The Est3 protein is a subunit of yeast telomerase

Timothy R. Hughes*†‡, Sara K. Evans§‡, Rodney G. Weilbaecher# and Victoria Lundblad*§#

***EST1*, *EST2*, *EST3* and *TLC1* function in a single pathway for telomere replication in the yeast *Saccharomyces cerevisiae* [1,2], as would be expected if these genes all encode components of the same complex. Est2p, the reverse transcriptase protein subunit, and TLC1, the templating RNA, are subunits of the catalytic core of yeast telomerase [3–5]. In contrast, mutations in *EST1*, *EST3* or *CDC13* eliminate telomere replication *in vivo* [1,6–8] but are dispensable for *in vitro* telomerase catalytic activity [2,9]. Est1p and Cdc13p, as components of telomerase and telomeric chromatin, respectively, cooperate to recruit telomerase to the end of the chromosome [7,10]. However, Est3p has not yet been biochemically characterized and thus its specific role in telomere replication is unclear. We show here that Est3p is a stable component of the telomerase holoenzyme and furthermore, association of Est3p with the enzyme requires an intact catalytic core. As predicted for a telomerase subunit, fusion of Est3p to the high affinity Cdc13p telomeric DNA binding domain greatly increases access of telomerase to the telomere. Est1p is also tightly associated with telomerase; however, Est1p is capable of forming a stable TLC1-containing complex even in the absence of Est2p or Est3p. Yeast telomerase therefore contains a minimum of three Est proteins for which there is both *in vivo* and *in vitro* evidence for their role in telomere replication as subunits of the telomerase complex.**

Addresses: *Program in Cellular and Molecular Biology, and Departments of §Biochemistry and Molecular Biology and #Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA.

Present address: †Rosetta Inpharmatics, Kirkland, Washington 98034, USA.

‡T.R.H. and S.K.E. contributed equally to this work.

Correspondence: Victoria Lundblad
E-mail: lundblad@bcm.tmc.edu

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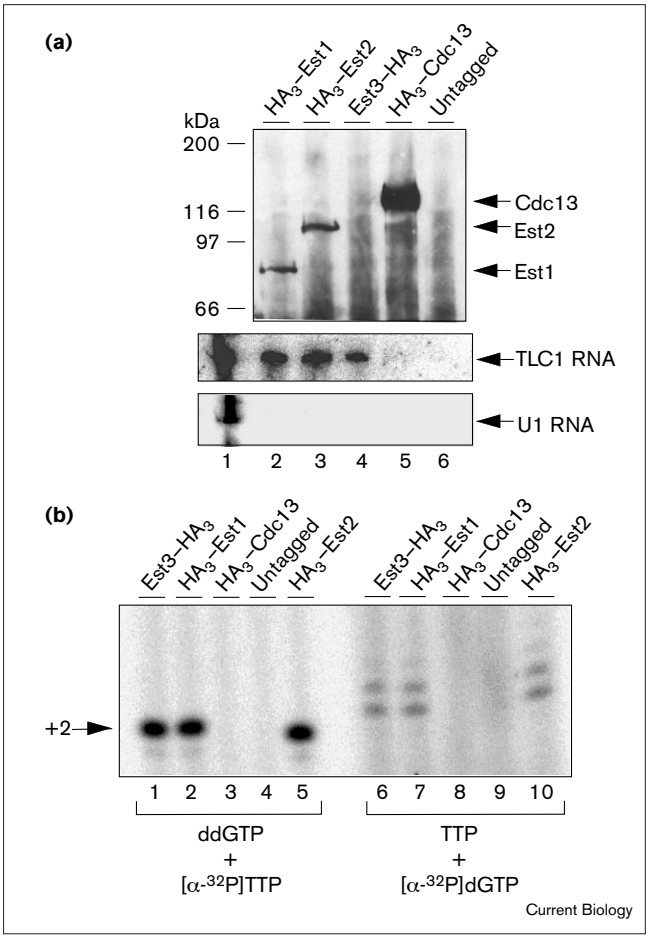
Results and discussion

To address whether Est3p is a component of telomerase, we examined whether it is associated with the TLC1 RNA and with enzyme activity, in parallel with Est1p, Est2p and Cdc13p. To avoid artifactual associations that might arise from over-expression, we constructed a set of strains containing epitope-tagged versions of each gene integrated into the respective endogenous locus under the control of the native promoter. The identical hemagglutinin $(HA)_3$ epitope was introduced into a region of each protein such that the activity of the tagged protein was not overtly affected; little or no alterations in telomere length were observed in strains bearing integrated versions of HA_3–*EST1*, HA_3–*EST2*, *EST3*–HA_3 or HA_3–*CDC13* (see Supplementary material).

Immunoprecipitates (IPs) from extracts prepared from these four individually tagged strains were assayed for the presence of the relevant recombinant protein, the TLC1 RNA subunit and telomerase activity (Figure 1). HA_3–Est1, HA_3–Est2, and HA_3–Cdc13 proteins were detected in the IPs (Figure 1a), but were not visible in the starting extracts (data not shown), presumably due to the low levels of each of these proteins when expressed under their native promoter. The TLC1 RNA was readily detectable in Est1p, Est2p and Est3p IPs by northern analysis, but was not present in IPs prepared from an untagged strain (Figure 1a). The interactions with TLC1 were specific for the telomerase RNP, since the U1 small nuclear RNP (snRNP) RNA was not present in each IP (Figure 1a).

This interaction between the Est proteins and TLC1 reflected an association with an active telomerase complex, since enzyme activity was identified in each of the Est protein IPs (Figure 1b). Two related assays, which have previously been used to monitor telomerase from *S. cerevisiae* [2,4,9], were used to assess enzyme activity. In the first assay, primer extension by telomerase was conducted in the presence of the chain-terminating ddGTP nucleotide, thereby concentrating the signal into a '+2' product and providing a sensitive means of detecting enzyme activity [2,4]. Telomerase activity was readily observed in each Est protein IP using this assay (Figure 1b, lanes 1–5). In several repetitions of this experiment, the levels of enzyme activity correlated directly with the levels of TLC1 and varied by no more than two-fold when compared among the three Est protein IPs (data not shown), as predicted if these three proteins, each containing the identical HA_3 epitope, are subunits of the same complex. Enzyme activity was also monitored using a non-terminating reaction (Figure 1b, lanes 6–10) that generates a ladder of reaction products representing the

Figure 1



Est1, Est2 and Est3 proteins associate with an active telomerase enzyme complex. **(a)** Extracts from an isogenic set of strains, each bearing the same HA_3 epitope tag on a single gene as indicated, were immunoprecipitated and analyzed by western blotting to detect HA tagged protein (top) and northern blotting to detect TLC1 RNA (middle) or U1 RNA (bottom). HA_3–Est1 (strain TVL288; lane 2); HA_3–Est2 (TVL292; lane 3); Est3–HA_3–GST (TVL293; lane 4); HA_3–Cdc13 (TVL290; lane 5); untagged (AVL78; lane 6). TLC1 and U1 RNA levels in crude extracts prepared from an untagged strain, prior to immunoprecipitation, are shown in lane 1; the amount of extract represents 4% of the amount loaded in the IP lanes. Lanes 2–6 are equivalently loaded, as assessed by total protein. The Est3–HA_3–GST polypeptide co-migrates with the antibody heavy chain (which is not shown) and is not detectable on this particular blot. **(b)** Immunoprecipitates from each tagged strain were assayed for telomerase activity with the primer substrate (5′-TGTGGTGTGTGT-GGG-3′), using either a chain terminating reaction in the presence of ddGTP (lanes 1–5; the band representing the +2 product is indicated) or non-chain terminating reaction (lanes 6–10). The strains used in this experiment are identical to those used in (a), with the exception of the Est3–HA_3 strain (TVL307). Telomerase activity was RNase A-sensitive and primer-dependent in both sets of assays (data not shown).

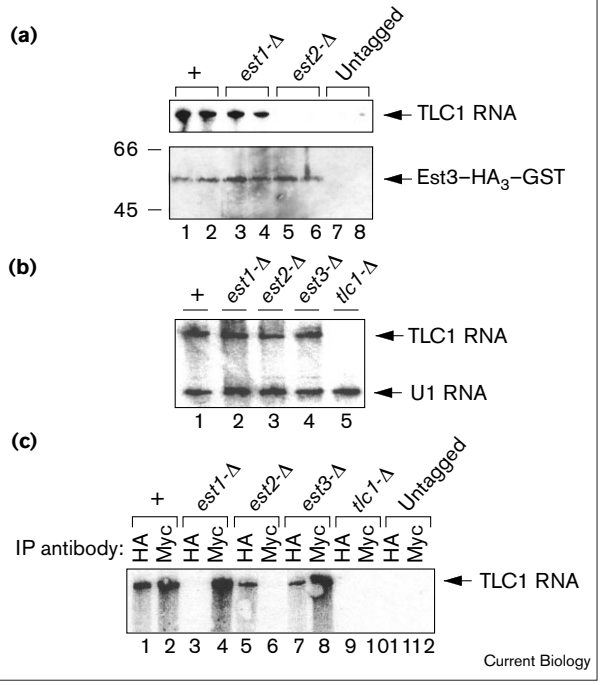
incomplete extension of a primer across the TLC1 template [9,11]. Telomerase activity was again detected in HA_3–Est1p and Est3p–HA_3 IPs at roughly the same levels as observed in HA_3–Est2p IPs (compare lanes 6 and 7 with lane 10). In both protocols, IPs were subjected to relatively stringent wash conditions (0.4 M NaCl) prior to enzyme assays. Therefore, Est3p is tightly associated with an active telomerase complex, indicating that Est3p plays its role in telomere replication as a subunit of telomerase. These experiments also show that Est1p is a stable component of the enzyme, confirming and extending previous observations which showed that Est1, when overexpressed, was associated with the TLC1 RNA [12–14].

In contrast, neither the TLC1 RNA subunit (Figure 1a) nor enzyme activity (Figure 1b) were detectable in Cdc13p IPs performed under the same high stringency conditions. Even under less stringent conditions, telomerase activity was not detected in Cdc13p IPs (data not shown). Thus, Cdc13p is not tightly associated with telomerase, although these observations do not exclude the possibility of a transient interaction.

To determine the requirements for interaction of Est3p with telomerase, we examined whether Est3p association with a TLC1-containing complex could be retained in the absence of either Est1p or Est2p. Deletion of *EST1* or *EST2* did not affect the level of Est3–HA_3–GST protein recovered from whole cell extracts on glutathione beads (Figure 2a). In addition, the steady state levels of TLC1 RNA, which were normalized to the levels of U1 RNA, were also unchanged in extracts prepared from strains that lacked either Est1p, Est2p or Est3p (Figure 2b). When immunoprecipitated, Est3p retained association with a TLC1-containing complex in a strain deleted for *EST1* (Figure 2a). However, the ability of Est3p to associate with TLC1 was abolished in the absence of the Est2p subunit (Figure 2a), indicating that an intact catalytic core is a requirement for the interaction of Est3p with the telomerase complex. Whether this reflects a direct interaction between the Est2 and Est3 proteins has not yet been determined; two hybrid tests intended to detect such an interaction have been negative so far (D.K. Morris and V.L., unpublished observation).

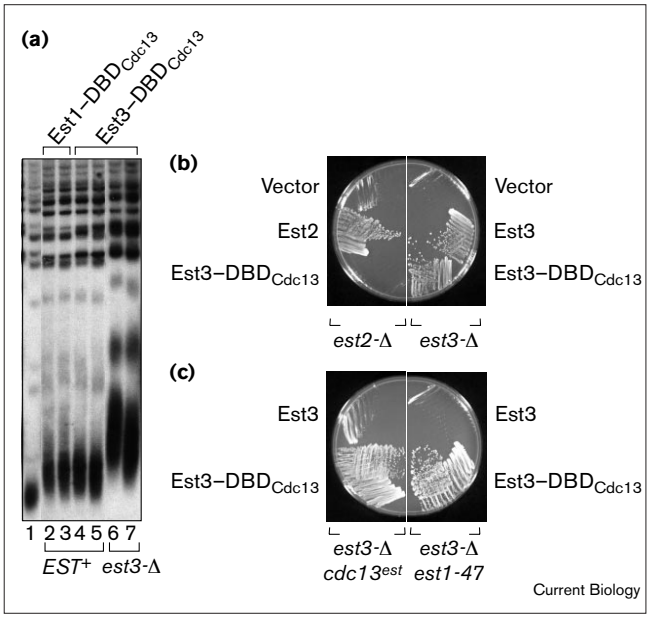
We similarly investigated the requirement for the association of Est1p with telomerase. For these experiments, we constructed a strain containing differentially tagged versions of Est1p and Est2p (HA_3–Est1p and Myc_3–Est2p) integrated into the genome and expressed under the native *EST1* or *EST2* promoters. Est3p was not required for the interaction of Est1p with the enzyme (Figure 2c), indicating that Est1p and Est3p associate with telomerase independently of each other. Unlike Est3p, however, the Est1 protein was capable of forming a TLC1-containing complex even in the absence of the catalytic Est2p subunit (Figure 2c), indicating that Est1p and Est2p interact with TLC1 independently of one another. Similar results for Est2p- and Est3p-independent association of

Figure 2



The association of Est3p with the telomerase RNP requires an intact catalytic core. **(a)** Top: extracts prepared from an Est3–HA_3–GST strain (TVL293) or deletion derivatives of TVL293 were immunoprecipitated, and TLC1 RNA was detected by northern blotting. TVL293 (lanes 1,2); *est1-Δ* derivative of TVL293 (lanes 3,4); *est2-Δ* derivative of TVL293 (lanes 5,6); untagged (AVL78; lanes 7,8). Bottom: extracts were purified on glutathione sepharose and analyzed by western blotting with an antibody to detect the HA_3-tagged Est3 protein; the GST-pulldown assay was used to determine levels of the Est3 protein, since Est3p comigrated with the immunoglobulin heavy chain when immunoprecipitated. **(b)** Extracts prepared from *est1-Δ* (lane 2), *est2-Δ* (lane 3), *est3-Δ* (lane 4) and *tlc1-Δ* strains (lane 5), in parallel with an *EST*$^+$ strain (lane 1), were analyzed by northern blotting to detect TLC1 RNA and U1 RNA, followed by quantification of the relative RNA levels by PhosphorImager analysis. The abundance of TLC1 RNA was reduced by no more than three-fold in the three *EST* deletion strains, relative to the ratio of the two RNAs in a wild-type strain. **(c)** Extracts from TVL300 (containing HA_3–Est1p and Myc_3–Est2p) or deletion derivatives of strain TVL300 were immunoprecipitated with either anti-Myc or anti-HA antibodies and analyzed by northern blotting to detect *TLC1* RNA. TVL300 (lanes 1,2); *est1-Δ* derivative of TVL300 (lanes 3,4); *est2-Δ* derivative of TVL300 (lanes 5,6); *est3-Δ* derivative of TVL300 (lanes 7,8); *tlc1-Δ* derivative of TVL300 (lanes 9,10); untagged (AVL78; lanes 11,12). The variations in TLC1 signal in HA versus Myc immunoprecipitations are due, at least in part, to the fact that the Myc_3 tag is a more efficiently immunoprecipitated epitope, as Myc_3–Est2p co-immunoprecipitates more telomerase RNA than HA_3–Est2p (data not shown).

Figure 3



The Est3–DBD_{Cdc13} fusion increases access of telomerase to the chromosome terminus. **(a)** Genomic Southern blot hybridized with a telomere-specific probe, as described previously [1]; DNA was prepared after ~50 generations of growth following plasmid transformation. *EST*$^+$ control (lane 1); *EST*$^+$/pVL1120 (Est1–DBD_{Cdc13}; lanes 2,3); *EST*$^+$/pVL1292 (Est3–DBD_{Cdc13}; lanes 4,5); *est3-Δ*/pVL1292 (Est3–DBD_{Cdc13}; lanes 6,7); all plasmids were single copy CEN vectors and expressed under the native *EST* gene promoter. **(b)** Growth after approximately 50 generations of *est2-Δ* or *est3-Δ* strains harboring either vector or single copy plasmids expressing the appropriate wild-type Est protein or the Est3–DBD_{Cdc13} fusion. **(c)** Growth after ~50 generations of an *est3-Δ cdc13-2*^{est} strain or an *est3-Δ est1-47* strain with plasmids expressing the wild-type Est3 protein or the Est3–DBD_{Cdc13} fusion. These strains expressing the Est3–DBD_{Cdc13} fusion have been propagated a further 75 generations with no signs of senescence.

Est1p with a TLC1 complex have been reported, using an over-expressed LexA–Est1 protein fusion [14].

We have previously shown that telomerase access to the telomere can be greatly augmented by fusing the high affinity DNA binding domain of Cdc13p (DBD_{Cdc13}) to Est1p, which results in substantial telomere lengthening [10]. The demonstration that Est3p, like Est1p, is also telomerase-associated predicts that fusion of the DBD_{Cdc13} to Est3p should similarly enhance telomerase access and consequently increase telomere length. Indeed, an Est3–DBD_{Cdc13} fusion, expressed under the *EST3* promoter and on a single copy plasmid, elongated telomeres in a wild-type strain, comparable to the lengthening conferred by the Est1–DBD_{Cdc13} fusion (Figure 3a). This fusion protein complemented a strain in which *EST3* had been deleted (*est3-Δ*; Figure 3b), demonstrating that the Est3 portion of the fusion was still functional. Telomere elongation was further enhanced in an *est3-Δ* strain (Figure 3a), presumably due to the absence of the competing wild-type Est3 protein. The effect on telomere length was telomerase-dependent, as the Est3–DBD_{Cdc13} fusion failed to bypass the telomere replication defect of an *est2-Δ* strain (Figure 3b). Therefore, telomerase can be delivered to the

chromosome end by fusing either of two telomerase-associated proteins, Est1p or Est3p, to the DBD_{Cdc13}. This is a specific consequence of joining a telomerase subunit to the DBD_{Cdc13}, since expression of either the DBD_{Cdc13} alone or as a fusion to two other proteins implicated in telomere length maintenance (Stn1 and Pol1) [15,16] does not result in telomere elongation (S.K.E., A. Chandra, E. Pennock and V.L., unpublished observations).

We further tested the ability of the Est3–DBD_{Cdc13} fusion to confer enzyme access in the absence of the telomerase-mediating functions of Cdc13p and Est1p. Two previously described mutations, *cdc13-2^{est}* and *est1-47*, appear to specifically compromise the ability of telomerase to access the end of the chromosome [10]. This is based in part on previous observations showing that first, the *cdc13-2^{est}* mutant phenotype can be bypassed by the Est1–DBD_{Cdc13} fusion, and second, the telomere replication defect of the Est1-47 protein can be bypassed by fusion of Est1-47p to the DBD_{Cdc13} [10]. The Est3p–DBD_{Cdc13} fusion was similarly effective in bypassing the senescence phenotype of both the *cdc13-2^{est}* and *est1-47* mutations (Figure 3c). This indicates that fusing the DBD_{Cdc13} to a telomerase-associated protein can alleviate the requirement for the telomerase accessing functions of either Cdc13 or Est1. However, the Est3–DBD_{Cdc13} fusion was not capable of rescuing the telomere replication defect of an *est1-Δ* null strain, and an Est1–DBD_{Cdc13} fusion was similarly unable to rescue an *est3-Δ* strain (data not shown). Thus, neither fusion is sufficient to bypass the complete function of the other protein.

The demonstration that the Est3 protein is a component of telomerase completes the prediction for the functions of *EST1*, *EST2*, *EST3* and *TLC1* based on PPgenetic observations. Previous epistasis analysis had shown that these four genes function in a single pathway for telomere replication, as would be expected if these genes each encode subunits of the same enzyme complex [1,2]. The work presented here, combined with previous observations, shows that all four genes encode components of telomerase. A role for the Est1 protein subunit in telomerase recruitment has been previously proposed, acting as a bridging protein between telomerase and the telomere. Additional genetic and biochemical investigation should help reveal the precise biochemical activity of the Est3 telomerase protein subunit.

Supplementary material

Supplementary material including details of strain construction and additional methodological procedures is available at http://current-biology.com/supmat/supmatin.htm.

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