE gels by comparison with an RNase A serial dilution series.

^{1}H-^{15}N minimal chemical shift perturbation analysis of Est3

^{15}N-labeled *S. cerevisiae* Est3 bearing an N-terminal $(His)_{10}$ tag was expressed in *E. coli* BL21(DE3) cells in minimal growth medium containing 1.5 g/l of $(^{15}NH_4)_2SO_4$. Following lysis, the His-tagged protein was isolated with Ni–NTA agarose beads (Qiagen) and size-exclusion chromatography to yield final a sample concentration of ~200 μM. ^{1}H-^{15}N TROSY-HSQC spectra were collected on Varian Inova 800-MHz nuclear magnetic resonance (NMR) spectrometer at the W.M. Keck High Field NMR facility at the University of Colorado, Boulder, using standard Varian pulse sequences with minor modifications. Overnight spectra were obtained for identical samples of ^{15}N-labeled *S. cerevisiae* Est3 in the presence and absence of 40-fold molar excess of a 17-mer telomeric oligomer (5′-GTGTG GGTGTGGTGTGG-3′) obtained from Integrated DNA Technologies and used without further purification. Spectra were collected at 25°C equipped with a salt-tolerant HCN cryoprobe. NMR data were processed in NMRPipe and spectra overlaid using CcpNmr analysis software.

RESULTS

Loss of Est3 function in *S. castellii* results in a telomere replication defect

In order to generate an *est3-Δ* strain of *S. castellii*, the *EST3* gene was cloned by functional complementation of an *S. cerevisiae est3-Δ* strain (described in 'Materials and Methods' section), and used to generate an *est3-Δ::kanMX6* disruption cassette. A haploid *S. castellii* strain was transformed with this cassette and isolates were recovered that had replaced the wild-type *EST3* gene with *est3-Δ::kanMX6*. The resulting *S. castellii est3-Δ* strain exhibited a senescence phenotype that was strikingly similar to that of an *S. cerevisiae* strain, which also lacks *EST3*: a growth defect was apparent by 50–75 generations, with senescence becoming pronounced by 100 generations (Figure 1A). As expected, telomere length was shorter in the *est3-Δ* strain and showed a progressive decline with continued propagation (Figure 1B and Supplementary Figure S1). These observations, combined with the ability of the *S. castellii EST3* gene to partially complement the telomere replication defect of an *S. cerevisiae est3-Δ* strain (34), indicate that the *S. castellii* and *S. cerevisiae* Est3 proteins perform functionally equivalent roles in telomere replication in these two species. The *S. castellii est3-Δ* strain was maintained with a covering *EST3* plasmid; isolates that had newly lost the *EST3* plasmid were used for the biochemical experiments presented below.

S. castellii telomerase activity does not exhibit a primer-specific defect in the absence of Est3

To monitor *S. castellii* telomerase activity, extracts were prepared according to the protocol described by Cohn and Blackburn (21) and monitored for telomerase activity in the presence of radiolabeled dGTP and unlabeled dCTP and dTTP. Single-stranded 16-mer telomeric oligomers could be elongated, in an RNase-sensitive manner, by the addition of >30 nt, with products exhibiting an 8-nt periodicity. This periodicity corresponds to translocation of the elongating primer on the template RNA; the identity of this pause position was confirmed by the pattern of products generated in the presence of the chain-terminating dideoxynucleotide analog ddTTP (ref. 21; Figure 1C; Supplementary Figure S2).

The behavior of telomerase activity from partially fractionated extracts prepared from *S. castellii EST3* and *est3-Δ* strains was assessed with a panel of seven 16-mer telomeric primers (Figure 2A and Supplementary Figure S3). Somewhat surprisingly, enzyme activity from the *est3-Δ* strain appeared to be substantially diminished, relative to wild-type activity. This was an unexpected result, as telomerase activity could be readily detected in extracts prepared from an *S. cerevisiae est3-Δ* strain (22; Lee,J.S. and Lundblad,V., unpublished data). The reduction in activity was reproducible, based on more than a dozen experiments that compared independently prepared extracts from wild type and *est3-Δ*: in every case, elongation of telomeric primers was reduced in the absence of the Est3 telomerase subunit. One possible explanation for the apparent reduction in activity might be the presence of an inhibitor in the *est3-Δ* extracts that masked enzyme activity; however, addition of an *est3-Δ* extract to a wild-type extract did not inhibit wild-type telomerase activity (data not shown), arguing against this possibility. Activity was not restored in the *est3-Δ* extracts when longer primers (32 nt) were used, nor was activity responsive to several variations in reaction

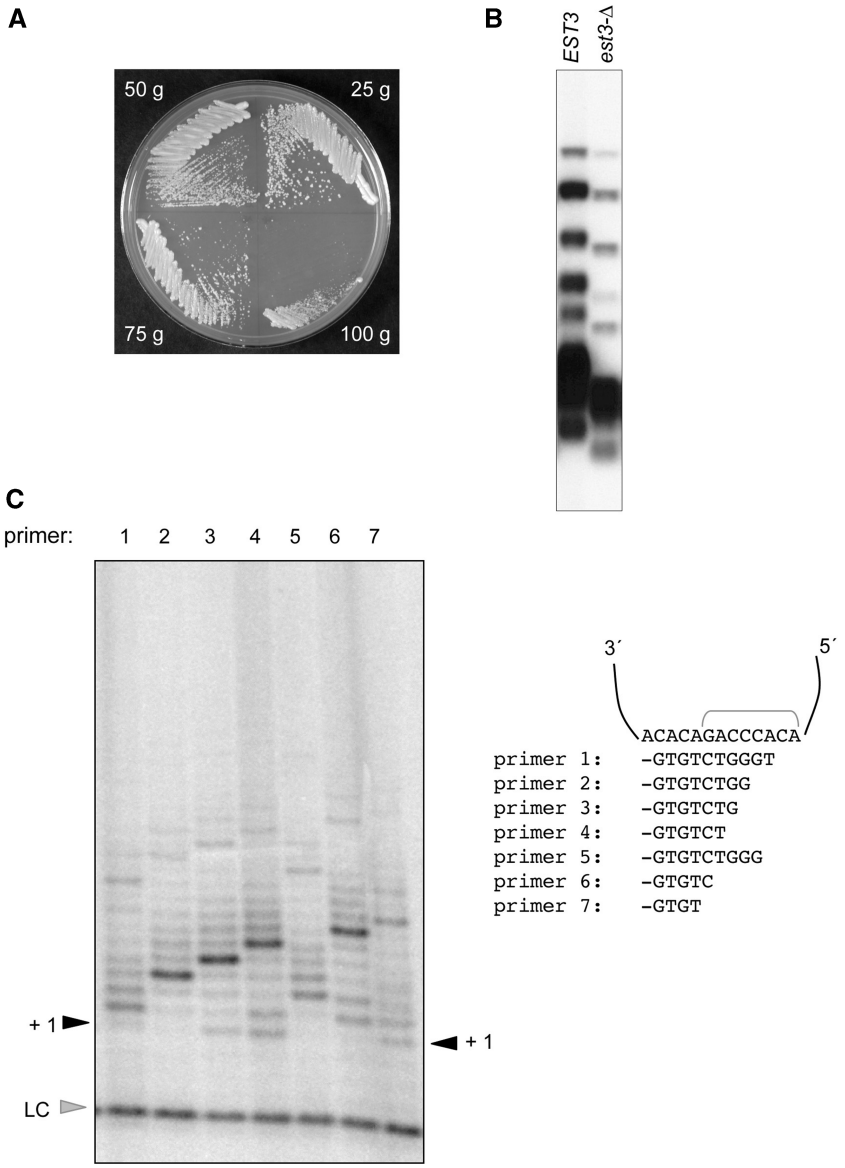

Figure 1. An *est3-Δ* strain of *S. castellii* is defective for telomere replication. (**A**) Successive streak-outs of an *S. castellii est3-Δ*, following loss of a covering *EST3* plasmid. (**B**) Southern blot analysis of genomic DNA from *EST3* and *est3-Δ* strains of *S. castellii* digested with HindIII and probed with a radiolabeled $(TCTGGGTG)_{10}$ oligomer. (**C**) Telomerase activity from a wild-type *S. castellii* strain, with a panel of 16-mer single-stranded telomeric oligomers; the position of the 3′ terminus of each oligo when annealed with the template region of the *S. castellii* telomerase RNA is indicated. LC = loading control (a [γ-^{32}P]-labeled 12-mer oligomer).

conditions (data not shown). The reduction in activity also did not appear to reflect a primer-specific defect, as no notable differences could be observed in the activity of telomerase isolated from an *est3-Δ* extract with seven primers that spanned the template of the *S. castellii* telomerase RNA (Figure 2A and Supplementary Figure S3).

As an alternative means of assessing the effects of the Est3 protein on telomerase, enzyme activity was monitored in a chain termination assay, in the presence of either ddTTP or ddGTP. This assay has the advantage that reaction products are concentrated in essentially a single band, providing an easier direct comparison between mutant and wild-type extracts. Telomerase activity was assayed under exactly the same conditions as in Figure 2A, except that dTTP or dGTP was replaced by the equivalent dideoxynucleotide analog. Figure 2B shows the results with four primers, where elongation was terminated by the addition of ddTTP. This more sensitive assay clearly demonstrated that the telomerase preparations from the *est3-Δ* extracts were catalytically active, consistent with prior results in *S. cerevisiae* (22). However, *S. castellii* telomerase activity displayed a modest decrease in activity in the absence of the Est3 protein: activity was reduced 2–3-fold in *est3-Δ* extracts with each of the four oligos, relative to wild type (Figure 2C). This behavior was not specific to termination by ddTTP; when elongation by primers 5 and 6 in the presence of ddGTP was examined, a similar ~2-fold decline in activity in the

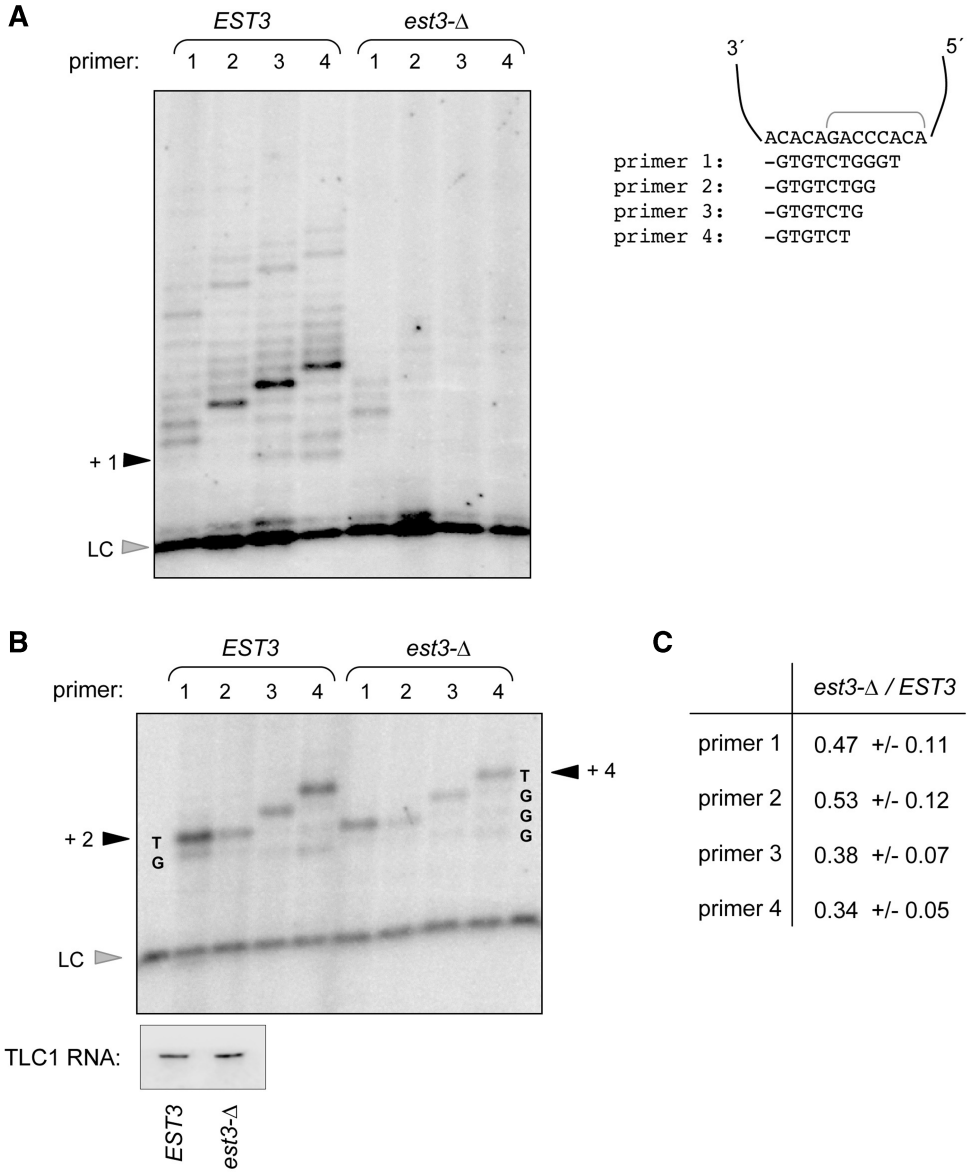

Figure 2. Assessing *S. castellii* telomerase activity from wild-type and *est3-Δ* strains. (**A**) Telomerase assay, with primers 1–4, comparing enzyme activity from partially fractionated wild-type and *est3-Δ* extracts, assayed as described in Figure 1C and 'Materials and Methods' section. (**B**) Telomerase assay with the same set of primers, in the presence of the chain-terminating dideoxynucleotide analog ddTTP. Northern analysis indicates the levels of the *S. castellii* TLC1 RNA in the wild-type and *est3-Δ* eluates used for this experiment. The relative level of activity with these four primers was reproducible; for example, signal with primer 1 was always ~2-fold more intense than that of primer 2, with telomerase isolated from both wild-type and *est3-Δ* strains. (**C**) The ratio of signal recovered from *est3-Δ* extracts, relative to *EST3*, for primers 1 through 4. Signal was calculated by monitoring the + 2 band for primers 1 and 2, and the + 3 and + 4 bands for primers 3 and 4, respectively, and normalizing to the loading control. Results are calculated from three independent experiments, using freshly prepared extracts from *EST3* and *est3-Δ* strains for each repeat.

absence of Est3 was observed (data not shown). Thus, the six primers examined in this chain termination assay behaved the same in response to loss of the Est3 protein. This argues against a role for *S. castellii* Est3 in mediating primer specificity in telomerase activity, as was previously observed for telomerase activity isolated from *est3-Δ* derivatives of *C. albicans* (29).

The Est3 protein does not bind telomeric single-strand DNA

Although telomerase lacking the Est3 subunit did not exhibit a primer-specific defect, loss of the Est3 protein might confer a general reduction in the ability of telomerase to interact with telomeric primers. This possibility was raised by a prior study which reported that an *S. cerevisiae* GST-Est3 fusion protein, when expressed in *E. coli* and affinity purified, exhibited an apparent (albeit weak) ability to bind telomeric DNA substrates, as assessed by gel shifts (36). We re-examined this, using NMR chemical shift perturbation, which should reveal chemical shift changes at proximal amide sites, as well as any sites that undergo a conformational change, upon binding of DNA. This has several advantages over a gel-shift assay, particularly when probing for weak

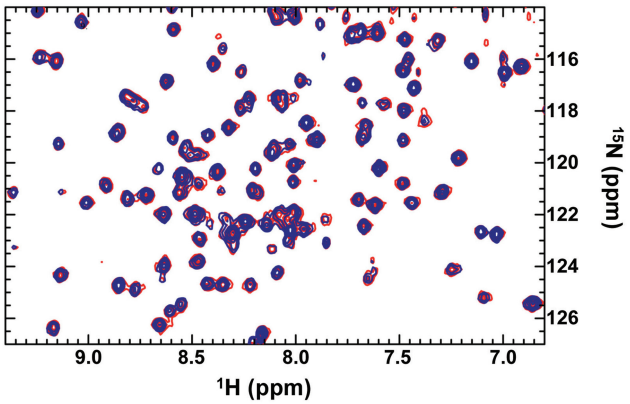

Figure 3. The *S. cerevisiae* Est3 protein does not bind single-stranded telomeric DNA. Superposition of ^{1}H-^{15}N HSQC-TROSY spectra of ^{15}N-labeled *S. cerevisiae* Est3 (200 μM) in the presence (blue) and absence (red) of 40-fold excess of a 17-mer single-strand telomeric oligomer.

binding. NMR is very sensitive to even a small degree of binding, since it is a true equilibrium technique and not impacted by fast off rates characteristic of weak binding interactions. In addition, this approach provides the ability to probe for binding at very high concentrations of protein and single-stranded DNA. Finally, because the spectrum of the Est3 protein is monitored for a change upon addition of single-stranded DNA, this unequivocally establishes that we are probing the activity of the correct protein. In contrast, when assessing a very weak DNA-binding activity by gel shifts, it is difficult to rule out that a positive result is not due to a contaminating protein in the extract.

The ^{15}N-HSQC spectra of ^{15}N-labeled *S. cerevisiae* Est3 (200 μM) were obtained in the presence and absence of a 17-mer telomeric oligomer. Superposition of these spectra revealed no chemical shift changes at any of the 160 resolved crosspeaks in the protein upon addition of single-stranded DNA, even at 8 mM (Figure 3). We conservatively estimate that we would be able to detect as little as 10% bound species, placing a minimal limit on the binding affinity of Est3 for single-stranded DNA in the high millimolar range. These observations indicate that any interaction between Est3 and telomeric single-stranded DNA is weaker than ~10 mM, arguing against a role for the Est3 protein in binding telomeric substrates.

Telomerase activity in *est3-Δ* extracts can be restored by exogenously added Est3 protein

The reduced activity observed in the *S. castellii est3-Δ* extracts might be due to altered assembly of the telomerase enzyme in the absence of Est3; for example, the Est3 protein could be required to mediate binding of Est2 to the TLC1 RNA or otherwise contribute to RNP assembly. If so, this predicts that addition of purified Est3 protein to extracts prepared from an *est3-Δ* strain would not restore telomerase activity. To test this, the *S. castellii* Est3 protein, bearing a C-terminal Flag epitope, was expressed in *E. coli* and purified by immunoprecipitation. When this affinity-purified Est3-Flag protein preparation was added to extracts prepared from the *S. castellii est3-Δ* strain, telomerase activity was restored to wild type levels (Figure 4A). This did not appear to be due to stimulatory effects of the exogenously added Est3 protein, as no enhancement of enzyme activity was observed when the Est3 protein was added to extracts prepared from a wild-type extract, even at >10-fold excess of the amounts required to restore activity to an *est3-Δ* extract (Figure 4B). This result indicates that the DEAE eluate prepared from both wild-type and *est3-Δ* extracts contains an intact catalytic core (which is also consistent with the fact that the level of the TLC1 RNA subunit was equivalent in telomerase preparations from *EST3* and *est3-Δ* strains; Figure 4A and Supplementary Figure S3).

This assay was also dependent on whether the exogenously added Est3 protein was capable of associating with the telomerase RNP, as revealed by the behavior of two mutant versions of the *S. castellii* Est3 protein (Est3-D168A and Est3-D168R) in this assay. These two mutations were chosen based on prior work examining the effect of missense mutations in the *S. cerevisiae* Est3 protein on telomerase association, using co-immunoprecipitation with the TLC1 RNA (26). In these experiments, the *S. cerevisiae* Est3-D164A protein (D164 is the equivalent residue to D168 in *S. castellii*) retained wild-type levels of association, whereas the Est3-D164R protein had a greatly reduced association with the *S. cerevisiae* telomerase RNP (20; Supplementary Figure S4A). The ability to form a complex with the telomerase RNP was mirrored in the *S. castellii* add-back assay: the Est3-D168A protein behaved like wild-type Est3, with regard to restoration of telomerase activity, whereas the Est3-D168R was unable to restore activity (Supplementary Figure S4B).

The above results argue that this add-back assay provides a sensitive means of probing whether mutant Est3 proteins that still retain association with telomerase would exhibit a reduction in telomerase activity, similar to that displayed by the *est3-Δ* null strain. We therefore examined the behavior of a fully defective Est3 protein, bearing a separation-of-function mutation in a highly conserved arginine residue (R110 or R112, in the *S. cerevisiae* or *S. castellii* proteins, respectively). Previous work demonstrated that *S. cerevisiae est3-R110E* and *est3-R110A* strains exhibit a pronounced *in vivo* telomere replication defect, even though the Est3-R110E and Est3-R110A mutant proteins, which are expressed at levels comparable to that of wild type, remain bound to the telomerase complex (26). The severity of the defect of the *est3-R110E* and *est3-R110A* mutant strains—which is equivalent to that of the null phenotype—indicate that mutations in this residue impair a key biochemical property of the Est3 protein; consistent with this, the *S. castellii* Est3-R112A was also defective *in vivo* (Supplementary Figure S5). Therefore, we examined *S. castellii* telomerase activity following addition of affinity purified *S. castellii* Est3-R112E-Flag and Est3-R112A-Flag mutant proteins to an *est3-Δ* extract. As shown in Figure 5 and Supplementary Figure S5, these two mutant proteins were indistinguishable from wild type in their ability to restore telomerase activity.

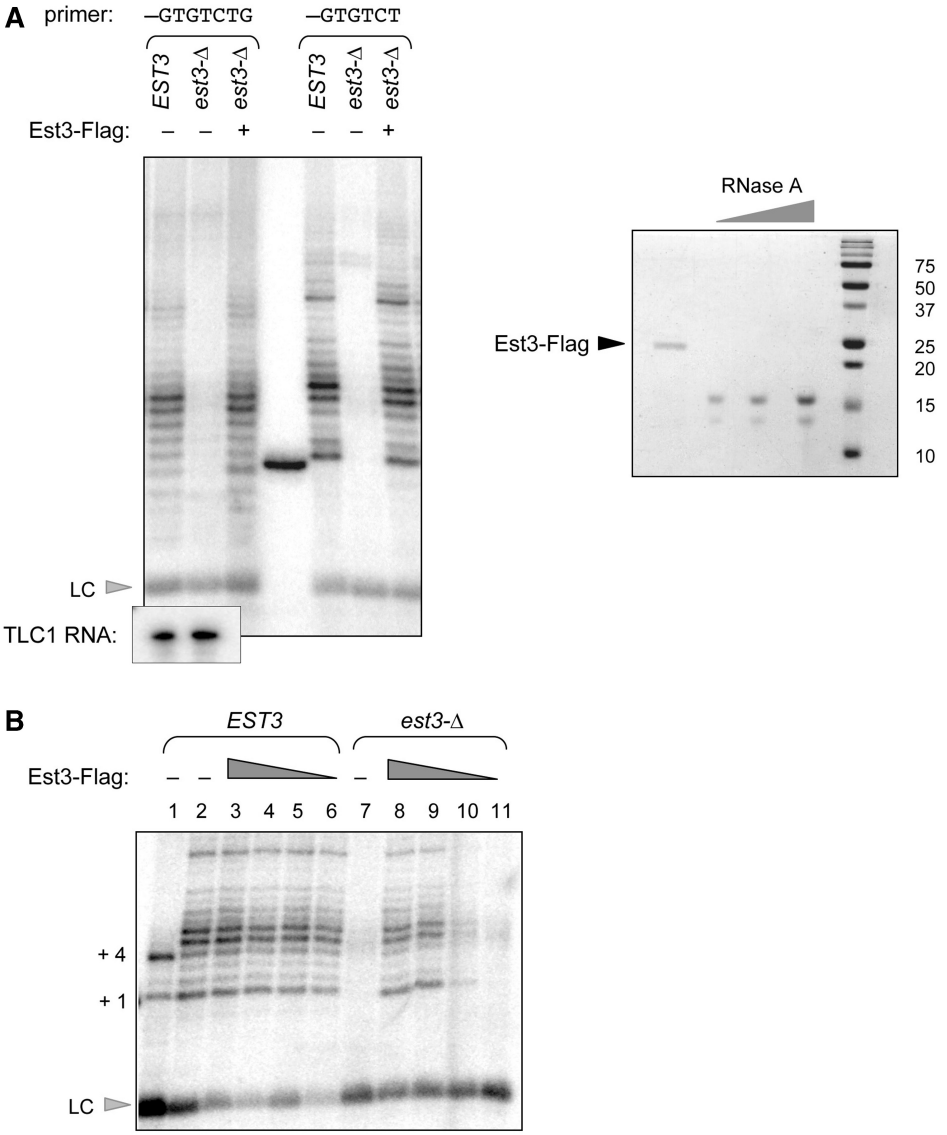

Figure 4. Exogenously added *S. castellii* Est3 protein can restore telomerase activity. (**A**) Telomerase activity from wild-type and *est3-Δ* extracts was examined with primer 3 (first three lanes) and primer 4 (last three lanes); affinity-purified *S. castellii* Est3-Flag protein was added to the telomerase reactions at ~500 nM, as estimated by the RNaseA standards (0.5, 0.25 and 0.125 μg) shown in the Coomassie-stained gel on the right. The middle lane contains a [γ-^{32}P]-labeled 15-mer oligomer used as an additional size marker control. Northern analysis indicates the levels of the *S. castellii* TLC1 RNA in the wild-type and *est3-Δ* eluates used for this experiment. (**B**) Ten-fold serial dilutions of affinity-purified *S. castellii* Est3-Flag protein were added to 40 μl telomerase reactions with wild-type and *est3-Δ* extracts, assessed with primer 4; LC = loading control ([γ-^{32}P]-labeled 10-mer oligomer); lane 1, telomerase activity in the presence of ddTTP. The concentration of Est3-Flag protein added to lanes 4–6 and lanes 8–11 was 100 nM, 10 nM, 1 nM and 0.1 nM. Since the stoichiometry of the telomerase complex in these reactions was not determined, we do not know whether the recombinant Est3 protein was added in molar excess (nor do we know what fraction of the affinity purified Est3-Flag protein is properly folded and/or biologically active). However, the reduction in telomerase activity between lanes 9 and 10, which correspond to 10 and 1 nM protein, respectively, provides an empirical estimate of the amount of Est3 protein required to restore activity to an *est3-Δ* extract. This can be used to demonstrate that when 10-fold excess of this empirically determined amount of Est3-Flag protein is added to a wild-type telomerase extract (lane 3), there is no enhancement of activity, when compared to the wild-type telomerase reaction shown in lane 2.

Thus, we conclude that the surface of Est3 that is defined by this arginine residue (and presumably adjacent residues) performs a function which is essential for Est3 function but which is independent of telomerase activity.

The Est3 protein associates with telomerase in an Est2-dependent manner

The previously shown results raise the possibility that the apparent reduction in telomerase activity in the *S. castellii est3-Δ* strain might not be the result of the loss of a specific biochemical property of the Est3 protein. An alternative possibility is that the effects are due to non-specific effects on the catalytic core of the enzyme, as a result of (partial) destabilization of the Est2 catalytic subunit when the Est3 protein is not present. Such a model implies a direct interaction between the Est2 and Est3 proteins. However, the prior published literature is contradictory on this point. Experiments that examined the pattern of co-immunoprecipitation of *S. cerevisiae* Est3 with TLC1,

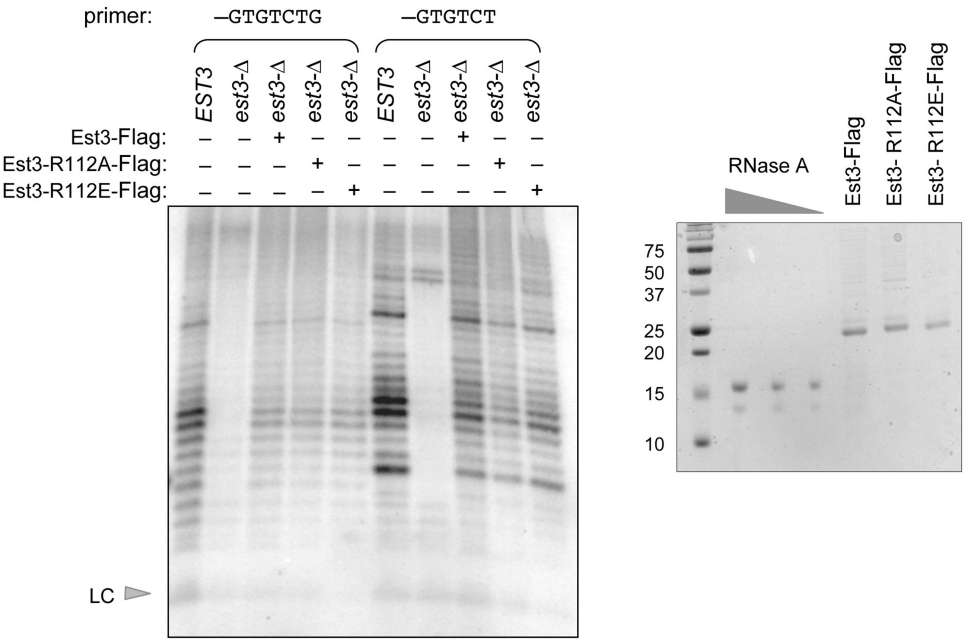

Figure 5. The invariant R112 (R110 in *S. cerevisiae*) of Est3 is not required for telomerase activity. Telomerase activity from wild type and *est3-Δ* extracts were examined with primers 3 and 4 (lanes 1–5 and lanes 6–10, respectively), with ~500 nM of wild type *S. castellii* Est3, Est3-R112A and Est3-R112E added back to the *est3-Δ* telomerase reactions, as assessed by the Coomassie-stained gel shown on the right. Supplementary Figure S5, which shows a serial dilution series of these three proteins added back to *est3-Δ* telomerase reactions, provides further support that these three proteins exhibit equal activity in restoring enzyme activity.

in the presence or absence of the Est1 and Est2 subunits, led to the conclusion that association of Est3 with the telomerase complex was Est2-dependent and Est1-independent (11). However, this observation was contradicted by a subsequent report, which concluded that association of the *S. cerevisiae* Est3 subunit with the telomerase complex was dependent on Est1 (37).

Therefore, we reexamined this issue, using several approaches. First, we examined the pattern of association of the three Est proteins in *S. cerevisiae* cells expressing both the wild-type TLC1 RNA and either of two mutant RNAs (*tlc1-47* and *tlc1-59*). A number of studies have established that Est1 and Est2 independently associate with TLC1, through separate structures on the RNA. Est1 interacts with TLC1 through a bulged stem–loop, and removal of this 5-nt bulge (referred to as *tlc1-47* here) eliminates the ability of Est1 (but not Est2) to bind to the telomerase RNA (16). Est2 interacts with a separate region of the RNA (38), and association of Est2 (but not Est1) is eliminated by a 65-nt deletion (*tlc1-59*) in this region (39). If the Est3 protein binds the telomerase RNP through an interaction with Est2, as originally proposed, then Est3 should exhibit the same pattern of association with TLC1-47 and TLC1-59 as the Est2 protein. To test this, two *S. cerevisiae* strains were constructed (YVL3187 and YVL3188), which co-expressed a version of the wild-type TLC1 RNA (TLC1-Δ148-440) as well as either of the two mutant RNAs. The deletion of ~350 non-essential nucleotides allows the TLC1-Δ148-440 RNA to be readily distinguished from the full-length mutant RNAs; as shown previously, Est1 and Est2 retain full association with the TLC1-Δ148-440 RNA (38). These two strains also contained tagged versions of the three Est proteins: YVL3187, which expressed the mutant TLC1-47 RNA, contained Est1-$(myc)_{13}$ and Est3-$(Flag)_3$, whereas YVL3188, which expressed the mutant TLC1-59, contained Est2-$(myc)_{13}$ and Est3-$(Flag)_3$. Parallel anti-myc and anti-FLAG immunoprecipitations were performed with both strains, and the immunoprecipitates were examined by northern analysis for the pattern of association with the two sets of TLC1 RNAs present in each strain. As expected, the Est1 and Est2 proteins exhibited a roughly 50-fold reduction in their ability to associate with TLC1-47 or TLC1-59, respectively, relative to TLC1-Δ148-440 (39). Notably, Est3 exhibited the same behavior as the Est2 protein, as would be predicted if these two proteins directly interact: co-precipitation of the Est3 protein with TLC1-59 was dramatically reduced, whereas Est3 retained association with the TLC1-47 RNA (Figure 6A).

We also repeated an experiment from the study by the Friedman laboratory (37), which examined the relative association of the Est1 and Est3 proteins with a different mutant RNA, TLC1-Δ535-770, which is impaired for binding the Est1 protein, but not the Est2 protein (38). Our experimental design employed the same strategy as that employed above: two *S. cerevisiae* strains were constructed which co-expressed tagged versions of Est1 and Est3, or Est2 and Est3, as well as the full-length TLC1 and TLC1-Δ535-770 RNAs. Immunoprecipitation, followed by northern analysis, demonstrated that Est3 retained wild-type levels of association with TLC1-Δ535-770, whereas, within the exact same strain, Est1 had lost association with TLC1-Δ535-770 (Figure 6B). As expected,

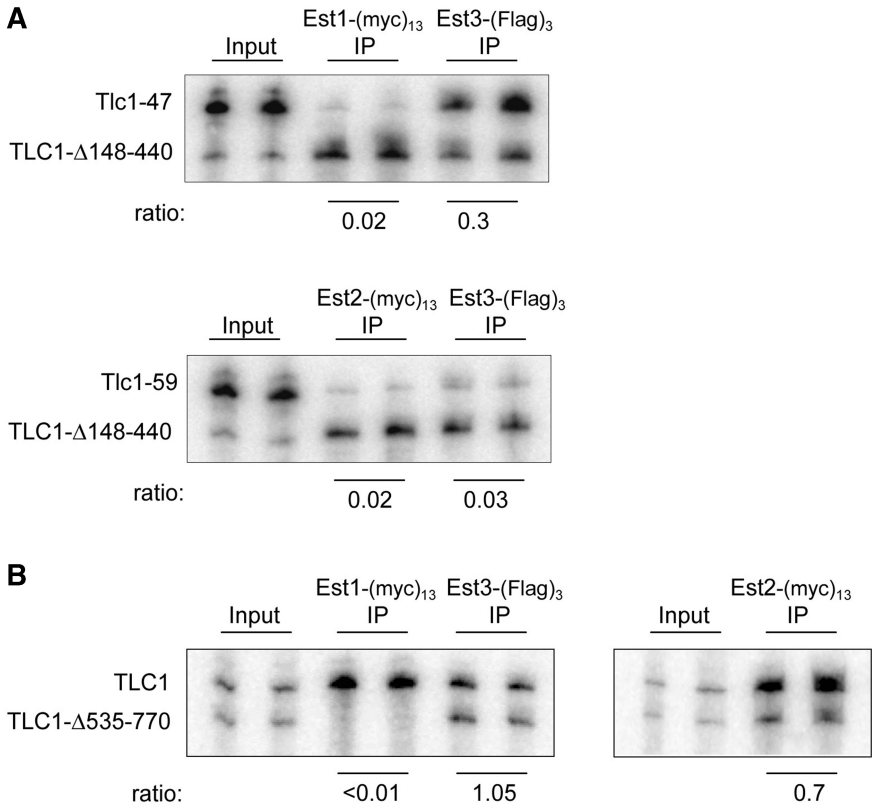

Figure 6. Association of Est3 with the *S. cerevisiae* telomerase complex is Est2-dependent. (**A**) Anti-myc and anti-Flag immunoprecipitations of Est1-$(myc)_{13}$ and Est3-$(Flag)_3$ (YVL3187, upper panel) or Est2-$(myc)_{13}$ and Est3-$(Flag)_3$ (YVL3187, lower panel) were examined for association with the indicated TLC1 RNAs by northern analysis, as described previously (39). The efficiency of co-immunoprecipitation of all three Est proteins with the wild-type TLC1 RNA was between 12 and 18%. (**B**) Anti-myc and anti-Flag immunoprecipitations of Est1-$(myc)_{13}$ and Est3-$(Flag)_3$, expressed in the same strain, were monitored for association with TLC1 versus TLC1-Δ535-770, in parallel with Est2-$(myc)_{13}$ association with the same two RNAs.

the Est2 protein could be co-immunoprecipitated with both the wild type TLC1 and TLC1-Δ535-770 RNAs. These observations with TLC1-Δ535-770 are consistent with our original report (11), as well as the experiments with the mutant TLC1-47 and TLC1-59 RNAs shown in Figure 6A, but inconsistent with prior results by the Friedman laboratory (37), which reported that the Est3 protein had lost association with the TLC1-Δ535-770 RNA. We suspect that the experimental design employed here, where the pattern of Est1 and Est3 association with mutant and wild-type RNAs was examined in the same strain, which provides an internal control to ensure that the predicted results for Est1 and Est2 were as expected, may explain the differing conclusions.

DISCUSSION

This study, together with our previous investigations on the role of the Est3 telomerase subunit in telomere function, combines the strengths of two related model organisms, *S. cerevisiae* and *S. castellii*. Est3 is a rapidly diverging protein at the primary amino acid sequence (26), such that the percent identity between the *S. cerevisiae* and *S. castellii* proteins is only 46%. Nevertheless, the *S. castellii EST3* gene partially compensates for the loss of Est3 function in *S. cerevisiae* (34), supporting the idea that observations in both organisms can contribute to models about the role of Est3 in telomere replication. Therefore, we exploited the ability of the *S. castellii* telomerase to elongate an oligo through several rounds of translocation, in contrast to the non-processive activity of the *S. cerevisiae* telomerase enzyme (21). Our initial observations, using the standard telomerase elongation assay, indicated that the *S. castellii* Est3 protein might make a substantial contribution to enzyme activity. However, when telomerase activity was monitored in the presence of a dideoxynucleotide analog, the consequence of the loss of the Est3 protein was much more modest. We suggest that the difference in the results obtained in these two assay conditions can be explained by the cumulative effects of a modest reduction at each nucleotide addition step, which can translate to an apparent dramatic reduction in telomerase activity. This raises a cautionary note when interpreting potential differences in telomerase activity between two different genotypes, when relying solely on assay conditions that monitor multiple rounds of elongation.

This study raises the issue of why an *S. castellii* telomerase complex lacking the Est3 subunit exhibits a decrease in nucleotide addition. One possibility is that

this effect is the result of non-specific destabilization of the complex due to the absence of a telomerase subunit, as mentioned already. Alternatively, the Est3 protein may contribute a specific biochemical property to enzyme catalysis, for example as a processivity factor. The *S. castellii* telomerase assay provides a sensitive assay for potentially differentiating between these possible models. Since the ability to add telomeric repeats onto a primer through multiple rounds of elongation is fully restored by the exogenous addition of Est3 protein affinity purified from *E. coli*, this provides a biochemical means of assessing the properties of separation-of-function mutations in Est3. Such an approach alleviates the potential concern that a defect in this assay is simply due to destabilization in the absence of the Est3 subunit, rather than loss of a specific property such as processivity. To pursue this, we turned to a cluster of residues on the predicted surface of the *S. cerevisiae* Est3 protein which exhibit an *in vivo* telomere replication defect when mutated (26). In particular, strains expressing missense mutations in the invariant R110 residue (R112 in *S. castellii*) display an Est$^-$phenotype that is indistinguishable from that of an *est3-Δ* null strain, even though the mutant Est3-R110A and Est3-R110E proteins remain associated with the telomerase RNP at wild-type levels. Furthermore, the properties of strains that overexpress either of these two mutant proteins are consistent with the proposal that mutations at amino acid R110 confer a defect in a specific biochemical property of the Est3 protein, rather than a defect due to an unfolded protein (26). Notably, however, addition of the *S. castellii* Est3-R112A or Est3-R112E proteins back to a telomerase extract prepared from an *est3-Δ* strain fully restored telomerase activity. Thus, we conclude that Est3 performs an essential function in telomere replication, as defined by R110 (or R112 in *S. castellii*), which is distinct from any potential contribution that Est3 might make to enzyme activity.

The observations reported in this study differ in one key aspect from prior work investigating the role of Est3 in *C. albicans* telomerase activity. In contrast to the lack of primer-specific defects in the absence of Est3 reported here, the Est3-deficient *C. albicans* telomerase exhibits substantial primer-specific differences (29), even with primers that align at comparable positions on the templates of the *C. albicans* and *S. castellii* telomerase RNAs (Supplementary Figure S6). This disparity may be a reflection of the fact that the *C. albicans* telomerase assays monitored addition onto primers that were only 12 nt in length. Previous work has shown that the *Tetrahymena* enzyme, which is normally highly processive *in vitro*, is non-processive with 10–12-nt primers (40). Thus, if the *C. albicans* enzyme active site is partially destabilized by the loss of the Est3 telomerase subunit, such an effect may be further exaggerated with very short telomeric primers. The unusually long (28 nt) length of the *C. albicans* RNA template (41) may also impose special requirements that differentiate the behavior of the *C. albicans* telomerase from telomerases of other species.

This study also addresses several contradictory observations, in *S. cerevisiae* and *C. albicans*, with regard to how Est3 associates with the telomerase complex (11,29,37). A common approach that has been used in *S. cerevisiae* has been to examine the pattern of association of tagged versions of Est1, Est2 and Est3 with TLC1 or with each other, comparing wild type with strains deleted for one of the *EST* genes (11,17,37). However, past work in our lab has indicated that such experiments can be problematic in telomerase-defective strains that are undergoing senescence, as false negatives can result from immunoprecipitations performed with cultures that are severely senescent (42), presumably due to a high degree of cell inviability. Thus, such experiments require strain manipulations to minimize senescence, as well as ensuring that the immunoprecipitations are performed well above detection limits. To alleviate this potential problem, we instead employed a set of strains that expressed mutant versions of TLC1 that were impaired for association with either Est1 or Est2, in the presence of a functional copy of TLC1, thereby avoiding any problems with senescence. Since Est1 and Est2 independently associate with TLC1, via separate structures on the RNA, this provides an internal control for assessing whether Est3 is dependent on either Est1 or Est2 for its association. The results of this experiment clearly establish that Est3 relies on Est2 in order to assemble with the telomerase complex, suggesting that Est2 and Est3 directly interact. If so, this provides a potential explanation for the reduction in telomerase activity observed when the Est3 telomerase subunit is missing.

However, the results presented in this study do not rule out the possibility that Est3 contributes a specific biochemical activity, such as processivity. Speculation about this possibility is driven by prior observations indicating that the Est3 protein consists of a predicted OB-fold, which exhibits potential structural similarity to the OB-fold found in the mammalian TPP1 protein (26,27). TPP1 is one subunit of a telomere end-binding complex, called POT1/TPP1 (43), and *in vitro* studies have shown that this heterodimeric complex acts as a processivity factor for human telomerase (44). However, there are some notable differences between Est3 and TPP1. Defects in these two proteins result in substantially different *in vivo* consequences (45–47), consistent with the fact that Est3 is a subunit of telomerase, whereas TPP1 is a subunit of an end protection complex. Furthermore, although the very small Est3 telomerase protein consists solely of the predicted OB-fold, TPP1 is a larger protein possessing additional domains. These distinctions argue that, despite the predicted structural similarities in their OB-folds, Est3 and TPP1 are not functional orthologs of each other and instead may reflect the repeated usage of OB-folds in telomere-associated proteins.

Although the behavior of the mutant Est3 proteins that were analyzed in this study does not support a role for Est3 in processivity, it is possible that additional function(s) of Est3 are yet to be identified, particularly since the entire surface of Est3 has not yet been completely surveyed by mutagenesis (26). If so, the *S. castellii* telomerase assay presented here provides a sensitive

assay for assessing whether mutant Est3 proteins defective for a postulated second function will influence the ability of telomerase to elongate telomeric primers *in vitro*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Jure Piskur for providing us with the initial *S. castellii* strain used in these studies, and we thank Geoffrey Armstrong for assistance with the NMR data collection.

FUNDING

National Institutes of Health AG11728 (to V.L.); National Science Foundation 0617956 (to D.S.W.); and National Institutes of Health T32 GM08759 (to T.R.). Funding for open access charge: National Institutes of Health AG11728.

Conflict of interest statement. None declared.

REFERENCES

1. Smogorzewska,A. and de Lange,T. (2004) Regulation of telomerase by telomeric proteins. *Ann. Rev. Biochem.*, **73**, 177–208.
2. Marcand,S., Brevet,V. and Gilson,E. (1999) Progressive *cis*-inhibition of telomerase upon telomere elongation. *EMBO J.*, **18**, 3509–3519.
3. Teixeira,M.T., Arneric,M., Sperisen,P. and Lingner,J. (2004) Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states. *Cell*, **117**, 323–335.
4. Marcand,S., Gilson,E. and Shore,D. (1997) A protein-counting mechanism for telomere length regulation in yeast. *Science*, **275**, 986–990.
5. Smogorzewska,A., van Steensel,B., Bianchi,A., Oelmann,S., Schaefer,M.R., Schnapp,G. and de Lange,T. (2000) Control of human telomere length by TRF1 and TRF2. *Mol. Cell. Biol.*, **20**, 1659–1668.
6. van Steensel,B. and de Lange,T. (1997) Control of telomere length by the human telomeric protein TRF1. *Nature*, **385**, 740–743.
7. Chang,M., Arneric,M. and Lingner,J. (2007) Telomerase repeat addition processivity is increased at critically short telomeres in a Tel1-dependent manner in *Saccharomyces cerevisiae*. *Genes Dev.*, **21**, 2485–2494.
8. Nugent,C.I., Hughes,T.R., Lue,N.F. and Lundblad,V. (1996) Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science*, **274**, 249–252.
9. Mitchell,J.R., Wood,E. and Collins,K. (1999) A telomerase component is defective in the human disease dyskeratosis congenita. *Nature*, **402**, 551–555.
10. Venteicher,A.S., Meng,Z., Mason,P.J., Veenstra,T.D. and Artandi,S.E. (2008) Identification of ATPases pontin and reptin as telomerase components essential for holoenzyme assembly. *Cell*, **132**, 945–957.
11. Hughes,T.R., Evans,S.K., Weilbaecher,R.G. and Lundblad,V. (2000) The Est3 protein is a subunit of yeast telomerase. *Curr. Biol.*, **10**, 809–812.
12. Lingner,J., Hughes,T.R., Shevchenko,A., Mann,M., Lundblad,V. and Cech,T.R. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*, **276**, 561–567.
13. Singer,M.S. and Gottschling,D.E. (1994) TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science*, **266**, 404–409.
14. Zappulla,D.C. and Cech,T.R. (2004) Yeast telomerase RNA: a flexible scaffold for protein subunits. *Proc. Natl Acad. Sci. USA*, **101**, 10024–10029.
15. Dandjinou,A.T., Levesque,N., Larose,S., Lucier,J.F., Abou Elela,S. and Wellinger,R.J. (2004) A phylogenetically based secondary structure for the yeast telomerase RNA. *Curr. Biol.*, **14**, 1148–1158.
16. Seto,A.G., Livengood,A.J., Tzfati,Y., Blackburn,E.H. and Cech,T.R. (2002) A bulged stem tethers Est1p to telomerase RNA in budding yeast. *Genes Dev.*, **16**, 2800–2812.
17. Evans,S.K. and Lundblad,V. (2002) The Est1 subunit of *Saccharomyces cerevisiae* telomerase makes multiple contributions to telomere length maintenance. *Genetics*, **162**, 1101–1115.
18. Beernink,H.T., Miller,K., Deshpande,A., Bucher,P. and Cooper,J.P. (2003) Telomere maintenance in fission yeast requires an Est1 ortholog. *Curr. Biol.*, **13**, 575–580.
19. Leonardi,J., Box,J.A., Bunch,J.T. and Baumann,P. (2008) TER1, the RNA subunit of fission yeast telomerase. *Nat. Struct. Mol. Biol.*, **15**, 26–33.
20. Webb,C.J. and Zakian,V.A. (2008) Identification and characterization of the *Schizosaccharomyces pombe* TER1 telomerase RNA. *Nat. Struct. Mol. Biol.*, **15**, 34–42.
21. Cohn,M. and Blackburn,E.H. (1995) Telomerase in yeast. *Science*, **269**, 396–400.
22. Lingner,J., Cech,T.R., Hughes,T.R. and Lundblad,V. (1997) Three Ever Shorter Telomere (*EST*) genes are dispensable for in vitro yeast telomerase activity. *Proc. Natl Acad. Sci. USA*, **94**, 11190–11195.
23. Pennock,E., Buckley,K. and Lundblad,V. (2001) Cdc13 delivers separate complexes to the telomere for end protection and replication. *Cell*, **104**, 387–396.
24. Bianchi,A., Negrini,S. and Shore,D. (2004) Delivery of yeast telomerase to a DNA break depends on the recruitment functions of Cdc13 and Est1. *Mol. Cell*, **16**, 139–146.
25. Chan,A., Boule,J.B. and Zakian,V.A. (2008) Two pathways recruit telomerase to *Saccharomyces cerevisiae* telomeres. *PLoS Genet.*, **4**, e1000236.
26. Lee,J., Mandell,E.K., Tucey,T.M., Morris,D.K. and Lundblad,V. (2008) The Est3 protein associates with yeast telomerase through an OB-fold domain. *Nat. Struct. Mol. Biol.*, **15**, 990–997.
27. Yu,E.Y., Wang,F., Lei,M. and Lue,N.F. (2008) A proposed OB-fold with a protein-interaction surface in *Candida albicans* telomerase protein Est3. *Nat. Struct. Mol. Biol.*, **15**, 985–989.
28. Singh,S.M. and Lue,N.F. (2003) Ever shorter telomere 1 (EST1)-dependent reverse transcription by *Candida* telomerase *in vitro*: evidence in support of an activating function. *Proc. Natl Acad. Sci. USA*, **100**, 5718–5723.
29. Hsu,M., Yu,E.Y., Singh,S.M. and Lue,N.F. (2007) Mutual dependence of *Candida albicans* Est1p and Est3p in telomerase assembly and activation. *Eukaryot. Cell*, **6**, 1330–1338.
30. Kabaha,M.M., Zhitomirsky,B., Schwartz,I. and Tzfati,Y. (2008) The 5' arm of *Kluyveromyces lactis* telomerase RNA is critical for telomerase function. *Mol. Cell. Biol.*, **28**, 1875–1882.
31. Greider,C.W. and Blackburn,E.H. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature*, **337**, 331–337.
32. Greider,C.W. (1991) Telomerase is processive. *Mol. Cell. Biol.*, **11**, 4572–4580.
33. Petersen,R.F., Langkjaer,R.B., Hvidtfeldt,J., Gartner,J., Palmen,W., Ussery,D.W. and Piskur,J. (2002) Inheritance and organisation of the mitochondrial genome differ between two *Saccharomyces* yeasts. *J. Mol. Biol.*, **318**, 627–636.
34. Chappell,A.S. (2004) Molecular and phylogenetic analysis of the *Saccharomyces cerevisiae EST3* and *TLC1* telomerase subunits. Thesis Dissertation. Baylor College of Medicine, Houston, Texas.
35. Gao,H., Cervantes,R.B., Mandell,E.K., Otero,J.H. and Lundblad,V. (2007) RPA-like proteins mediate yeast telomere function. *Nat. Struct. Mol. Biol.*, **14**, 208–214.
36. Sharanov,Y.S., Zvereva,M.I. and Dontsova,O.A. (2006) *Saccharomyces cerevisiae* telomerase subunit Est3p binds DNA

and RNA and stimulates unwinding of RNA/DNA heteroduplexes. *FEBS Lett.*, **580**, 4683–4690.
37. Osterhage,J.L., Talley,J.M. and Friedman,K.L. (2006) Proteasome-dependent degradation of Est1p regulates the cell cycle-restricted assembly of telomerase in *Saccharomyces cerevisiae*. *Nat. Struct. Mol. Biol.*, **13**, 720–728.
38. Livengood,A.J., Zaug,A.J. and Cech,T.R. (2002) Essential regions of *Saccharomyces cerevisiae* telomerase RNA: separate elements for Est1p and Est2p interaction. *Mol. Cell. Biol.*, **22**, 2366–2374.
39. Chappell,A.S. and Lundblad,V. (2004) Structural elements required for association of the *Saccharomyces cerevisiae* telomerase RNA with the Est2 reverse transcriptase. *Mol. Cell. Biol.*, **24**, 7720–7736.
40. Lee,M.S. and Blackburn,E.H. (1993) Sequence-specific DNA primer effects on telomerase polymerization activity. *Mol. Cell. Biol.*, **13**, 6586–6599.
41. Hsu,M., McEachern,M.J., Dandjinou,A.T., Tzfati,Y., Orr,E., Blackburn,E.H. and Lue,N.F. (2007) Telomerase core components protect *Candida* telomeres from aberrant overhang accumulation. *Proc. Natl Acad. Sci. USA*, **104**, 11682–11687.
42. Evans,S.K. (2000) Analysis of *EST1* and *CDC13* as regulators of telomerase access in *Saccharomyces cerevisiae*. Thesis Dissertation. Baylor College of Medicine, Houston, Texas.
43. de Lange,T. (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.*, **19**, 2100–2110.
44. Wang,F., Podell,E.R., Zaug,A.J., Yang,Y., Baciu,P., Cech,T.R. and Lei,M. (2007) The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature*, **445**, 506–510.
45. Lendvay,T.S., Morris,D.K., Sah,J., Balasubramanian,B. and Lundblad,V. (1996) Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional *EST* genes. *Genetics*, **144**, 1399–1412.
46. Hockemeyer,D., Palm,W., Else,T., Daniels,J.P., Takai,K.K., Ye,J.Z., Keegan,C.E., de Lange,T. and Hammer,G.D. (2007) Telomere protection by mammalian Pot1 requires interaction with Tpp1. *Nat. Struct. Mol. Biol.*, **14**, 754–761.
47. Miyoshi,T., Kanoh,J., Saito,M. and Ishikawa,F. (2008) Fission yeast Pot1-Tpp1 protects telomeres and regulates telomere length. *Science*, **320**, 1341–1344.