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# Analysis of Telomerase in *Candida albicans*: Potential Role in Telomere End Protection

Sunitha M. Singh,[1] Olga Steinberg-Neifach,[1] I. Saira Mian,[2] and Neal F. Lue[1]*

*Department of Microbiology and Immunology, W. R. Hearst Microbiology Research Center, Weill Medical College of Cornell University, New York, New York 10021,[1] and Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720[2]*

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**Telomerase is a ribonucleoprotein reverse transcriptase responsible for the maintenance of one strand of telomere terminal repeats. Analysis of the telomerase complex in the budding yeast *Saccharomyces cerevisiae* has revealed the presence of one catalytic protein subunit (Est2p/TERT) and at least two noncatalytic components (Est1p and Est3p). The genome of the pathogenic yeast *Candida albicans* contains putative orthologues of all three telomerase components. Disruption of each homologue resulted in significant but distinct telomere dysfunction in *Candida*. Similar to *S. cerevisiae*, the *Candida EST3* disruption strain exhibits progressive telomere loss over many generations, at a rate that is consistent with incomplete replication. In contrast, telomeres in both the *Candida TERT* and *EST1* disruption strains can contract rapidly, followed by partial or nearly complete recovery, suggesting a defect in telomere "capping." We propose that these two telomerase subunits may participate in the protection of chromosomal ends in *Candida*. Analysis of telomerase-mediated primer extension in vitro indicates that only the TERT protein is absolutely essential for enzyme activity. Our results support the conservation of telomerase protein components beyond the catalytic subunit but reveal species-specific phenotypic alterations in response to loss of individual telomerase component. We also identify potential homologues of Est1p in phylogenetically diverse organisms. The Est1p sequence family possesses a conserved N-terminal domain predicted to be structurally related to tetratricopeptide repeat-containing proteins.**

Telomeres are specialized nucleoprotein structures that maintain the integrity of chromosomal termini from fusion and recombination and promote chromosomal end replication (4, 16). In most organisms, telomeric DNA consists of short repetitive sequences that are rich in G residues on the 3′-end-containing strand. These repeats are maintained by a ribonucleoprotein (RNP) known as telomerase, which acts as an unusual reverse transcriptase (RT) by using a small segment of an integral RNA component as the template to synthesize the G-rich strand of telomere repeats (23). The regulation of telomere length and telomerase activity has been shown to be pivotal in the control of cellular life span.

Components of the telomerase enzyme complex have been analyzed in a variety of organisms, including ciliated protozoa, yeast, and mammals (6, 11, 46). Two components are essential for the polymerization activity: an RNA that acts as the template, and a protein subunit that catalyzes nucleotide addition. Telomerase RNAs found in ciliated protozoa, in addition to having a short templating region, share a common secondary structure (33). Telomerase RNAs from yeast and mammals are considerably larger and, within each group, conserved structural elements can be identified based on phylogenetic and mutational analysis (9, 54). The catalytic protein subunit, generically known as telomerase RT (TERT), is a ca. 100 to 130 kDa evolutionarily conserved polypeptide that contains essential RT-like motifs (13, 34). Extensive mutagenesis analysis of TERT in a variety of systems provides compelling support for the notion that TERT mediates nucleotide addition through an RT-like mechanism (2, 5, 7, 43, 47, 56).

Several groups of telomerase-associated polypeptides have been identified by using either biochemical or genetic tools, including (i) p80 and p95 from *Tetrahymena* and related proteins in mammals; (ii) molecular chaperones such as Hsp90 and p23; and (iii) RNA-binding proteins such as La, Sm proteins, dyskerin, a Staufen homologue, and the ribosomal L22 protein (1, 12, 20, 25, 27, 30, 42, 44, 45, 52). Several of these proteins have been shown to be required for telomerase RNP formation or stability and telomere length maintenance in vivo. However, with few exceptions, these "noncatalytic" components of telomerase do not appear to participate directly in the telomere extension function of telomerase in vivo. Two notable exceptions to this generalization are Est1p and Est3p in the budding yeast *Saccharomyces cerevisiae* (28, 55). 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 dispensable for in vitro activity (10, 32). Further studies implicate Est1p in the recruitment of the telomerase complex to telomere ends in vivo (17, 48). However, the extent of evolutionary conservation for Est1p and Est3p are not known.

To broaden knowledge of telomerase components and regulation, we investigated the opportunistic fungal pathogen *Candida albicans*, a significant fraction of whose genome has been sequenced. *C. albicans* is an attractive model system for investigating telomere physiology for several reasons. First, unlike *S. cerevisiae* and *Schizosaccharomyces pombe*, whose

* Corresponding author. Mailing address: Department of Microbiology and Immunology, W. R. Hearst Microbiology Research Center, Weill Medical College of Cornell University, 1300 York Ave., New York, NY 10021. Phone: (212) 746-6506. Fax: (212) 746-8587. E-mail: nflue@med.cornell.edu.



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telomeres consist of short irregular repeats ($TG_{1-3}$), *C. albicans* possesses a regular, 23-bp terminal repeat (40). Second, the overall lengths of *C. albicans* telomeres can be greatly perturbed by changing growth conditions, a finding suggestive of novel regulatory mechanisms (40). Finally, although *C. albicans* is ordinarily the cause of mild cutaneous infections, it can engender life-threatening systemic infections in immunocompromised patients. Thus, in addition to revealing potentially interesting aspects of telomere regulation, investigating *C. albicans* telomeres may lead to the identification of useful therapeutic targets. In this report, we queried the *C. albicans* database with *S. cerevisiae* telomerase genes and identified three potential protein components of the *C. albicans* telomerase complex (named CaTERT, CaEst1p, and CaEst3p). Analysis of knockout strains indicates that CaTERT, CaEst1p, and CaEst3p are all required for normal telomere maintenance. CaTERT and CaEst1p appear to have an additional function in telomere end protection. Only CaTERT is absolutely required for telomerase activity as measured by a primer extension assay in vitro. These results indicate that at least two "noncatalytic" components of the telomerase complex are conserved between *Saccharomyces* and *Candida*. PSI-BLAST and hidden Markov model (HMM) based analyses of CaEst1p revealed sequence homologues in a phylogenetically diverse group of organisms. The N-terminal region of the Est1p family possesses a conserved domain predicted to be structurally related to tetratricopeptide repeat (TPR)-containing proteins.

## MATERIALS AND METHODS

**Strains and plasmids.** *C. albicans* BWP17 (*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*; a gift from A. Mitchell [Columbia University]) was derived from CAI4 as previously described (57).

The disruption plasmids (pBME-EST1, pBME-TERT, and pBME-EST3) were all derived from pBME101 (also a gift from A. Mitchell), which contains the UAU1 marker (15). For each disruption plasmid, two fragments (each ca. 500 to 1,000 bp in length) from the 5′ and 3′ region of the gene to be replaced were obtained by PCR and cloned upstream and downstream of the UAU1 marker of pBME101 (in between the *Kpn*I and *Xba*I and between the *Sac*II and *Sac*I sites, respectively). The precise boundaries of the PCR fragments are as follows (with the numbers designating the nucleotide residue number in relation to the first nucleotide of the putative open reading frame [ORF]): for pBME-EST1, −623 to +197 and +1757 to +2677; for pBME-TERT, +1 to +768 and +1738 to +2598; for pBME-EST3, −316 to +264 and +604 to +1584. Each disruption plasmid was designed to replace >80% of the ORF and is likely to result in a null allele.

**Transformation and selection.** *C. albicans* transformations were carried out as previously described by using pBME plasmids linearized with *Kpn*I and *Sac*I (57). Transformants were selected first on SD-Arg plates, and colonies were tested for correct integration of the UAU1 cassette by PCR. Correct integrants were incubated in YPD-Uri, grown to saturation, and plated onto SD-Arg-Ura plates. Homozygous mutant or triplication strains were identified by PCR (for CaTERT and CaEST1 disruptions) or Southern blotting (for CaEST3 disruption).

**Sequence comparison.** All sequences used in comparative analysis, except for the *C. albicans* homologues, were obtained from National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Sequence data for *C. albicans* was obtained from the Stanford Genome Technology Center website (http://www-sequence.stanford.edu/group/candida).

**Determination of telomere length.** BWP17 and disruption clones were propagated either by repeated dilution of liquid culture or by repeated restreaking of single colonies on plates. For repeated dilution, a 5-ml culture was inoculated with a single colony and grown at 30°C to saturation. A small aliquot the culture was then diluted into 5 ml of fresh medium (1:5,000- or 1:10,000-fold dilution), and the new culture grown again to saturation. For repeated restreaking, the relevant strain was streaked for the growth of well-isolated colonies on a fresh plate and incubated at 30°C for 2 days. A single colony was then picked for streaking on yet another fresh plate. The number of cell divisions was estimated to be ca. 12 to 13 for each dilution and ca. 25 for each restreaking. Chromosomal DNA was isolated by using the "smash-and-grab" protocol, digested with one or more restriction enzymes, and electrophoretically separated on a 0.7 to 0.9% agarose gel. After capillary transfer to nylon membranes, telomere-containing fragments were detected by hybridization with a 5′-end-labeled oligonucleotide containing two copies of the *C. albicans* telomere repeat (58).

**Purification of and assay for *C. albicans* telomerase.** Whole-cell extracts of *C. albicans* and active telomerase fractions were prepared essentially as previously described for *S. cerevisiae* (10, 36). Briefly, *C. albicans* cultures were grown in YPD-Uri (80 μg/ml) to an optical density of 1.0. Cells were harvested, resuspended in TMG-15(0), and lysed by vortexing with glass beads. Extracts were clarified by centrifugation, and the soluble fraction loaded onto a DEAE-agarose column. The column was washed with TMG-10(400), and active telomerase fractions were obtained by eluting the column with TMG-10(900). A typical telomerase reaction was carried out in a 30-μl volume containing the following: 10 mM Tris-HCl (pH 8.0), 2 mM magnesium acetate, ~150 mM sodium acetate (contributed by the protein fraction), 1 mM spermidine, 1 mM dithiothreitol, 5% glycerol (contributed by the protein fraction), 5 μM primer oligodeoxynucleotides, 5- to 10-μl column fractions, and combinations of labeled and unlabeled nucleotides (present at 0.2 and 50 μM, respectively). Primer extension products were processed and analyzed by gel electrophoresis as previously described (36). Signals were quantified by using a PhosphorImager system (Molecular Dynamics). For quantification of activity, the signals from all labeled and RNase-sensitive products were summed.

## RESULTS

**Analysis of the *C. albicans* database reveals three putative protein components of the telomerase complex.** BLAST searches (TBLASTN) of the *C. albicans* database using the *S. cerevisiae* Est1p, Est2p/TERT, and Est3p sequences as queries revealed the existence of a single homologue for each of the three proteins. We provisionally designated the *Candida* proteins CaEst1p, CaTERT, and CaEst3p. In pairwise BLAST comparisons, the matches have E values of $3 \times 10^{-12}$, $5 \times 10^{-39}$, and 0.004, respectively. The use of CaEst1p, CaTERT, and CaEst3p as queries also leads to the identification of the *S. cerevisiae* proteins as homologues. In the most current assembly, the *Candida* genes corresponds to ORF6.4411, ORF6.6287, and ORF6.8659 and encode proteins of 612, 867, and 210 amino acids, respectively. Taken together, these results suggest that the *C. albicans* genes are likely to be orthologues of *S. cerevisiae* telomerase genes. Metz et al. have independently identified and cloned *CaTERT* (41).

**Both CaTERT and CaEst1p are required for normal telomere maintenance in *C. albicans*.** To determine the physiologic function of CaTERT, CaEst1p, and CaEst3p, we employed a recently developed gene disruption strategy that utilizes a genetic construct named *UAU1. C. albicans* is a naturally diploid organism, requiring the disruption of two alleles in order to create a null strain. The *UAU1* construct (*ura3-ARG4-ura3*) contains an intact *ARG4* gene flanked on both sides by truncated *URA3* segments. The two *URA3* segments share a duplicated region that can recombine to yield a functional *URA3* gene, leading simultaneously to the excision of *ARG4* (Fig. 1A) (15). Thus, homozygous mutants can be obtained after a single transformation by successively selecting for $Arg^+$ and $Arg^+$/$Ura^+$ segregants. For reasons that are not clear, this procedure often resulted in the isolation of $Arg^+$/$Ura^+$ segregants that have retained a wild-type locus (despite the presence of two disrupted loci). These segregants possess three copies of a gene locus and have been referred to as triplication segregants. It was shown previously that a *UAU1* insertion in nonessential genes yields both homozygous and triplication segregants, but

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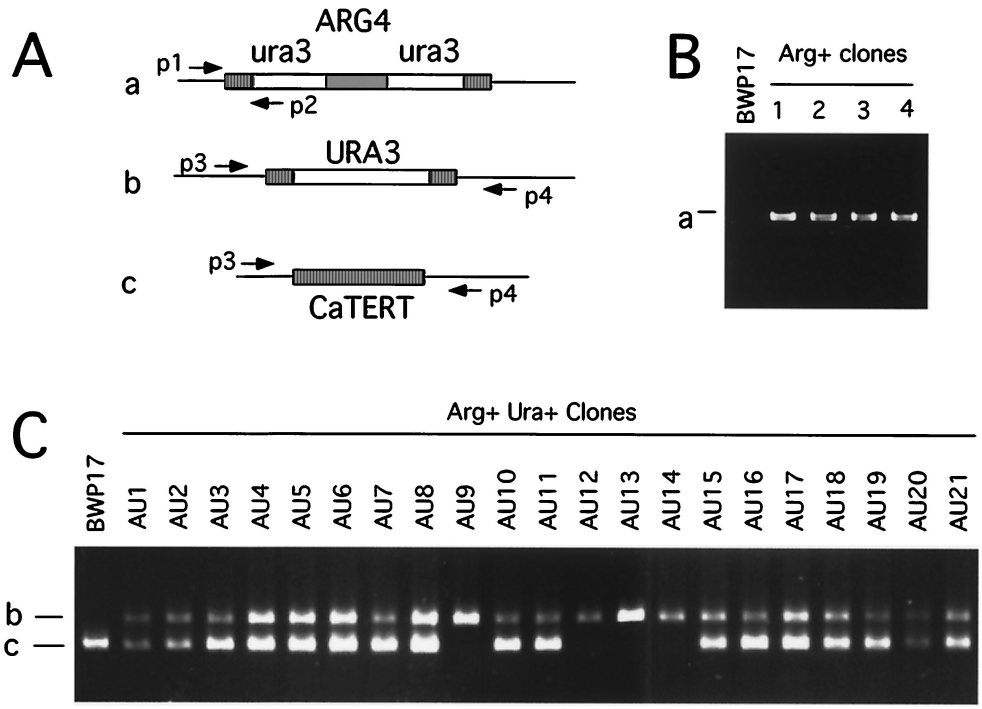


FIG. 1. Disruption of the *CaTERT* gene loci. (A) Schematic illustration of the arrangement of the locus before and after disruption and of the PCR primers used for characterizing the loci. The *URA3* and *ARG4* portions of the *UAU1* cassette are shown as open and filled horizontal bars, respectively. The regions of the *CaTERT* gene incorporated in the disruption cassette, as well as those replaced by the cassette, are shown as bars with vertical hatch marks. (B) PCR analysis of the *CaTERT* locus was performed on the BWP17 and four $Arg^+$ clones with primers (P1 and P2) designed to identify correct integration of the *UAU1* cassette (fragment a). See the upper diagram in panel A. (C) PCR analysis of the *CaTERT* locus was performed on the BWP17 and 22 $Arg^+/Ura^+$ clones with primers (P3 and P4) designed to identify the undisrupted locus (fragment c) and the *URA3*-disrupted locus (fragment b). See the middle and lower diagrams in panel A.

an insertion in essential genes yields only triplication segregants (15).

PCR fragments flanking the *CaEST1*, *CaTERT*, and *CaEST3* genes were cloned upstream and downstream of the *UAU1* marker, and the resulting cassettes used to disrupt the respective genes. After transformation and each round of selection, the relevant loci were analyzed by PCR or Southern blotting. As shown in Fig. 1B and Table 1, $Arg^+$ colonies can be easily obtained after the first round of selection, and correct integration of the *UAU1* marker occurs at high frequencies (60 to 100%). As expected, the second round of selection leads to the isolation of many $Arg^+/Ura^+$ segregants in all three disruption studies. Surprisingly, the ratios of homozygous to triplication segregants differ significantly between the disruption experiments. The ratios for the *CaTERT* and *CaEST3* experiment (~1:4 and ~2:1) are similar to that reported for other nonessential genes (~1: 2) (Fig. 1C). However, all of the $Arg^+/Ura^+$ clones from the *CaEST1* experiment were found to be homozygous mutant segregants (Table 1). The reason for the discrepancy is not understood.

TABLE 1. Summary of *UAU1*-mediated gene disruption in *Candida*

| Disruption | Frequency of correct integration after Arg selection[a] | Frequency of homozygous mutant[a] |
|---|---|---|
| *CaTERT* | 4/4 | 4/21 |
| *CaEST1* | 4/6 | 16/16 |
| *CaEST3* | 12/12 | 16/24 |

[a] Number of correct integrations or homozygous mutants/total number as indicated for each column. The state of the locus was determined by PCR (for *CaTERT* and *CaEST1* disruption) or by Southern blotting. (for *CaEST3* disruption).

To determine whether CaTERT, CaEst1p, and CaEst3p are involved in telomere maintenance, we first analyzed telomere dynamics in the parent strain (BWP17). Chromosomal DNA was treated with combinations of restriction enzymes (e.g., *Alu*I and *Nla*III) that do not recognize the *C. albicans* telomere repeats, fractionated in agarose gels, and the terminal restriction fragments (TRFs) detected in Southern assays with an oligonucleotide probe. As shown in Fig. 2A, the majority of fragments detected by the telomere probe are sensitive to exonuclease treatment of chromosomal DNA, a finding consistent with their terminal localization. As a control, the status of an internal fragment from the *CaEST1* locus was also assayed by hybridization to a specific probe. Quantitative analysis of hybridization signals indicates that the putative TRFs are at least 10 times more sensitive to degradation than the internal fragment, as predicted (Fig. 2B). The same results were obtained with other combinations of restriction enzymes, suggesting that the TRFs detected consist mostly of the terminal repeats (data not shown). Interestingly, the TRFs in the BWP17 strain have an average length of ~2 kb, considerably longer than the ca. 400- to 700-bp telomeres previously reported for the WO-1 cells grown at the same temperature (40).

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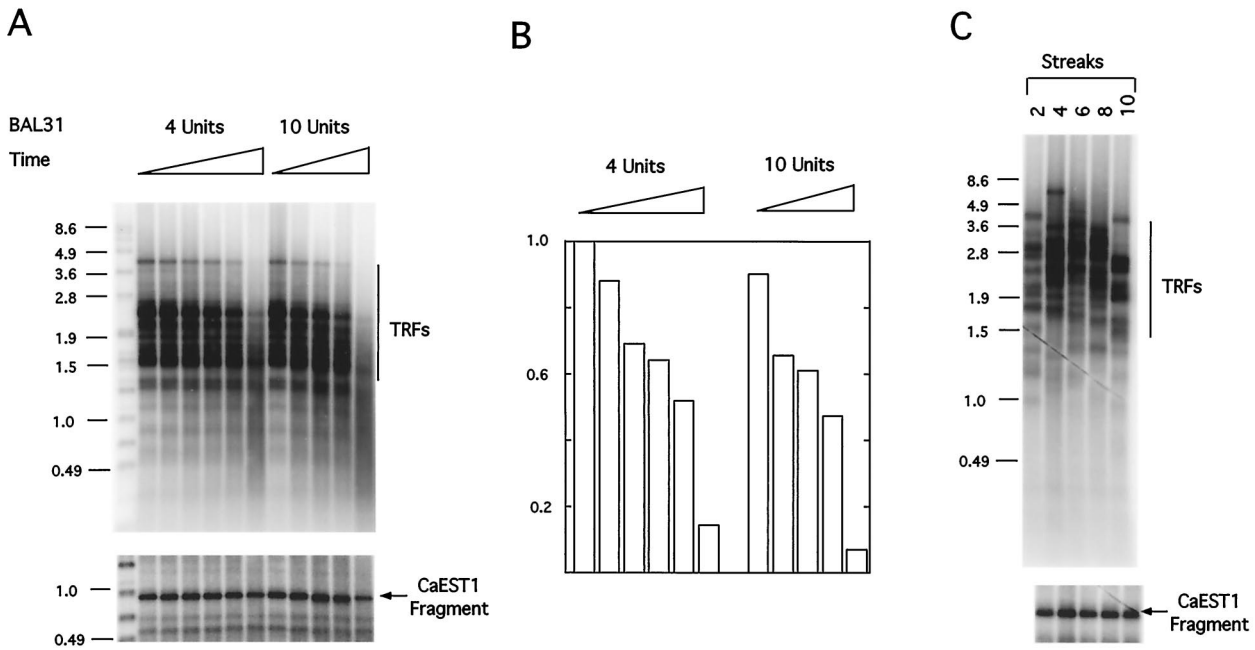


FIG. 2. Telomere length dynamics in the parent strain. (A) Chromosomal DNA was prepared from BWP17, digested with either 4 or 10 U of BAL-31 for different durations (0, 10, 20, 30, 50, 100, 0, 10, 20, 30, and 70 min for the samples from left to right), further digested with *Alu*I and NlaIII, fractionated on a 0.7% agarose gel, and probed with either a telomere repeat oligonucleotide (upper panel) or a PCR fragment from the *CaEST1* locus (lower panel). The positions of the telomere TRFs and the *CaEST1* restriction fragment are indicated by a vertical line and an arrow, respectively. (B) The ratios of the hybridization signals for TRFs to those for the *CaEST1* fragment from panel A were quantified and plotted. (C) BWP17 cells were restreaked multiple times on YPD-Uri plates. Chromosomal DNAs were then prepared from five different streaks, digested with *Alu*I and *Nla*III, and analyzed as in panel A.

We then investigated the dynamics of the TRFs in the wild-type strain over many generations to obtain the basis for comparison with telomerase-deficient strains. For these analyses, clones were propagated either by repeated restreaking of single colonies on plates or by repeated dilution of liquid culture. The first method is more sensitive at detecting changes in a small fraction of cells, whereas the second method is more reflective of telomere dynamics in a population of cells. Interestingly, with the restreaking method, the sizes of the TRFs were found to be quite dynamic such that it is not possible to follow the fate of a specific TRF over many generations (Fig. 2C). In contrast, with the dilution method, telomeres are more heterogeneous in size, but their length distribution is more stable overtime (data not shown and Fig. 3B and 4A). The dynamics of *Candida* TRFs suggests that that telomerase may carry out very selective extension of individual telomeres or that recombination may be somewhat active at chromosome termini. Despite the telomere size heterogeneity and size change over time, the overall intensity of hybridization signal vary by $<20\%$ when standardized against a control fragment (Fig. 2C and data not shown), indicating that the total amount of telomere repeats is relatively stable.

We then analyzed homozygous mutants and some triplication strains for alterations in telomere lengths. Because in most organisms, loss of telomerase function leads to progressive telomere shortening (rather than immediate telomere loss), we passaged individual clones for extended periods by using either the restreaking or dilution method and tested telomere lengths at different time points. The results obtained by either method are similar except that more discreet telomere fragments and more dynamic telomere behaviors can be observed in the restreaking samples. In terms of cell division, each dilution represents ca. 12 to 13 generations of growth, whereas each restreaking represents ca. 25 generations. As shown in Fig. 3A, analysis of two homozygous Δ*CaTERT* mutant clones indicates significant telomere loss. Quantification of the telomere hybridization signals against internal controls revealed substantial loss in the two knockout strains even after the second streak. As much as 75% of telomere repeats are lost in the homozygous mutant strain up to the 10th streak (Fig. 3A). Further passages did not result in additional reduction in average telomere lengths or loss of telomeric hybridization signal (data not shown). Instead, the telomere size appears to fluctuate around a shorter average than that in wild-type cells. Importantly, the loss of telomeres does not appear to be uniform or progressive over time, as one would have expected based on studies in *S. cerevisiae*. Although slow-growing colonies can occasionally be observed, none of the clones exhibited a consistent senescence phenotype as reported for the *S. cerevisiae est2*Δ strains (data not shown). Taken together, these results suggest that in *Candida*, an active recombination machinery is able to maintain telomeres in the absence of telomerase and prevent the progressive loss of telomeres prior to the onset of senescence.

The homozygous Δ*CaEST1* mutant clones showed milder telomere length defects upon repeated passage. DNAs prepared from some passages showed sudden telomere loss, similar to the aforementioned Δ*CaTERT* clones. However, subse-

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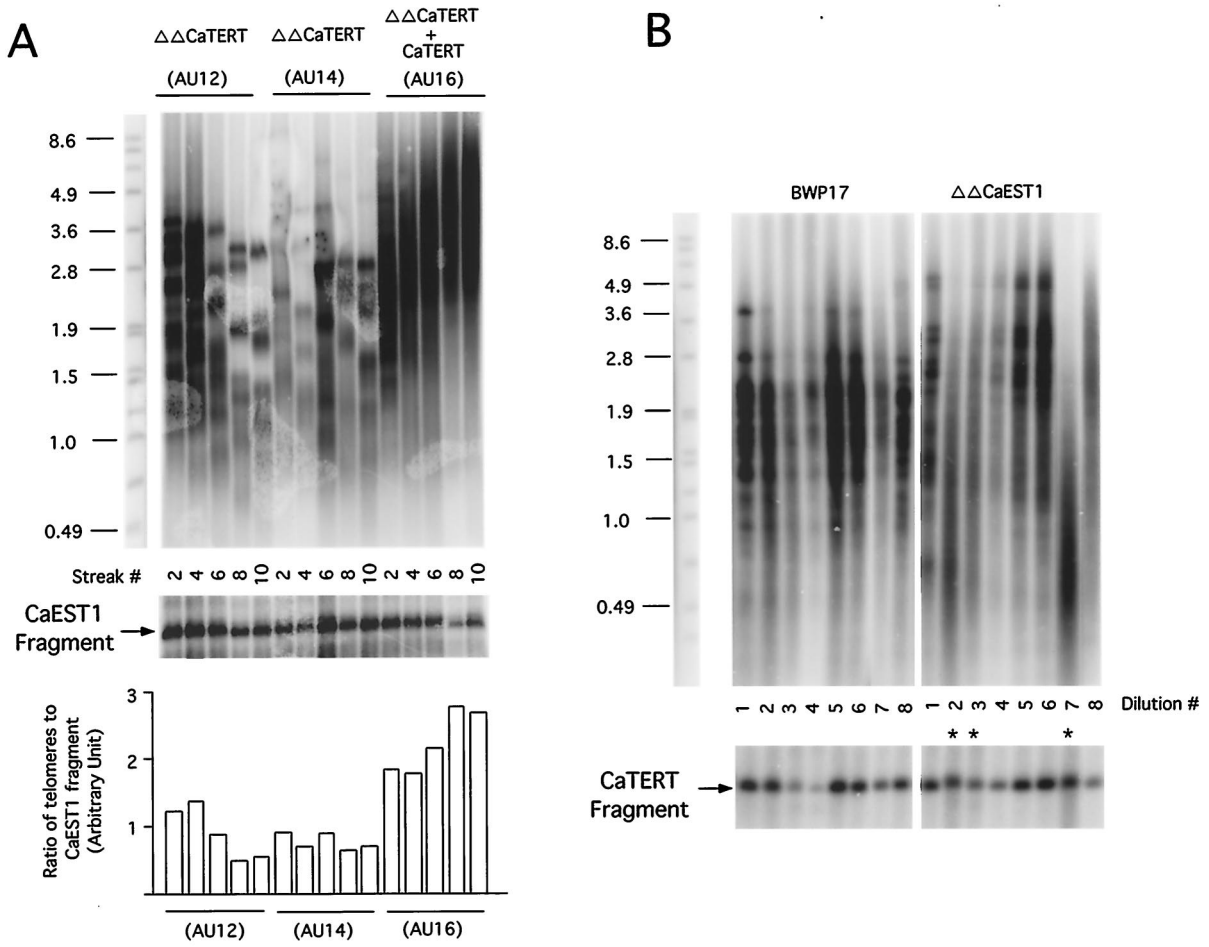


FIG. 3. Telomere length dynamics in *CaTERT* and *CaEST1* disruption clones. (A) Chromosomal DNA was prepared from homozygous *CaTERT* knockout (Δ*CaTERT*) or triplication (Δ*CaTERT*+*CaTERT*) strains after multiple restreaks on plates, digested with *Alu*I and *Nla*III, and analyzed for TRFs as described in Fig. 2. As a control for the amount of DNA loaded, the blot was stripped and reprobed for a fragment at the *CaEST1* locus. The numbers of streaks before DNA isolation are indicated in between the two panels. The ratios of the hybridization signals for TRFs to those for the *CaEST1* fragment are quantified and plotted at the bottom. (B) Chromosomal DNA was prepared from the parent strain (BWP17) and a homozygous *CaEST1* knockout strain (Δ*CaEST1*) after multiple 1:5,000 dilutions in liquid culture, digested with *Alu*I and *Nla*III, and analyzed for TRFs as described in Fig. 2. As a control for the amount of DNA loaded, the blot was stripped and reprobed for a fragment at the *CaTERT* locus. It is evident that some of the apparent variations in the intensities of TRFs can be ascribed to the amount of DNA applied to the gel. The numbers of dilutions before DNA isolation are indicated at the bottom. The DNA samples that show apparent telomere shortening are marked by asterisks.

quent streaks often manifested nearly complete recovery of telomere lengths and hybridization signals. For example, for one of the clones, DNAs prepared from the second, third, and seventh dilution had shorter telomeres, whereas those from other dilutions had nearly wild type-length telomeres (Fig. 3B). The apparent variation in the intensity of hybridization was due to differences in the amount of DNA loaded (as measured by reprobing the blot for a chromosomal fragment within the *CaTERT* gene) (Fig. 3B). Analysis of other clones gave similar results. Thus, it appears that, in contrast to *S. cerevisiae*, the loss of CaEst1p has less effect on telomere maintenance in vivo than the loss of CaTERT.

Interestingly, the homozygous Δ*CaEST3* mutant clones exhibited yet another distinct telomere phenotype, namely, steady shortening over many generations without evidence of sudden loss (Fig. 4). This behavior was apparent in both the restreak and dilution samples. Furthermore, analysis of the size of multiple telomere fragments over time (indicated by arrows in Fig. 4) consistently yielded the contraction rate of ca. 3 to 4 bp/generation, a finding very much in accord with studies of other budding and fission yeasts that have suffered loss of telomerase. Overall, these results suggest that in Δ*CaEST3* clones telomeres are lost entirely due to incomplete replication, but that in Δ*CaTERT* and Δ*CaEST1* clones other processes may intervene that further contribute to the loss.

**Only CaTERT is essential for telomerase activity in vitro.** The polymerization activity of telomerase can be assayed in vitro when it is provided with single-stranded telomere oligonucleotide primers and nucleotide triphosphates. Earlier studies suggest the telomerase complex from budding and fission yeast can be consistently isolated in high-salt eluates from DEAE columns. We therefore tested a comparable fraction prepared from *C. albicans* for its ability to catalyze nucleotide addition in "primer extension" reactions. The *Candida* telome-

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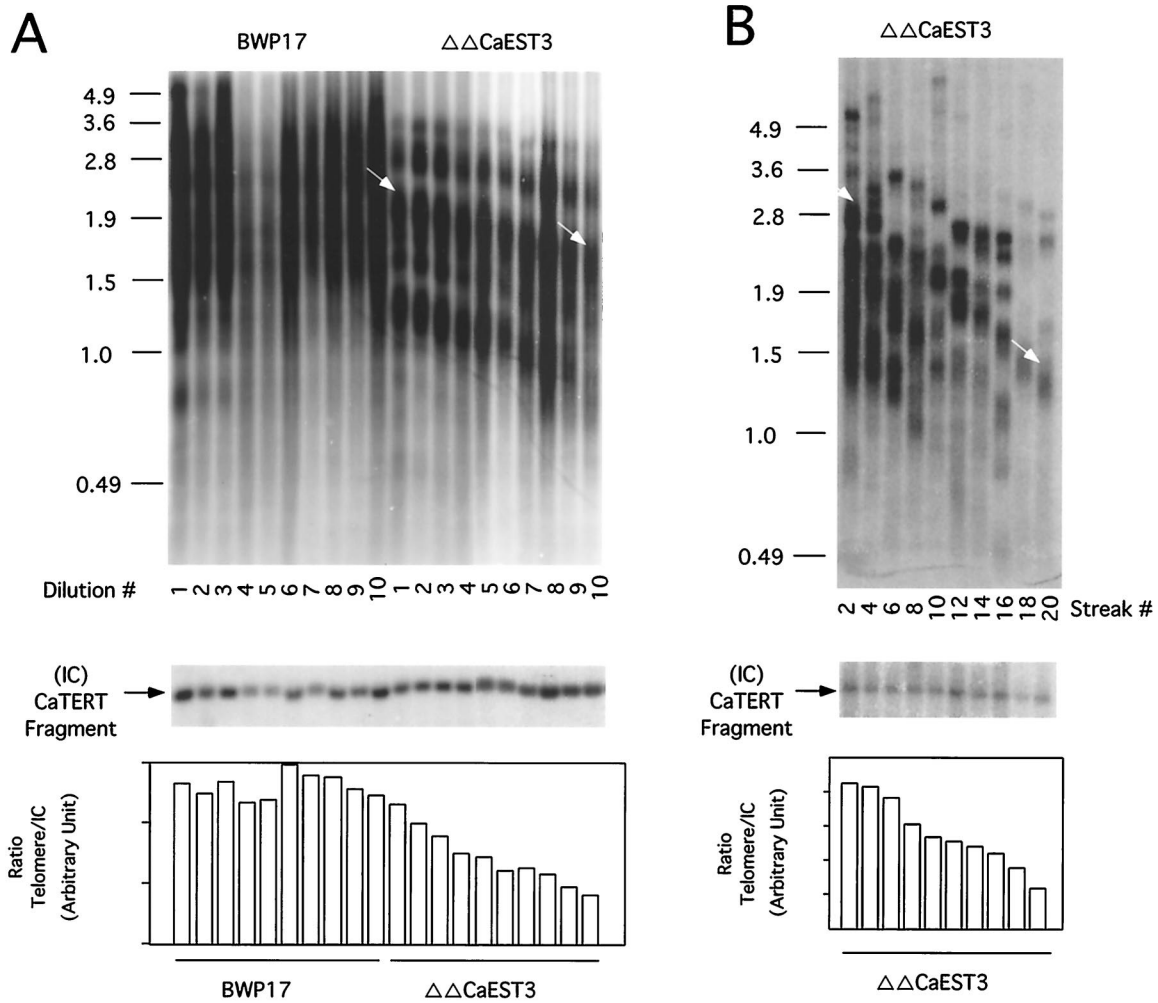


FIG. 4. Telomere length dynamics in *CaEST3* disruption clones. (A) Chromosomal DNA was prepared from the parent strain (BWP17) and a homozygous *CaEST3*-knockout strain (ΔΔ*CaEST3*) after multiple 1:10,000 dilutions in liquid culture, digested with *Alu*I and *Nla*III, and analyzed for TRFs as described in Fig. 2. The telomere fragments used for calculation of telomere shortening rate are indicated by arrows. As a control for the amount of DNA loaded, the blot was stripped and reprobed for a fragment at the *CaTERT* locus. The numbers of dilutions before DNA isolation are indicated in between the two panels. The ratios of the hybridization signals for TRFs to those for the *CaTERT* fragment were quantified and are plotted at the bottom of the figure. (B) Chromosomal DNA was prepared from a homozygous *CaEST3* knockout strain (ΔΔ*CaEST3*) after multiple restreaks on plates, digested with *Alu*I and *Nla*III, and analyzed for TRFs as described in Fig. 2. The telomere fragments used for calculation of telomere shortening rate are indicated by arrows. As a control for the amount of DNA loaded, the blot was stripped and reprobed for a fragment at the *CaTERT* locus. The numbers of restreaks before DNA isolation are indicated between the two panels. The ratios of the hybridization signals for TRFs to those for the *CaTERT* fragment were quantified and are plotted at the bottom of the figure.

rase RNA sequence has not been reported but, based on analysis of other budding yeast telomerase, is likely to contain a segment that is capable of encoding slightly more than one copy of the telomere repeat unit. For illustrative purposes, we show a hypothetical RNA template segment in Fig. 5A. (Although the precise permutation of the hypothetical template sequence may differ from the correct one, the sequences added by telomerase should be as illustrated.) Two 12-nucleotide primers designed to align to different regions of the template were incubated with DEAE fractions in the presence of different combination of nucleotide triphosphates, and the labeled and extended products were analyzed by gel electrophoresis and visualized with a PhosphorImager. A primer extension activity can be readily identified in these assays. Similar to telomerase from other budding and fission yeast, the *Candida* activity is relatively nonprocessive, catalyzing the addition of only a few nucleotides to the starting primer even when all four deoxynucleotides are present (10, 19, 35). As expected for telomerase, the activity is abolished by RNase A pretreatment and synthesizes a defined sequence corresponding to the *Candida* telomere repeat (Fig. 5B). For example, for primer 1, up to two nucleotides are added to the starting primer in the presence of dTTP alone, whereas in the presence of dTTP and ddGTP, a third nucleotide can be added (Fig. 5B, lanes 2, 3, 5, and 6). With the combination of dTTP, dGTP, and ddATP, up to eight nucleotides can be incorporated, again as predicted (Fig. 5B, lane 8). Two observations were somewhat unanticipated. First, the addition of dCTP to the other three nucleotides did not result in significant extension beyond the "primer+8" position (Fig. 5B, lane 1), suggesting that

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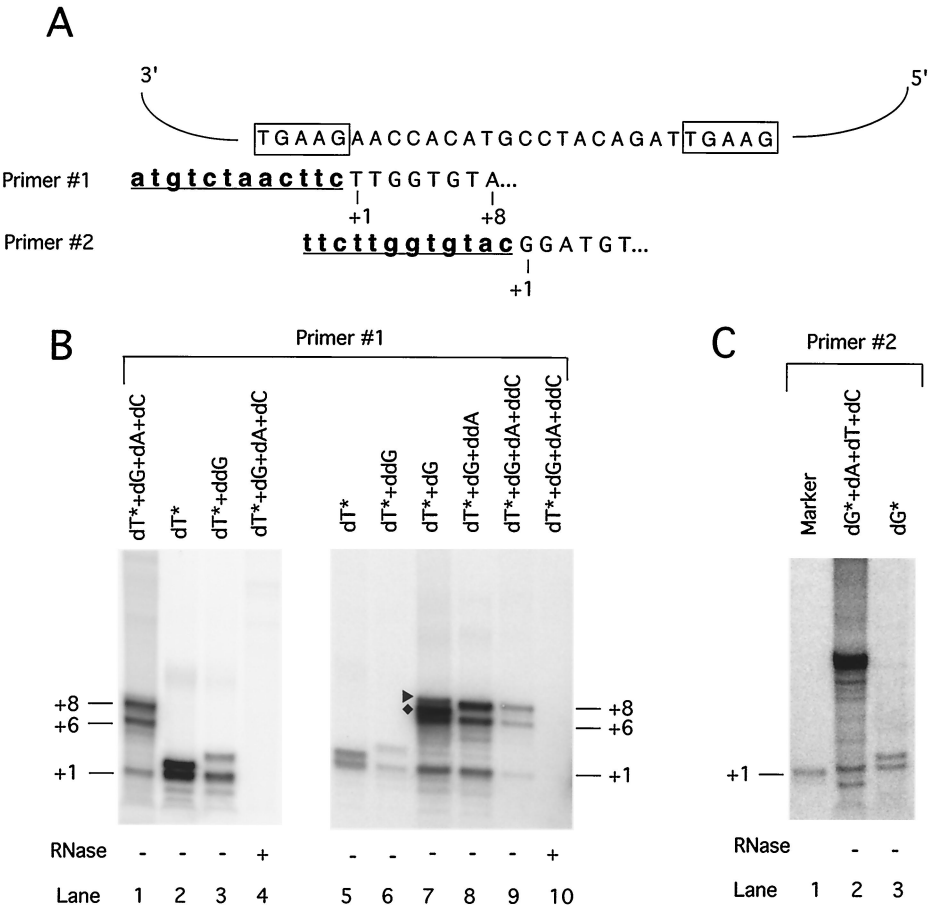


FIG. 5. Development of a primer extension assay for *C. albicans* telomerase. (A) A hypothetical model for the *Candida* telomerase RNA template region and its interaction with two primer oligonucleotides is shown. The duplicated regions at the 3′ and 5′ end of the template are shown in rectangular boxes. The sequences that are expected to be added by telomerase are also illustrated. (B) DEAE fractions are prepared from BWP17 and tested for their ability to extend primer 1 by using different combinations labeled (indicated by an asterisk) and unlabeled deoxynucleotide triphosphates as indicated at the top. Some fractions were pretreated with RNase prior to the assay as indicated at the bottom. The primer+7 and primer+8 product for the dTTP+dGTP reaction are indicated by a diamond and a triangle, respectively. (C) DEAE fractions are prepared from BWP17 and tested for their ability to extend primer 2 by using different combinations labeled (indicated by an asterisk) and unlabeled nucleotide triphosphates as indicated at the top.

telomerase has a tendency to pause or terminate at this position. (The lack of a "primer+9" band in the dTTP+dGTP+dATP+ddCTP reaction can be similarly rationalized [Fig. 5B, lane 9].) Second, the combination of dTTP and dGTP yielded a primer+8 product, which is one nucleotide longer than anticipated (Fig. 5B, marked by a triangle). This may be due to "slippage" of the enzyme relative to the RNA template such that the active site reutilized an rC or rA residue as a template for nucleotide addition. That the primer+8 product was somewhat anomalous was consistent with the presence of a strong primer+7 product in this assay (Fig. 5B). Enzyme "slippage" has also been observed in the case of *Saccharomyces* telomerase (10, 50). For primer 2, with a different 3′ end, up to two nucleotides can be added in the presence of dGTP alone, again as predicted based on the *Candida* telomere repeat. Taken together, these results indicate that our primer extension protocol can be used to measure the activity of *C. albicans* telomerase.

DEAE column fractions were prepared from both a homozygous Δ*CaTERT* mutant and a triplication clone and then tested in primer extension assays. As shown in Fig. 6B, the triplication fraction had robust primer extension activity, whereas the homozygous mutant fraction was completely devoid of activity. The use of fractions from other homozygous and triplication clones and other telomeric primers gave identical results. Furthermore, mixing experiments ruled out the presence of inhibitors in the mutant fraction (Fig. 6B). CaTERT therefore appears to be essential for the polymerization activity of telomerase, a finding consistent with its catalytic role in DNA synthesis.

In contrast, extracts from several independent Δ*CaEST1* and Δ*CaEST3* clones were found to be capable of mediating primer extension in our assay. As shown in Fig. 6C and D, parallel analysis of wild-type and mutant fractions with two different primers did not reveal large differences in the level of DNA synthesis. Altering primer concentrations or the amounts of extracts also failed to uncover large differences in the level of DNA synthesis (Fig. 6C and data not shown). These observa-

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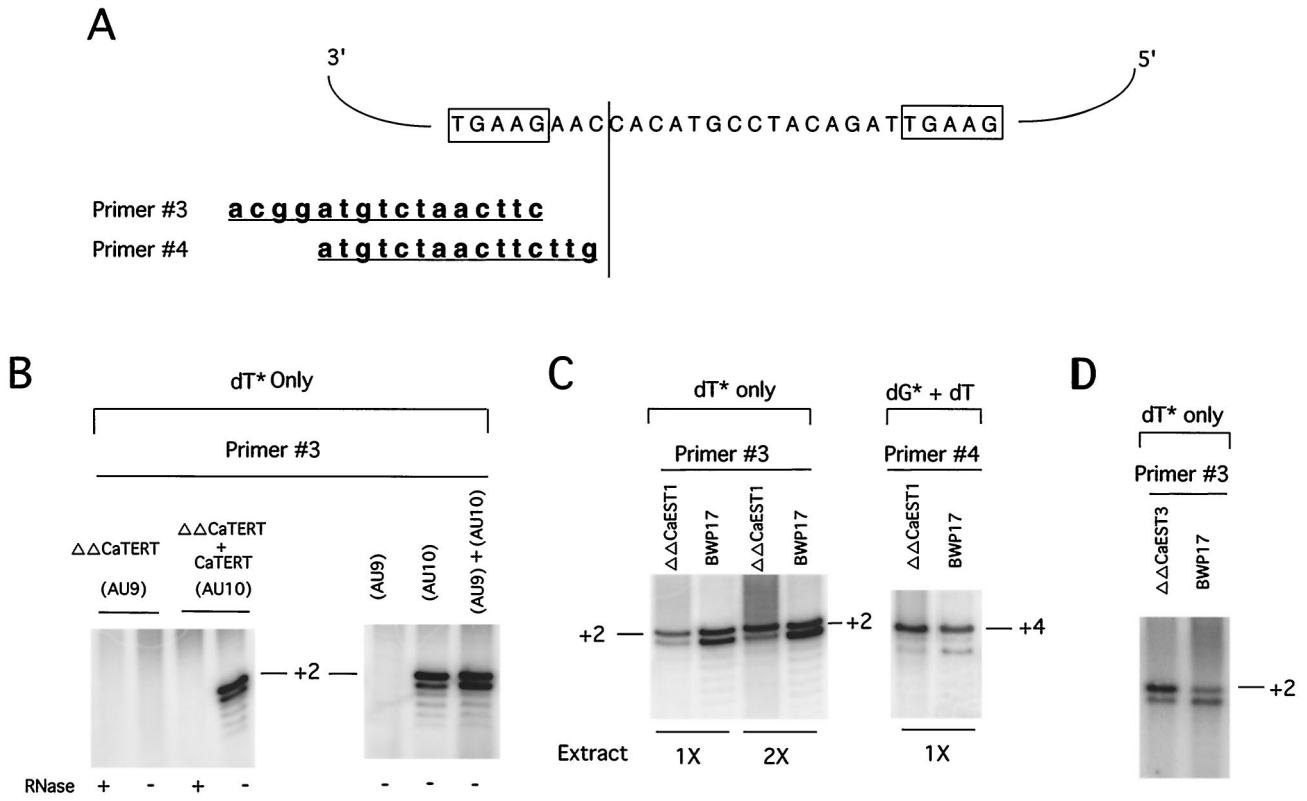


FIG. 6. Telomerase activity assays for mutant strains. (A) Hypothetical model for the *Candida* telomerase RNA template region and its interaction with two primer oligonucleotides. The duplicated regions at the 3′ and 5′ end of the template are shown in rectangular boxes. (B) DEAE fractions were prepared from a homozygous *CaTERT* knockout strain (AU9, Δ*CaTERT*) and a triplication strain (AU10, Δ*CaTERT*+*CaTERT*) and tested for their ability to extend primer 3 in the presence of labeled dTTP alone. Some fractions were pretreated with RNase prior to the assay as indicated at the bottom. A mixing experiment was also done to investigate the potential existence of inhibitors in the AU9 fraction. (C) DEAE fractions are prepared from BWP17 and a homozygous *CaEST1* knockout strain (Δ*CaEST1*), and tested for their ability to extend primers 3 and 4 by using different combinations of labeled (indicated by an asterisk) and unlabeled nucleotide triphosphates as indicated at the top. For primer 3, the assays were performed with either 5 μg (once) or 10 μg (twice) of protein fraction, with very similar results. (D) DEAE fractions are prepared from BWP17 and a homozygous *CaEST3* knockout strain (Δ*CaEST3*) and tested for their ability to extend primer 3 with labeled dTTP.

tions support the notion that CaEst1p and CaEst3p are not absolutely essential for the polymerization activity of telomerase, a finding consistent with earlier studies in *S. cerevisiae*.

**The presence of Est1p-like but not Est3p-like proteins in phylogentically diverse organisms.** The phenotypic defects of the Δ*CaEST1* and Δ*CaEST3* mutant suggest that these proteins may perform a conserved function in telomere maintenance. We used CaEst1p as the query for a PSI-BLAST search and identified sequence homologues in organisms ranging from *S. pombe* to *Neurospora crassa* to humans (Fig. 7B). Not all of these homologues are likely to be Est1p orthologues in light of the existence of *Saccharomyces* Ebs1p, an Est1p-like protein that has a lesser role in telomere maintenance (60). Using a combination of PSI-BLAST and HMMs, we defined and characterized a conserved domain in the N-terminal region of members of the Est1p sequence family. We used an HMM for this conserved Est1p domain to search a database of protein sequences of known and unknown three-dimensional structures. The results revealed similarity to several TPR-containing proteins including human Pex5p (a peroxisome receptor) and Ogt1 (UDP-*N*-acetylglucosamine-peptide *N*-acetylglucosaminyl transferase) (Fig. 7B). Since the alpha-helical TPRs are typically part of modules involved in protein-protein interactions (14, 21), the conserved Est1p TPR domain may perform a similar function.

In contrast to Est1p, additional homologues of Est3p can only be identified in closely related fungi such as *Saccharomyces paradoxus*, *Saccharomyces servazzii*, and *Zygosaccharomyces rouxii* (data not shown). In addition, unlike the *S. cerevisiae EST3* gene, which requires a translational frameshift for the synthesis of full-length protein, the *Candida* homologue comprises a single continuous ORF. These results suggest that if Est3p is conserved in distantly related organisms, then the degrees of sequence similarity must be very low.

## DISCUSSION

**The interplay between telomerase and recombination at *Candida* telomeres.** Studies in a number of systems have revealed at least two major mechanisms for telomere maintenance: a telomerase-based pathway and a recombination-based pathway (37, 53). In telomerase-positive cells, telomerase appears to be the preferred mechanism. However, depending on the particular system, recombination at telo-

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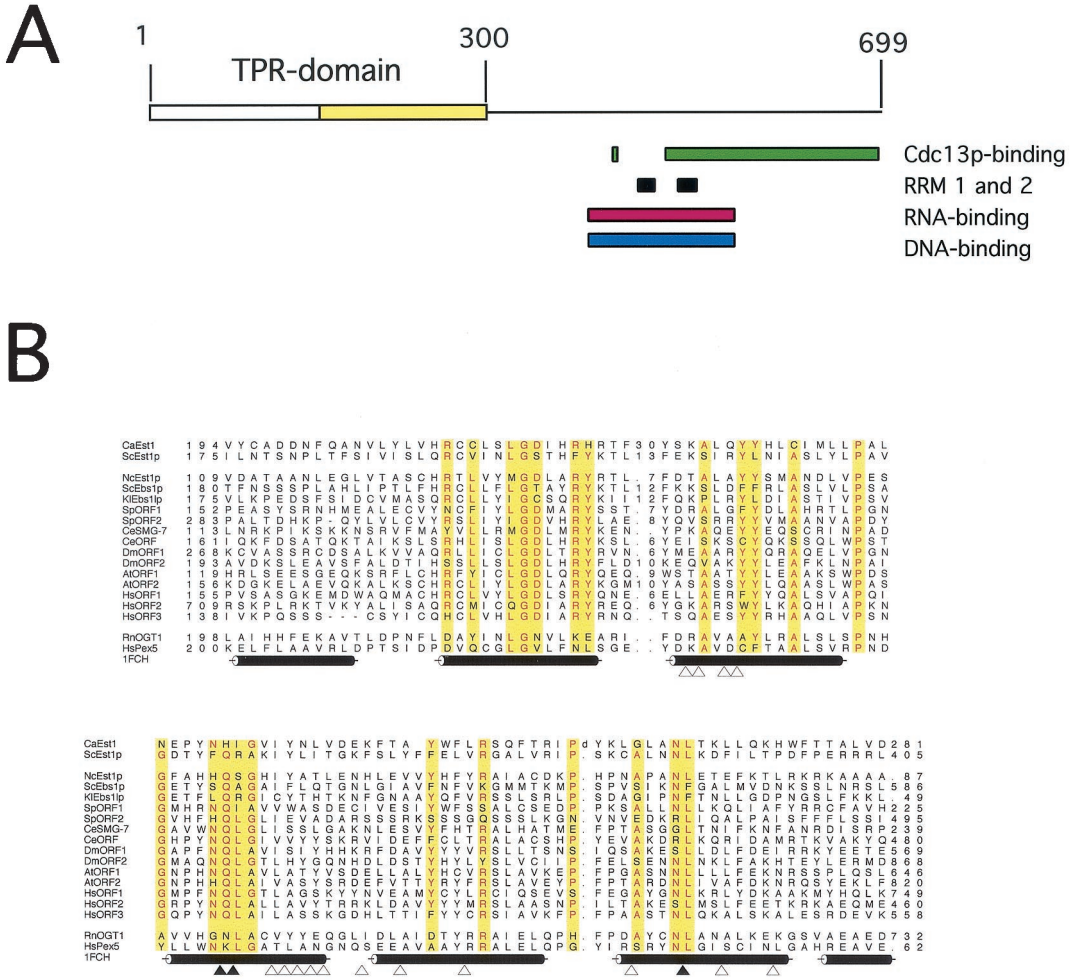


FIG. 7. Organization of Est1p and alignment of Est1p homologues. (A) A schematic diagram of ScEst1p illustrating its domain organization and regions with defined biochemical activity (48, 51, 55, 60). The conserved N-terminal TPR domain present in the Est1p sequence family consists of two tandem sets of TPRs, each being roughly 100 amino acids in length and similar at the amino acid sequence level. The second and more conserved half of the TPR domain is shown in yellow. (B) HMM-generated multiple sequence alignment of the region depicted in yellow in panel A. Conserved residues are shown in red on a yellow background. Sequences labeled CaEst1 to HsORF3 are members of the Est1p sequence family, whereas RnOGT1 (rat O-GlcNAc transferase) and HsPex5 (human peroxin 5) are TPR-containing proteins. For HsPex5, cylinders denote the locations of alpha-helices in the three-dimensional structure, triangles mark amino acids that interact with the peptide ligand, and filled triangles indicate residues conserved in the Est1p family (note that HsPex5 aligns well with the entire Est1p TPR domain and not just the region shown). Numbers to the left, right, and in the middle denote the numbers of amino acids not shown. Protein phosphatase 5 (RCSB 1A17) and Hop (1ELW), two other TPR motif-containing proteins of known structure, align well with the region shown but are not displayed for clarity. The sequences depicted are as follows: CaEst1, *C. albicans* Est1; ScEst1p, *S. cerevisiae* Est1p (databank code NP_013334); NcEst1p, *N. crassa* Est1-like ORF (contig 1.442); ScEbs1p, *S. cerevisiae* EST1-like bcy1 suppressor Ebs1 (NP_010492); KlEbs1lp, *Kluyveromyces lactis* putative EBS1-like protein (AF324496_1); SpORF1, *S. pombe* SPBC2D10.13 (T40116); SpORF2, *S. pombe* C2F12.03C (YB33_SCHPO); CeSMG-7, *Caenorhabditis elegans* smg-7 (NP_501033); CeORF, *C. elegans* Y54F10AL.2 (NP_497566); DmORF1, *Drosophila melanogaster* CG6369 (AAF56380); DmORF2, *D. melanogaster* anon-34Ea (AE003406_38); AtORF1, *Arabidopsis thaliana* At1g28260 (NP_174147); AtORF2, *A. thaliana* At5g19400 (NP_197441); HsORF1, *Homo sapiens* KIAA1089 (XP_044148); HsORF2, *H. sapiens* C17orf31 (XP_029470); HsORF3, *H. sapiens* AK056035 (BAB71079); RnOGT1, *Rattus norvegicus* UDP-*N*-acetylglucosamine-peptide *N*-acetylglucosaminyltransferase 100-kDa subunit (OGT1_RAT); HsPex5/1FCH, *Homo sapiens* Pex5 TPR region in complex with peroxisomal targeting signal-1 (RCSB 1FCH).

meres can either be repressed in such cells or be coexistent (18, 22, 49). When telomerase is disrupted, continued growth of cells in culture leads to the selection of those that have become active in telomere recombination, although the efficiency of such activation events appears to vary from system to system (29, 38, 39, 53). In short, the interplay between telomerase and recombination at telomeres is probably complex and somewhat species specific.

Two of our observations suggest that, at *Candida* telomeres, recombination may play a greater role than usual. First, even in wild-type cells, the TRFs of *Candida* showed significant size heterogeneity and variability such that it is often difficult to track the behavior of a single telomere over time. Second, we were unable to detect an obviously senescent phenotype (progressive loss of cell viability) in any of the telomerase knockout strains. It is unlikely that senescent cells arose early enough

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(e.g., within the first one or two streaks) to avoid detection by our analysis, given that the average size of the telomeres are ~2 kb and that shortening occurred at a rate of 3 to 4 bp per generation in Δ*CaEST3* clones (Fig. 4). It is tempting to suggest that either recombination was already somewhat active in wild-type cells or that short telomeres easily become recombinogenic in *Candida* spp. Clearly, to address these possibilities, one would need to analyze telomeres in strains that are deficient in both telomerase and recombination.

**Potential function for CaEst1p and CaTERT in telomere "capping."** Earlier epistasis analysis in *S. cerevisiae* provides compelling support for the notion that all of the telomerase components act coordinately in a single genetic pathway (31, 55). Our observation that disruption of individual telomerase subunits in *Candida* resulted in distinct telomere dysfunction is therefore surprising. An attractive explanation invokes a novel role for CaEst1p and CaTERT in telomere end protection (independent of their telomere extension function). As pointed out earlier, the somewhat dynamic behavior of *Candida* telomeres and the lack of senescence in the absence of telomerase suggests that the "capping" of telomeres by other telomere-binding proteins (such as Rap1p, Ku, etc.) may be less complete in this yeast, and may require the binding of telomerase for more complete protection. In this scenario, CaEst1p and CaTERT, but not CaEst3p, are required for the binding of telomerase to telomere ends and for telomere protection. Thus, in the absence of CaEst1p or CaTERT, telomeres are more subject to sudden length changes due to recombination or degradation. In contrast, in the absence of CaEst3p, "capping" is still relatively intact, and telomeres shorten progressively due to the inability of telomerase to carry out its "telomere extension" function. It would be interesting to determine whether the *Candida* telomerase RNA is also required for this "end protection" function. The possibility that telomerase may participate in the capping of telomeres has been suggested before (3, 61). In any case, our findings underscore the possibility of uncovering distinct physiologic and biochemical functions of telomerase components in *Candida*.

**Evolutionary conservation of telomerase protein components.** Different telomerase protein components exhibit different levels of sequence conservation. The TERT protein is particularly well conserved, probably due to its role in mediating multiple catalysis-related functions. Quite a number of RT-like and TERT-specific motifs have been shown to be common to all member of this protein family, including CaTERT (8, 58). There is little doubt that CaTERT would possess the many biochemical activities that have been ascribed to other TERTs, such as RNA binding and nucleotide addition.

Members of the Est1p-like protein family exhibit a lower level of sequence conservation. As described earlier, the most generally conserved region is located near the N terminus of the polypeptides and bears a resemblance to TPR domain proteins. Based on known functions of TPR domains, we suggest that this region of Est1p may participate in protein-protein interactions. Interestingly, the well-characterized biochemical activities of *S. cerevisiae* Est1p, such as binding telomerase RNA, binding Cdc13p (a single-strand telomere end-binding protein), and binding telomeric DNA (51, 55, 60), have all been shown to involve C-terminal regions of the polypeptide (Fig. 7A), raising questions about the extent of conservation for these activities. However, the C terminus of CaEst1p is quite similar to *S. cerevisiae* Est1p. In fact, amino acid residues critical for both Cdc13p binding (Lys444) and RNA binding (Leu466, Ile494, Phe496, Phe511, and Asp513) in *S. cerevisiae* are either conserved or replaced with similar residues in *Candida* (data not shown). These biochemical activities of *S. cerevisiae* Est1p are therefore likely to be possessed by at least the CaEst1p homologue.

Est3p appears to be the least well-conserved protein component, having been detected so far only in *Saccharomyces* and *Candida*. Nevertheless, the existence of these functional homologues with minimal sequence similarity should encourage further studies aimed at understanding their structure and mechanisms.

The lack of growth defects in *Candida* strains with no telomerase activity suggests that telomerase components by themselves are not attractive targets for antifungal drug discovery. However, components of telomerase have been shown to be valid targets for developing anti-cancer therapeutics (24, 26, 59). Greater understanding of the multiple functions and activities of all of the conserved components in model systems such as *S. cerevisiae* and *C. albicans* should facilitate such a development.

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