Mutual Dependence of *Candida albicans* Est1p and Est3p in Telomerase Assembly and Activation

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Telomerase is an RNA-protein complex responsible for extending one strand of the telomere terminal repeats. Analysis of the telomerase complex in budding yeasts has revealed the presence of one catalytic protein subunit (Est2p/TERT) and at least two noncatalytic components (Est1p and Est3p). The TERT subunit is essential for telomerase catalysis, while the functions of Est1p and Est3p have not been precisely elucidated. In an earlier study, we showed that telomerase derived from a *Candida est1*-null mutant is defective in primer utilization in vitro; it exhibits reduced initiation and processivity on primers that terminate in two regions of the telomere repeat. Here we show that telomerase derived from a *Candida est3*-null mutant has nearly identical defects in primer utilization and processivity. Further analysis revealed an unexpected mutual dependence of Est1p and Est3p in their assembly into the full telomerase complex, which accounts for the similarity between the mutant enzymes. We also developed an affinity isolation and an in vitro reconstitution protocol for the telomerase complex that will facilitate future mechanistic studies.

Telomerase is a ribonucleoprotein (RNP) that is responsible for maintaining the terminal repeats of telomeres in most organisms (1, 3, 5, 7, 25). The catalytic core of telomerase consists minimally of two components: an RNA in which the template is embedded (named TER) and a reverse transcriptase (RT)-like protein that mediates catalysis (named TERT in general and Est2p in yeast). In addition, telomerases from different organisms have been shown to possess a number of accessory or regulatory subunits that promote telomerase RNP biogenesis and assembly. However, with few exceptions, these accessory components do not appear to participate directly in the telomere extension function of telomerase. Two notable exceptions to this generalization are Est1p and Est3p in the budding yeast *Saccharomyces cerevisiae* (12, 24). Both were identified through genetic screens and shown to act in the same pathway as telomerase RNA and TERT and to be subunits of the telomerase complex but not essential for in vitro activity. Est1p has been shown to bind telomerase RNA, telomeric DNA, and Cdc13p (a G-strand telomere-binding protein) (16, 26). Recent studies further implicate Est1p in promoting the recruitment of the telomerase complex to telomere ends in vivo (2, 9, 15). Est1p is also believed to perform a postrecruitment or activation function, but the biochemical nature of this function is obscure (8, 23). The assembly of Est3p into the telomerase complex is known to require both TERT and Est1p, but the mechanisms of this protein in promoting telomere extension are not understood (11, 14).

We recently identified putative orthologues of *TERT/EST2*, *EST1*, and *EST3* in the genome of the pathogenic fungus *Candida albicans*. Analysis of knockout strains indicates that the *Candida* homologues are all required for normal telomere maintenance. *C. albicans* EST2 was found to be essential for telomerase activity in vitro, consistent with a catalytic role for this protein (21). Unexpectedly, we uncovered a primer-specific impairment of function for the telomerase derived from an *est1Δ/est1Δ* strain in vitro. The mutant enzyme exhibited reduced abilities to catalyze nucleotide addition at two loci within the *Candida* telomere repeat (20). This represented the first instance in which a mutation in a noncore component of yeast telomerase was observed to alter the biochemical property of telomerase. In this report, we describe the development of an affinity pulldown protocol for isolating *Candida* telomerase for in vitro analysis. Using this protocol, we examined the properties of telomerase and found that the *est3Δ/est3Δ* mutant exhibited defects similar to those of the *est1Δ/est1Δ* mutant. Further analysis revealed an unexpected mutual dependence of Est1p and Est3p in their assembly into the full telomerase complex, which accounts for the primer specificity of both mutant enzymes. We propose that the previously suggested “activating” function of Est1p is due to its ability to promote Est3p assembly.

**MATERIALS AND METHODS**

*Strains.* *C. albicans* strain BWP17 (*ura3Δ::Imm434/ura3Δ::Imm434 his1Δ::hisG His1*/*hisG Arg1*/*hisG Arg1*/*hisG) was obtained from A. Mitchell (Columbia University) (27). The *Candida est1Δ/est1Δ* and *est3Δ/est3Δ* strains were constructed from BWP17 using URA-Blaster cassettes as previously described (22). For introduction of a FLAG/TAP-tagged EST2, we transformed *Candida* strains with the tagging plasmid PBS-CaEST2-TAG-URA linearized by BstEII. This plasmid was constructed as follows. First, a 3.5-kb PCR fragment containing the EST2 open reading frame (ORF) and upstream and downstream sequences was amplified by PCR from internal and *Candida* EST2-TAG-URA linearized by BstEII. This plasmid was constructed as follows. First, a 3.5-kb PCR fragment containing the EST2 open reading frame (ORF) and upstream and downstream sequences was inserted between the KpnI and PstI sites of pBluescript II KS(+) (29). The *Candida* est1Δ/est1Δ and est3Δ/est3Δ strains were constructed from BWP17 using URA-Blaster cassettes as previously described (22).

*Introduction of protein A-tagged *est1Δ*/*est1Δ* and *est3Δ*/*est3Δ* strains were constructed from BWP17 using URA-Blaster cassettes as previously described (22). For introduction of a FLAG/TAP-tagged EST2, we transformed *Candida* strains with the tagging plasmid PBS-CaEST2-TAG-URA linearized by BstEII. This plasmid was constructed as follows. First, a 3.5-kb PCR fragment containing the EST2 open reading frame (ORF) and upstream and downstream sequences was inserted between the KpnI and PstI sites of pBluescript II KS(+) (29). The *Candida* est1Δ/est1Δ and est3Δ/est3Δ strains were constructed from BWP17 using URA-Blaster cassettes as previously described (22).
EST1 and EST3, the strains were transformed with pBS-CaEST1-proA-HIS and pBS-CaEST3-proA-HIS linearized by SmaI. The plasmids were constructed as follows. First, a PCR fragment containing the EST1 (or EST3) ORF and upstream and downstream sequences was inserted between the HindIII and SalI sites of pBluescript II KS(+) (+). The C terminus of the ORF was then engineered to introduce an AfII and an NheI site. The protein A tag from pEZZ18 (GE Healthcare Inc.) was amplified by PCR and inserted between the AfII and NheI sites. Finally, a Sall-to-Apal fragment from pGEM-HIS1 (containing the HIS1 marker; a gift of A. Mitchell, Columbia University) was introduced to yield pBS-CaEST1-proA-HIS (or pBS-CaEST3-proA-HIS). Thus, each tagging plasmid contains the native gene fused at its C terminus to the tag and flanked by native 5′ and 3′ sequences. Proper integration of the tagged genes at the respective native chromosomal loci was confirmed by Southern blot analysis. Each tagged strain contains one copy of the tagged gene.

Telomere length analysis. Chromosomal DNAs were isolated from 4 to 5 ml of saturated C. albicans culture by the smash-and-grab method, digested with AluI and NlaIII, and fractionated in 0.9% agarose gels, and the telomere restriction fragments were detected by Southern blotting as previously described (21).

Purification of and assay for C. albicans telomerase. TMG(n) buffer (10 mM Tris- HCl [pH 8.0], 1.2 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol [n refers to the millimolar concentration of sodium acetate]) was used throughout the study. Whole-cell extracts of C. albicans and DEAE column fractions were prepared as previously described (20, 21). For isolation of TAP-tagged Candida telomerase on immunoglobulin G (IgG)-Sepharose beads, we followed a previously developed protocol for protein A-tagged systems. The protein was expressed using the plasmid and the TNT-coupled reticulocyte lysate system (Promega Inc.).

In vitro reconstitution of wild-type telomerase activity. For in vitro reconstitution, we used extracts derived from est2Δ/Δ and est3Δ/Δ strains. The est2Δ/est3Δ strain also carried one copy of FLAG3/TAP-tagged EST2 (in addition to two copies of untagged EST2) to facilitate purification. Approximately 3 mg each of the protein extracts was mixed at 22°C for 20 min in 1.2 ml TMG(0). The entire mixture was then subjected to IgG-Sepharose pulldown and a telomerase primer extension assay as described before (20). For in vitro synthesis of Candida Est3p, the EST3 ORF was first amplified by PCR and cloned between the BamHI and EcoRI sites of the pCITE-4a vector (Novagen Inc.) to give pCITE-CaEST3. The Candida EST1 gene encodes two CUG codons (codons 18 and 172), which are abnormally translated in this organism into Ser instead of Leu. These codons in pCITE-CaEST3 were mutated to UCG by the QuikChange protocol (Stratagene Inc.) to allow for expression of the native protein in heterologous systems. The protein was expressed using the plasmid and the TNT-coupled reticulocyte lysate system (Promega Inc.).

Analysis of the association of Est1p and Est3p with the telomerase core complex. IgG-Sepharose beads (45 µl) were pretreated with 100 µg tRNA in 1 ml TMG(0) at 4°C for 30 min to minimize nonspecific binding. After one wash in TMG(0), the beads were incubated with extracts (4.8 mg) in 1.2 ml TMG(500) and subjected to gentle rotation at 4°C for 2 h. The beads were then washed five times with TMG(800) and twice with TMG(0) and were divided into three equal aliquots. One aliquot each was subjected to Western blot analysis and a telomerase activity assay as previously described (4, 28). The last aliquot was treated with proteinase K and extracted with phenol-chloroform-isooamyl alcohol (25:24: 1), and the RNA was isolated by ethanol precipitation. The level of TER (Candida telomerase RNA) was then quantified by semiquantitative RT-PCR (2X RT-PCR master mix; USB Corporation) using primer pairs designed to amplify a 350-bp fragment (forward, 5′-CTCCATAATGCTAGTTGATGTTG-3′; reverse, 5′-CTCCACAGGTATCATCACAAATGTGG-3′). The linearity of the assay was confirmed by titrating the samples and by using different cycle numbers. The pulldown assays for EST1-proA and EST3-proA were each performed three or four times, and the results were highly reproducible.
FIG. 2. Telomerase from an est3Δ/est3Δ mutant exhibited primer-specific loss of primer extension activity in vitro. (A) Telomerases from the tagged BWP17 (wild-type [WT]) and est3Δ/est3Δ (est3-ΔΔ) strains were isolated by either DEAE chromatography or IgG-Sepharose pulldown and tested for primer extension activity using primers P6 and P20 in the presence of labeled dTTP. (B) Telomerases isolated from the tagged BWP17 and est3Δ/est3Δ strains were tested for primer extension activity using primers P3 to P23 in the presence of labeled dTTP. (C) A diagram showing the telomere repeat sequence and the RNA template used for primer extension activity.
RESULTS

Development of an affinity isolation procedure for Candida telomerase. We had earlier developed a partial purification procedure for C. albicans telomerase based on DEAE chromatography (21). However, because the procedure requires elution from the column using a high-salt buffer, it is possible that loosely bound subunits were lost. To develop an alternative purification procedure, we constructed an integrating plasmid with a URA3 marker and an EST2 gene fused at its C terminus to the FLAG$_3$ and TAP tag (17). The functionality of the tagged gene was tested by integrating the plasmid into an est2Δest2Δ strain and examining the lengths of telomeres in the resulting strain over many generations. As shown in Fig. 1A, the reconstituted strain exhibited stable telomere hybridization signals over time, in contrast to the progressive loss observed in the est2Δest2Δ mutant. While some telomeres in the reconstituted strain appear to shorten over time, this loss is apparently compensated by the emergence of long telomeres (Fig. 1A). The telomeres in the reconstituted strain were slightly shorter (lengths, ~1 to 3.5 kb) than those in the original parental strain, BWP17 (~2 to 5 kb) (21, 22). This reduction suggests that one copy of Candida EST2 may be unable to maintain telomeres in this strain background. Alternatively, tagging of the C terminus of EST2 might have resulted in partial loss of function. Interestingly, strain BWP17 was recently shown to be monozygous for EST1 due to a large chromosomal deletion (18), yet it maintains substantially longer telomeres than another commonly used laboratory strain, CA14 (6). Because of widespread aneuploidy in Candida laboratory strains, careful comparison of isogenic strains carrying different copy numbers of telomerase genes is clearly necessary to establish the dosage effect of telomerase genes on telomere length. Notably, the S. cerevisiae telomerase RNA gene was recently demonstrated to be haploinsufficient (13), providing motivation for further analysis of this issue in Candida. In any case, our results clearly show that the tagged EST2 gene is capable of complementing telomerase function in vivo.

We then examined the expression of FLAG$_3$/TAP-tagged Est2p in cell extracts by IgG-Sepharose pulldown and Western blotting. As expected, the tagged but not the untagged strain contained an ~115-kDa protein that reacted with anti-protein A antibodies (Fig. 1B). For comparison, we also analyzed concurrently the expression of a protein A-tagged S. cerevisiae Est2p and identified a protein of slightly greater mobility. The intensities of the Western blot signals suggest that the two telomerase catalytic subunits are expressed at comparable levels in S. cerevisiae and C. albicans. To determine if active Candida telomerase can be isolated on IgG-Sepharose, we subjected the treated beads to primer extension assays. As shown in Fig. 1C, beads incubated with an extract derived from the tagged strain contained much higher levels of RNase-sensitive primer extension products than those incubated with an extract from an untagged strain, consistent with specific binding of telomerase to IgG-Sepharose. Notably, some nonspecific binding of untagged telomerase to the beads can also be observed (Fig. 1C, lanes 1 and 2). This nonspecific binding can be further reduced by higher salt concentrations during the binding reaction (data not shown).

As reported previously, Candida telomerase purified by DEAE chromatography exhibits different activity levels on different primers (20). We compared the relative activities of DEAE- and IgG-Sepharose-derived telomerases using several different primers and found that they exhibited similar activity profiles, suggesting that the methods yielded telomerases of similar compositions and properties (Fig. 2A and data not shown). For the remainder of this study, we used exclusively IgG-Sepharose-bound telomerase.

Candida telomerase derived from the est3Δ/est3Δ mutant is defective in the utilization of specific primers in vitro. To examine the primer utilization properties of est3Δ/est3Δ telomerase, we adopted the approach that was used earlier for est1Δ/est1Δ telomerase (20). The wild-type and mutant enzymes were isolated from strains with FLAG$_3$/TAP-tagged Est2p by IgG-Sepharose pulldown and were subjected to primer extension assays using a series of 12-mer oligonucleotides (each with a different 3’ end) that correspond to all the

strains by IgG-Sepharose pulldown were tested for primer extension activity in vitro using a series of 12-nt primers that correspond to different permutations of the Candida telomere repeat. Pairs of representative assays are shown, and primer names are given at the top. (C) The activities obtained from assays such as those shown in panels A and B were quantified by a PhosphorImager. The ratios of wild-type to est3Δ/est3Δ telomerase activity on different primers were calculated, and the average ratios (and deviations) from two or three independent experiments were plotted (dark bars). For comparison, the results obtained for est1Δ/est1Δ (est1-ΔΔ) telomerase from an earlier study were also plotted (light bars).
different permutations of the Candida repeat (Fig. 2B). Each primer was designated by the position of the first nucleotide added. (Thus, primer P5 has a 3' end that corresponds to position 4 in Fig. 2C, and the first nucleotide added is dC at position 5.) To simplify the analysis, we assayed wild-type and mutant enzymes using just one labeled nucleotide such that only the primer 1 (or primer 2) product was generated. As shown in Fig. 2B and C, this “single-nucleotide-addition” assay revealed the existence of two regions within the telomere repeat (at positions 3 to 7 and positions 18 to 19) where the deletion of EST3 greatly reduced enzyme activity. At other positions, the loss of Est3p either had no effect or resulted in greater DNA synthesis, especially at position 23. Remarkably, this pattern of primer dependence closely mirrors that observed earlier for the telomerase derived from the est1Δ/est1Δ strain (Fig. 2C) (20).

Earlier studies of est1Δ/est1Δ telomerase revealed addition-ally an elongation or processivity defect in assays that allowed multiple nucleotide addition (20). To determine if this is true for est3Δ/est3Δ telomerase also, we tested the activities of wild-type and mutant enzymes by using an 8-nt primer to which 6 nt can be added in the presence of dCTP and dTTP (Fig. 3). These 6 nt correspond to an EST1- or EST3-responsive region in the single-nucleotide-addition assay (positions 2 to 7). Careful inspection of the banding pattern revealed not only an initiation defect (Fig. 3, compare lanes 1 and 2 with lanes 3 and 4) but also an elongation defect for the est3Δ/est3Δ mutant. Specifically, in the presence of labeled dCTP and unlabeled dTTP, the wild-type enzyme generated predominantly 1 and 3 products (lane 3), while the mutant enzyme generated predominantly +1 and +3 products (lane 5). Thus, both the initiation defect and the elongation defect of the est3Δ/est3Δ telomerase are recapitulated by the est3Δ/est3Δ telomerase.

Reconstitution of full telomerase activity from mutant extracts. The similarity in the primer extension defects of the est1Δ/est1Δ and est3Δ/est3Δ telomerases raises interesting
questions concerning the interaction between the two subunits. One possibility that could explain the similarity is that deletion of one subunit may lead to the destabilization and loss of the other. To address this possibility and to begin to develop an in vitro reconstitution protocol for *Candida* telomerase, we attempted to recover wild-type telomerase activity by mixing extracts derived from the est1Δ/est1Δ and est3Δ/est3Δ strains. To facilitate the analysis, we used the est3Δ/est3Δ strain that had been modified by the introduction of the EST2-FLAG/G/TAP allele. After the extracts were mixed, telomerase was isolated on IgG-Sepharose and subjected to primer extension assays using either an Est1- and Est3-hyperresponsive primer (P6) or a nonresponsive primer (P20) (Fig. 4A). As expected, the mutant telomerase failed to extend P6 but was quite active on P20 (Fig. 4A, lanes 1, 2, 5, and 6). Interestingly, significant activity could be detected on P6 in the mixed sample (Fig. 4A, lanes 3 and 4). This result indicates that the nondeleted component remained present in the extracts and could associate with the core components to reconstitute full telomerase activity. Notably, the inclusion of ATP in the mixing reaction did not enhance reconstitution, suggesting that assembly is not energy dependent (Fig. 4A and data not shown). We also attempted to recover wild-type telomerase by mixing partially purified DEAE fractions but were unable to detect activity on P6 in these assays (Fig. 4B). Together, our results suggest that Est1p and Est3p each remain stable in each other’s absence but that each may not associate with the telomerase complex without its partner.

To further test the requirement for functional reconstitution, we examined the ability of in vitro-synthesized Est3p to complement the est3Δ/est3Δ extract. Radioactively labeled *Candida* Est3p can be identified following a coupled transcription-translation reaction (Fig. 4C, left panel). Addition of an Est3p-containing lysate but not a control lysate) to the est3Δ/est3Δ extract followed by IgG-Sepharose pulldown resulted in a telomerase preparation that was active on P6 (Fig. 4C, center panel). In addition, the signals were proportional to the amount of Est3p lysate added (Fig. 4C, right panel). Thus, it appears that the only factor missing in the est3Δ/est3Δ extract is Est3p. The ability to incorporate in vitro-synthesized Est3p into the telomerase complex should greatly facilitate future structure-function analysis of *Candida* Est3p. As far as we are aware, these results represent the development of the first functional reconstitution assays for yeast telomerase regulatory components in vitro.

The association of Est3p with *Candida* telomerase depends on Est1p. The foregoing results suggest that in *C. albicans*, either Est1p or Est3p depends on the other regulatory subunit for incorporation into the telomerase complex. Indeed, a recent report demonstrates that in *S. cerevisiae*, the association of Est3p with telomerase is Est1p dependent, but not vice versa (14). To determine if this is also true for *C. albicans*, we generated a set of strains containing protein A-tagged Est3p and monitored its association with *Candida* telomerase RNA (TER) in the presence or absence of Est1p. Southern blot analysis revealed that the tagged *EST3* gene (EST3-proA) was capable of maintaining telomeres in the est3Δ/est3Δ background (Fig. 5), indicating that tagging did not abolish function. In addition, we found that a tagged protein of the expected size for Est3-proA was detected in extracts from transformed strains regardless of the presence or absence of Est1p (Fig. 6A). Thus, the stability of Est3p is not compromised in the absence of Est1p, in accordance with the reconstitution analysis. However, the level of Est3p-associated TER as judged by IgG-Sepharose pulldown assays was greatly reduced in the est1Δ/est1Δ strain background in three independent experiments (Fig. 6B and data not shown). Titration studies indicated that the amount of Est3p-associated TER was reduced at least 10-fold in the absence of Est1p (Fig. 6C). In contrast, the levels of TER in the starting extracts were unaffected by deletions of either *EST1* or *EST3* (Fig. 6A). The association between Est3p and TER likely reflected Est3p binding to active telomerase, because the levels of TER in the precipitates closely paralleled those of telomerase activity (Fig. 6B, compare the two panels). We conclude that, as in *S. cerevisiae*, the assembly of Est3p into telomerase in *C. albicans* is Est1p dependent. Notably, the levels of Est3-proA-associated TER and telomerase were reproducibly higher in the est3Δ/est3Δ strain background than in the BWP17 background (Fig. 6B, lanes 2 and 4). This difference was most likely due to the presence of two wild-type copies of *EST3* in BWP17, which resulted in the expression of untagged Est3p that could compete with protein A-tagged Est3p for association with telomerase.
The association of Est1p with Candida telomerase depends on Est3p. In S. cerevisiae, the association of Est1p with core telomerase is thought to depend primarily on its binding to telomerase RNA, and hence this association is not Est3p dependent (11, 14, 19). To determine if this is also the case for C. albicans, we investigated the requirement for Est1p association with telomerase by using the same strategy as that described above for Est3p. A set of strains containing protein A-tagged Est1p were generated and the association of the tagged protein with telomerase by using the same strategy as that described above for Est3p. A set of strains containing protein A-tagged Est1p were generated and the association of the tagged protein with TER in cell extracts examined. Western blot analysis indicates that the Est1-proA fusion protein was well expressed in all the strains (Fig. 7A). Moreover, telomere length analysis indicates that the tagged gene was capable of maintaining telomeres in the est1Δ/est1Δ background, indicating that tagging did not abolish function (data not shown). Surprisingly (in light of the S. cerevisiae results), we found in three independent experiments that the level of TER in the starting extracts was unaffected by deletions of either EST1 or EST3 (Fig. 7A). The complex detected between Est1p and TER likely represents active telomerase, because the levels of TER in the precipitates were proportional to those of telomerase activity (Fig. 7B & C). We conclude that, in contrast to S. cerevisiae, the assembly of Est1p into telomerase in C. albicans is Est3p dependent. Thus, the two critical regulatory subunits of Candida telomerase are mutually dependent in their association with the telomerase complex.

DISCUSSION

Est1p and Est3p are both required for full telomerase function in vitro. To our knowledge, this is the first instance where deletion of EST3 has been shown to affect the biochemical property of telomerase in vitro. Like Est1p, Est3p is required specifically for reverse transcription through two regions of the Candida telomerase RNA template. The mechanisms for this requirement are not understood, but it is tempting to speculate that the requirement reflects an important in vivo function of these regulatory factors. For example, the telomerase RNP must undergo conformational changes as it catalyzes successive nucleotide additions, because the template is dragged through the protein active site while other regions of RNA are bound by other domains of the protein. At certain template positions,
the ability of Est2p to engage in the requisite conformational change may be low and may require the function of regulatory factors. More studies are clearly necessary to understand the mechanistic basis of the stimulatory effects of Est1p and Est3p that we observed in vitro and to determine their functional relevance in vivo.

The nature of the “activation” function for Est1p. S. cerevisiae Est1p has been proposed to serve a “postrecruitment” or “activation” function based on two lines of evidence. First, even though Est2p was telomere bound in G1 as judged by chromatin immunoprecipitation, telomere extension occurred only in S/G2, when Est1p was recruited to chromosome ends (10, 23). However, because of the limited resolution of chromatin immunoprecipitation, it was unclear if recruitment of telomerase to telomere ends by Est1p in S/G2 was ruled out by this study. Second, certain mutant alleles of EST1 could not be suppressed by the artificial recruitment of telomerase (through a CDC13-EST1 fusion), suggesting that they were defective in a “nonrecruitment” function (8). Based on our earlier finding that Candida telomerase derived from est1\Delta est3\Delta strains was impaired in primer utilization, we had proposed that Est1p may directly interact with the 5’ region of substrate DNA and somehow activate the enzyme allosterically (20). This notion was consistent with the DNA-binding activity described for S. cerevisiae Est1p (26). However, the results described in this report as well as those of Osterhage et al. support an alternative hypothesis (14). Because Est1p is required for assembly of Est3p into the telomerase complex, the mutant alleles of EST1 that cannot be suppressed by artificial recruitment may in fact be defective in promoting Est3p assembly. In other words, Est3p is directly responsible for “activating” telomerase, whereas Est1p is indirectly responsible by recruiting Est3p. Further studies will be necessary to address the validity of this hypothesis for S. cerevisiae and C. albicans.

The “mutual assembly” function of Est1p and Est3p. Our observation that Candida Est3p is required for stable association of Est1p with telomerase is surprising in light of three earlier studies with S. cerevisiae (11, 14, 29), all of which reported that Est3p is dispensable for Est1p assembly. One reasonable possibility is that different tagging and isolation procedures might have affected the results. However, close scrutiny of two of the earlier reports suggests that the difference between the two yeasts may be one of degree rather than kind. Specifically, while it is not abolished, the association of Est1p with core telomerase appears to be significantly reduced by deletion of EST3 in S. cerevisiae (14, 29). Thus, we propose that the “mutual assembly” function of Est1p and Est3p may be conserved in budding yeasts. Interestingly, Est1p in S. cerevisiae is known to bind telomerase RNA (8, 19). Thus, a corollary of our proposal is that this protein-RNA interaction, while specific, must be of relatively low affinity such that it alone is insufficient to recruit all available Est1p into the telomerase complex. A plausible model for the assembly of the full telomerase complex thus entails a network of interactions between multiple telomerase components (Fig. 8). Minimally, we suggest that Est1p interacts with both telomerase RNA and Est3p, while Est3p makes contact with both Est1p and Est2p. Each contact may be of low affinity, but the total free energy of binding may be sufficient to support stable complex formation. This assembly system may have evolved to facilitate the regu-

FIG. 7. The association of Candida Est1p with active telomerase is Est3p dependent. (A) (Top) Extracts were prepared from a set of strains with or without protein A-tagged EST1 (EST1-proA). Total RNAs were isolated from the extracts and subjected to RT-PCR using primers designed to amplify a 350-bp fragment of TER. (Bottom) The same extracts were subjected to IgG-Sepharose pulldown followed by Western blot analysis using antibodies directed against protein A. (B) Extracts from the indicated strains were subjected to IgG-Sepharose pulldown. Total RNAs on the beads were isolated by proteinase K digestion and ethanol precipitation, followed by RT-PCR to detect TER. To facilitate quantitative comparison, the BWP17 EST1-proA and est1\Delta est3\Delta (est1-\Delta est3-\Delta) EST1-proA samples were subjected to threefold serial dilutions prior to RT-PCR. Thermocycling was performed for 20 or 23 cycles to ensure the linearity of the signals. (C) Est1-proA-associated telomerases from the indicated strains were isolated by IgG-Sepharose pulldown, followed by direct primer extension analysis using P20 as the primer.
lution of the enzyme. Indeed, several recent studies have demonstrated that the expression level and assembly of Est1p into telomerase are both cell cycle regulated, peaking in S phase (14, 23). Curiously, the level of Est1p in G1 is reduced only two- to threefold relative to the level in S phase, yet no Est1p-telomerase complex formation is detectable in G1. This drastic concentration dependence can be explained by the multiple low-affinity but cooperative interactions postulated by our model. A future challenge will be to reconstitute the assembly process in vitro and to rigorously determine the energetic contribution of each set of interactions between telomerase components. In this regard, the reconstitution system that we described represents a promising start.

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