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Functional Analysis of the Single Est1/Ebs1 Homologue in *Kluyveromyces lactis* Reveals Roles in both Telomere Maintenance and Rapamycin Resistance

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Est1 and Ebs1 in *Saccharomyces cerevisiae* are paralogous proteins that arose through whole-genome duplication and that serve distinct functions in telomere maintenance and translational regulation. Here we present our functional analysis of the sole Est1/Ebs1 homologue in the related budding yeast *Kluyveromyces lactis* (named *Kl*Est1). We show that similar to other Est1s, *Kl*Est1 is required for normal telomere maintenance *in vivo* and full telomerase primer extension activity *in vitro*. *Kl*Est1 also associates with telomerase RNA (Ter1) and an active telomerase complex in cell extracts. Both the telomere maintenance and the Ter1 association functions of *Kl*Est1 require its N-terminal domain but not its C terminus. Analysis of clusters of point mutations revealed residues in both the N-terminal TPR subdomain and the downstream helical subdomain (DSH) that are important for telomere maintenance and Ter1 association. A UV cross-linking assay was used to establish a direct physical interaction between *Kl*Est1 and a putative stem-loop in Ter1, which also requires both the TPR and DSH subdomains. Moreover, similar to *S. cerevisiae* Ebs1 (*Sc*Ebs1) (but not *Sc*Est1), *Kl*Est1 confers rapamycin sensitivity and may be involved in nonsense-mediated decay. Interestingly, unlike telomere regulation, this apparently separate function of *Kl*Est1 requires its C-terminal domain. Our findings provide insights on the mechanisms and evolution of Est1/Ebs1 homologues in budding yeast and present an attractive model system for analyzing members of this multifunctional protein family.

Telomeres are specialized nucleoprotein structures that maintain the integrity of eukaryotic chromosomal termini by protecting them from fusion and recombination and promoting their replication (for reviews, see references 12, 23 and 32). In most organisms, telomeric DNA consists of short repetitive sequences that are rich in G residues on the 3′ end-containing strand (G strand). The repeats on this strand are replenished by the telomerase ribonucleoprotein (RNP), whose essential polymerization function is mediated by two core components: a catalytic reverse transcriptase protein, telomerase reverse transcriptase (TERT), and a template-specifying RNA, telomerase RNA (TER) (for reviews, see references 2 and 30).

The telomerase RNP in the budding yeast *Saccharomyces cerevisiae* has been extensively characterized and contains two auxiliary protein components named Est1 and Est3. Both components are essential for telomere maintenance *in vivo* but dispensable for telomerase activity *in vitro*. A major function of Est1 is to promote the recruitment of the telomerase complex to chromosome ends (33). This function is believed to require a physical interaction between Est1 and the telomere end-binding protein Cdc13, as well as an interaction between Est1 and telomerase RNA. The domains of Est1 and its binding targets responsible for these interactions have been characterized to some extent, but not in great detail. For example, in *S. cerevisiae*, a bulged stem and surrounding nucleotides in telomerase RNA (Tlc1) are found to be necessary and sufficient for Est1-Tlc1 interaction, but the region of Est1 required for RNA binding is poorly understood (11, 38, 45). Besides recruitment, a second, activation function for Est1 is also evident from existing data, and recent studies suggest that this function is attributable to the ability of Est1 to promote the incorporation of Est3 into the telomerase RNP (11, 22, 31, 42, 43). In the absence of Est1, Est3 exhibits reduced association with telomerase core components in cell extracts and with telomeres *in vivo*. Despite these insights on Est1 functions, many questions remain with regard to the detailed biochemical properties of this protein and their physiologic relevance. For example, even though both genetic and biochemical evidence supports a direct physical interaction between Est1 and the telomere end-binding protein Cdc13, how this interaction promotes telomerase function remains incompletely understood (33, 34, 50). In addition, Est1 has been reported to possess moderate affinity and sequence specificity for telomeric G-strand DNA and to promote G-quadruplex formation, but the physiologic relevance of these activities remains unclear (10, 45, 53). Intriguingly, several mutations in Est1were recently observed to induce telomere lengthening, suggesting a previously unsuspected negative regulatory function with regard to telomere homeostasis (37). Evidently, more studies are necessary to achieve a full understanding of this critical and multifunctional protein.

Investigations of Est1 homologues have resulted in yet more questions concerning the mechanisms and evolution of this protein family. In *S. cerevisiae*, an *EST1* homologue named *EBS1* (Est1-like Bcy1 Suppressor) was shown to play a modest role in telomere maintenance but also to regulate translation and non-

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sense-mediated decay (NMD) (14, 24, 54). Specifically, the *ebs1*Δ mutant manifests higher levels of NMD-targeted RNAs and greater resistance to rapamycin, which inhibits the TOR signaling pathway that regulates cell growth and translation. In *Schizosaccharomyces pombe*, two Est1-like genes have been identified, only one of which is required in telomere maintenance (4). Notably, *Sp*Est1 also mediates telomerase recruitment but does so by interacting with the *S. pombe* telomere protein Ccq1 rather than a Cdc13 homologue (29). In humans, three *EST1*-like genes (EST1A, EST1B, and EST1C) have been identified. Two of these genes, EST1A and EST1B, have been implicated in telomere regulation, though their mechanisms of action appear to be distinct from that of *Sc*Est1 (35, 41). In particular, neither protein has been shown to mediate telomerase recruitment or telomerase RNP assembly. Interestingly, EST1A and EST1B are also known as SMG6 and SMG5 and have been implicated in NMD pathways. These proteins may regulate TERRA, which are telomere-encoded RNAs that modulate telomere lengths and chromatin structure (3, 9, 25). The involvement of diverse Est1 homologues (from yeasts to humans) in NMD suggests that this involvement may have occurred early in evolution.

Our laboratories have explored less frequently studied budding yeasts (i.e., yeasts other than *S. cerevisiae*) as alternative model systems for analyzing the mechanisms of telomere and telomerase proteins. We consider the effort worthwhile for two reasons. First, many *S. cerevisiae* telomere and telomerase proteins are difficult to express, purify, and analyze *in vitro*, thus limiting the possibilities for detailed mechanistic analysis. By screening homologues, we wish to identify biochemically tractable proteins and subject them to in-depth analysis. Second, we reasoned that an *in vitro* activity of a protein is more likely to be biologically relevant if it is conserved in evolution. By comparing the properties of orthologues to the prototypical member, we may be able to focus on features of the protein that are of greater general significance. In this study, we examined the mechanism and functions of the single Est1 homologue in the budding yeast *Kluyveromyces lactis* (*Kl*Est1), which we found to be quite amenable to both biochemical and genetic analyses. We showed that *Kl*Est1 is required for telomere maintenance *in vivo* and modulates the primer extension activity of telomerase in a primer-specific manner. We developed a cross-linking assay to analyze Est1-telomerase RNA interaction and characterized the regions and features of the two molecules required for direct mutual interaction. We also found that *Kl*Est1 confers rapamycin sensitivity and thus shares the functions of *Sc*Ebs1. A careful analysis of the Est1 family of proteins in *Saccharomycotina* yeasts (encompassing *Saccharomyces*, *Kluyveromyces*, and *Candida* species) revealed the existence of two Est1/Ebs1-like proteins only in organisms that are descendants of the ancestor with a duplicated genome. Our findings support the notion that both telomere and NMD regulations are ancestral functions of Est1 and that the existence of functionally distinct Est1 and Ebs1 in *S. cerevisiae* is the result of whole-genome duplication (WGD) followed by subspecialization.

MATERIALS AND METHODS

Construction and growth of *K. lactis* strains. The *K. lactis* strain 7B520 (*ura3-1 his2-2 trp1*) was used as the parental strain (49). A PCR-amplified *K. lactis EST1* gene was cloned into the XmaI/KpnI sites of pBS SK II+ to create pEbs1. The primers used for this were ATCCCGGGtactgcttctcccac cagaga and ACGGTACCcacctcgaatgcttttacgac, with the *K. lactis* sequences shown in lowercase. The 1,833-bp MfeI-NheI region within the *EST1* open reading frame (ORF) was then replaced with the EcoRI-XbaI *HisG-URA3-HisG* fragment from pBS-URA3-2 to create plasmid pΔEbs1-HUH. The *K. lactis est1*Δ mutant was constructed by transforming strain 7B520 with a fragment containing the disrupted *EST1* gene from pΔEbs1-HUH, followed by selection on synthetic defined (SD) agar plates lacking uracil. Independent transformants were then placed on medium containing 5-fluoroorotic acid (5-FOA) to select for cells that had undergone recombination to excise the *URA3* gene and one copy of *HisG*. Strains bearing various *EST1* mutant alleles were created by transforming the *est1*Δ mutant with the pCXJ18-EST1-TAP series of plasmids. Strains were all passaged at 30°C in either solid or liquid yeast extract-peptone-dextrose (YPD) medium (2% peptone, 1% yeast extract, 2% dextrose).

Plasmids and mutagenesis. For protein expression in *Escherichia coli*, we cloned *K. lactis EST1* fragments into pSMT3 to enable the expression of SUMO fusion proteins. PCR fragments encompassing the entire *EST1* open reading frame (ORF), the N-terminal domain (NTD) (amino acids [aa] 1 to 598), the TPR subdomain (aa 1 to 303), and the downstream helical subdomain (DSH) (aa 304 to 598) were cloned between the SacI and XhoI sites of pSMT3 vector to generate pSMT3-KlEST1, pSMT3-KlEST1$_{NTD}$, pSMT3-KlEST$_{TPR}$, and pSMT3-KlEST1$_{DSH}$, respectively. A FLAG tag was incorporated into each of the downstream primers used for PCR such that the recombinant proteins all carry a C-terminal FLAG tag. For *in vivo* analysis of *EST1* function, we cloned different alleles of *EST1* into the pCXJ18 shuttle vector, which carries the *URA3* marker. The vector was first modified by introducing the TAP tag between the SalI and HindIII sites to give pCXJ18-TAP (6, 36). A PCR fragment containing the *EST1* ORF and 550 bp of upstream sequence was then inserted between the KpnI and SalI sites of pCXJ18-TAP to give pCXJ18-KlEST1-TAP. Truncated *EST1* genes corresponding to the NTD and the TPR domain were generated by PCR using primers containing the KpnI and SalI site and used to substitute the corresponding fragment in pCXJ18-KlEST1-TAP to give pCXJ18-KlEST1$_{NTD}$-TAP and pCXJ18-KlEST1$_{TPR}$-TAP, respectively. To generate pCXJ18-KlEST1$_{DSH}$-TAP, two overlapping PCR fragments comprising the promoter region and the DSH subdomain were first amplified separately. The two fragments were both added to a second PCR mixture to synthesize a combined DNA fragment, which was then inserted into pCXJ18-TAP.

To analyze the roles of individual amino acids, site-specific mutagenesis was employed to create the RR (R111A and R118A), RHRQ (R111A, H117A, R118A, and Q119A), QKR (Q219A, K220A, and R223A), RR-QKR (R111A, R118A, Q219A, K220A, and R223A), KF (K522A and F529A), RR-KF (R111A, R118A, K522A, and F529A), and K467E mutants of *EST1*$_{NTD}$ in either pSMT3-KlEST1$_{NTD}$ or pCXJ18-KlEST1$_{NTD}$-TAP.

Sequence analysis. Est1 and Ebs1 homologues from *Saccharomyces*, *Kluyveromyces*, and *Candida* spp. were identified from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Broad Institute (http://www.broad.mit.edu/annotation/genome/candida_group/Blast.html), and SGD (http://www.yeastgenome.org/) databases. The multiple-sequence alignment was generated using the T-COFFEE server (http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) and displayed using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). The phylogeny for these homologues was investigated using Phylip (http://evolution.genetics.washington.edu/phylip/getme.html) and displayed using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Telomere length analysis. Chromosomal DNAs were isolated from 5 ml of saturated *K. lactis* culture by the smash and grab method (20), digested with EcoRI, and fractionated in 0.6 to 0.8% agarose gels. Following transfer to nylon membranes, the telomere restriction fragments were detected as previously described using an oligonucleotide probe that contains two copies of the *K. lactis* telomere repeat (5′-ACGGATTTGATTAGGTATGTGGTGTA CGGATTTGATTAGGTATGTGGTGT-3′). The hybridization was performed at 50°C to 60°C.

Partial purification of the *K. lactis* telomerase complex and analysis of telomerase activity. *K. lactis* telomerase fractions were generated by resolving whole-cell extracts (derived from the wild-type or an *est1*Δ

strain) over DEAE columns as previously described for *Candida albicans* (21, 40). The fractions were subjected to primer extension assays using 12-nucleotide (nt) primers and a single labeled nucleotide triphosphate that is needed for incorporation at the primer +1 position. For comparison of wild-type and *est1*Δ telomerases, fractions containing approximately equal levels of Ter1 (as measured by reverse transcriptase [RT]-PCR) were utilized. The primer extension products were resolved on denaturing polyacrylamide gels and analyzed using a Typhoon PhosphorImager (Molecular Dynamics) and the ImageQuant software (GE Healthcare).

RNA-protein cross-linking assay. The His_6-SUMO fusion proteins were expressed and purified essentially as previously described (52). Briefly, extracts were prepared from *E. coli* following IPTG (isopropyl-β-D-thiogalactopyranoside) induction and the fusion proteins were purified using Ni-nitrilotriacetic acid (Ni-NTA) chromatography. The fusion proteins were then cleaved by ULP1 and the untagged polypeptides were isolated from the His_6-SUMO tag by a second round of Ni-NTA affinity chromatography. The protein preparations were further purified by sedimentation over glycerol gradients (15% to 30%). ^{32}P-labeled RNA probes for the cross-linking assay were prepared by *in vitro* transcription using PCR fragments containing the T7 promoter placed upstream of wild-type and mutant Ter1-EBD (the putative Est1 binding domain of *K. lactis* telomerase RNA) sequences. Cross-linking reaction mixtures (20 μl) contained ^{32}P-labeled RNA probe, 12.5 ng/μl tRNA, 20 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), and 0.5 U/μl RNasin. Following incubation at 30°C for 30 min, the reaction mixtures were irradiated with 254-nm UV light on ice for 20 min and then treated with 250 ng/μl RNase A at 37°C for 30 min. The reaction mixtures were further incubated at 65°C for 5 min following the addition of SDS (4 μl of a 10% stock solution) and DTT (0.4 μl of a 1 M stock solution). The RNA-protein complexes were precipitated by adding 4 μg glycogen and 1.2 ml acetone at room temperature for 10 min, recovered by centrifugation, and then resolved by SDS-PAGE. The levels of cross-linking were analyzed using a Typhoon PhosphorImager and the ImageQuant software (GE Healthcare).

Analysis of Est1-Ter1 association in cell extracts. *K. lactis* whole-cell extracts were prepared and subjected to IgG-Sepharose pulldown as previously described with slight modifications (21). Briefly, cell extracts (5 mg) were incubated with IgG-Sepharose beads (45 μl) in 1.2 ml TMG(400) buffer (10 mM Tris-HCl, pH 8.0, 1.2 mM magnesium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol plus 400 mM sodium acetate) plus 0.05% Tween 20. The mixtures were rotated at 4°C for 2 h and the beads were washed five times in TMG(800) and twice in TMG(0) (21). The precipitated samples were then subjected to RT-PCR and Western blot analysis as previously described to quantify the levels of Ter1 and Est1, respectively (21). For RT-PCR, fragments corresponding to 201 bp of Ter1 and 385 bp of U1 (used as the control for nonspecific binding) were amplified by specific primer pairs (Ter1 forward, 5′-GCTATGACA ACAATACCTTTAGAAT-3′; Ter1 reverse, 5′-GATTAGGTATGTGGTG TACGGATTTG-3′; U1 forward, 5′-GTTTGTTGACCGGGTATGAGGT TTTC-3′; U1 reverse, 5′-CTCTCACCCATAAACCTGTAAACAAG-3′). P^{32}-labeled dCTP (1 μCi/4 nmol) was included in the reaction mixtures to allow for quantification of the amounts of PCR products by PhosphorImager analysis.

Analysis of rapamycin sensitivity and NMD transcripts. The *K. lactis* strains were grown in YPD to an optical density at 600 nm (OD_{600}) of 0.6 to 1.0. The cultures were diluted to an OD_{600} of 0.1, and 5-fold serial dilutions of these suspensions were spotted onto YPD plates containing 5 or 10 ng/ml rapamycin (LC laboratories). The plates were incubated at 30°C for 4 to 5 days and checked for growth. The NMD pathway was also assessed by measuring the levels of *CYH2* pre-mRNA and mRNA using RT-PCR. The sequences of the primers are as follows: forward primer for mRNA, 5′-GAAAGCACAGAGGTCACGTCTCAGCCGG-3′; forward primer for pre-mRNA, 5′-CAGAACCACAGGTTTACTAACAAACAC-3′; reverse primer, 5′-CAGACTTGGAAGCAGACTTCAAGTATTC-3′. P^{32}-labeled dCTP (1 μCi/4 nmol) was included in the reaction mixtures to allow for quantification of the amounts of PCR products by PhosphorImager analysis.

Homology modeling. The *Kl*Est1$_{NTD}$ sequence was submitted to the I-Tasser server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and 1YA0.pdb (the structure file for human SMG7 N terminus) was specified as the template for modeling. The resulting model was visualized and analyzed in Pymol (http://pymol.sourceforge.net/index.html).

RESULTS

***Kl*Est1 and other Est1/Ebs1 homologues in *Saccharomycotina*.** As described earlier, Est1 has been most extensively characterized in *S. cerevisiae*. However, many reported mechanisms of *Sc*Est1, including its nucleic acid-binding properties and its role in enhancing telomerase activity, have been controversial. In addition, recombinant expression and purification of *Sc*Est1 are reportedly difficult. We therefore screened other Est1/Ebs1 homologues as alternative targets of investigation. Our preliminary analysis indicated that the homologue encoded by the *K. lactis* genome (here referred to as *Kl*Est1) is particularly amenable to recombinant expression and purification. Because this organism has already been utilized extensively as a model system to analyze different aspects of telomere maintenance (17, 27), we decided to engage in a detailed analysis of *Kl*Est1.

Before proceeding with molecular genetic and biochemical investigation of *Kl*Est1, we performed multiple-sequence alignments and phylogenetic analysis to clarify the relationship of *Kl*Est1 to other Est1/Ebs1 homologues. As depicted in Fig. 1A, each member of the *Kluyveromyces* and *Candida* clades contains just one Est1/Ebs1-like protein. However, in the *Saccharomyces* clade, each genome contains both a presumptive *EST1* and an *EBS1* gene. Within this clade, the *EST1* and *EBS1* genes form well-defined monophyletic groups, suggesting a duplication of an ancestral gene prior to speciation of the *Saccharomyces* members. Interestingly, *Saccharomyces* species are known to be descendants of an ancestor that underwent whole-genome duplication (48). Indeed, syntenic analysis of available yeast genomes supports the notion that *EST1* and *EBS1* arose as a consequence of WGD and can be considered ohnologs (5). The Ebs1 proteins in *Saccharomyces* are also considerably larger than Est1 proteins, with the former ranging from 870 aa to 1,023 aa and the latter ranging from 528 aa to 767 aa. This size difference can be attributed to the larger C-terminal domain (CTD) in Ebs1 proteins; the remaining portions of the Est1 and Ebs1 proteins align well to one another and are presumably comprised of an α-helical domain (Fig. 1B). Specifically, the crystal structure of an N-terminal fragment of human EST1C (also known as SMG7), which aligns well to the N terminus of the yeast Est1/Ebs1 proteins, revealed a predominantly α-helical protein comprised of an N-terminal TPR subdomain and a downstream helical subdomain (DSH) (15). In the *Kluyveromyces* clade (*Kluyveromyces waltii*, *K. lactis*, *Saccharomyces kluyveri*, and *Ashbya gossypii*), the Est1/Ebs1-like proteins have extended CTDs that align well to the *Saccharomyces* Ebs1 proteins. Overall, the sequence and phylogenetic analyses suggest that *Kl*Est1 may perform the functions mediated by both Est1 and Ebs1 in *S. cerevisiae*.

***Kl*Est1 is required for telomere maintenance *in vivo* and telomerase activity *in vitro*.** To determine if *KlEST1* is involved in telomere maintenance, we constructed an *est1* null strain and characterized its growth and telomere phenotypes. As predicted for a telomerase gene (26, 28), the *est1* null mutant strain exhibited growth senescence and telomere attrition (Fig. 2A and data not

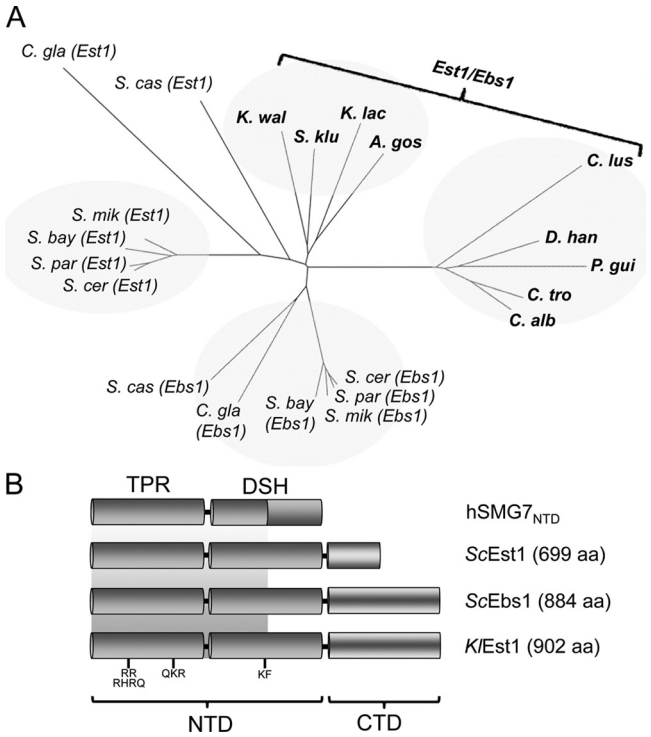

FIG 1 Phylogenetic analysis and domain comparison of Est1/Ebs1 homologues in budding yeast. (A) The relationships between the yeast Est1/Ebs1 homologues were analyzed using the neighbor-joining method and the results were plotted using FigTree. The abbreviations are as follows: *A. gos*, *Ashbya gossypii*; *C. gla*, *Candida glabrata*; *C. alb*, *Candida albicans*; *C. lus*, *Candida lusitaniae*; *C. tro*, *Candida tropicalis*; *D. han*, *Debaromyces hansenii*; *K. lac*, *Kluyveromyces lactis*; *K. wal*, *Kluyveromyces waltii*; *P. gui*, *Pichia guilliermondii*; *S. bay*, *Saccharomyces bayanus*; *S. cas*, *Saccharomyces castellii*; *S. cer*, *Saccharomyces cerevisiae*; *S. klu*, *Saccharomyces kluyveri*; *S. mik*, *Saccharomyces mikata*; *S. par*, *Saccharomyces paradoxus*. (B) The domain structures of the indicated Est1/Ebs1 homologues are depicted. The crystal structure of the N-terminal domain of human SMG7 ($hSMG7_{NDT}$) revealed a TPR subdomain and a downstream helical subdomain (DSH). The shaded background is used to illustrate the fact that the first half but not the second half of $hSMG7_{DSH}$ aligns well to the yeast homologues.

shown; see Fig. S1A and B in the supplemental material). The growth defects of the *est1*Δ mutant clones were variable. In one serially passaged clone, for example, some small colonies can be seen ~75 to 100 generations after gene deletion, followed by apparent recovery and subsequently more uniform and severe growth defects at ~175 generations (see Fig. S1A in the supplemental material). Other clones senesced and recovered at other time points during passage (data not shown). This heterogeneous behavior is very similar to that of the *K. lactis ter1*Δ mutant (28). As predicted, Southern analysis of the mutant revealed progressive telomere shortening (see Fig. S1B in the supplemental material). Overall, these findings support the notion that Est1 is essential for telomerase-mediated telomere maintenance in *K. lactis*.

We then examined the activity of telomerase derived from the wild-type and mutant strains by primer extension assays using a series of 12-nt primers that represent different regions and permutations of the *K. lactis* telomere repeat unit. Each primer was designed to test the ability of telomerase to initiate extension at a defined position along the RNA template (Fig. 2B). To simplify the interpretation of results, we added just a single labeled nucleotide triphosphate to each reaction mixture (39). For most primers, we were able to detect RNase-sensitive products of the predicted size(s) (Fig. 2B and data not shown). Consistent with previous findings, we observed very little DNA synthesis on primers 24 and 25, whose 3′ ends anneal close to the 5′ template boundary of Ter1 (Fig. 2D) (16, 44). Interestingly, we observed a variable reduction of primer extension activity in the fractions derived from the *est1*Δ strain (Fig. 2C and D). For example, the ability of the mutant enzyme to mediate nucleotide addition was similar to that of the wild-type enzyme at position 1 within the telomere repeat but was reduced 5-fold at position 5 (Fig. 2C). The magnitude of activity reduction was especially severe (>10-fold) at positions 7 and 8. However, at most positions, the mutant enzyme was ~2- to 5-fold less active than the wild-type enzyme. These results indicate that *Kl*Est1 affects the activity of telomerase in a DNA substrate-dependent manner, as has been observed for *Candida albicans* Est1 (39). The telomere length and telomerase activity defects of the *K. lactis EST1* deletion mutant argue that this gene is indeed orthologous to *S. cerevisiae EST1*.

The N terminus of *Kl*Est1 is sufficient for maintaining telomeres. To identify the regions of Est1 required for telomere length maintenance, we introduced full-length or truncated TAP-tagged *EST1* alleles into an *est1*Δ mutant and monitored the length of telomeres in the resulting strains by Southern blotting. The truncation variants tested include the entire N terminus of the protein (NTD, aa 1 to 598), as well as the TPR and the DSH subdomains (aa 1 to 303 and aa 304 to 598, respectively). As predicted, TAP-tagged full-length *EST1* restored telomeres of the *est1*Δ mutant to nearly wild-type lengths (Fig. 3A). The strains reconstituted with $EST1_{NTD}$ contained similarly long telomeres, indicating that CTD is dispensable. In contrast, the strains reconstituted with $EST1_{TPR}$ or $EST1_{DSH}$ exhibited shorter-than-wild-type telomeres, indicating that both subdomains are required for telomere maintenance.

Two previous studies identified multiple residues of *S. cerevisiae* Est1 required for its association with telomerase RNA and for telomere maintenance (11, 54). One study revealed clusters of charged residues within the TPR subdomain, whereas the other uncovered basic and hydrophobic residues in the DSH subdomain as being important. (The DSH subdomain was previously proposed to contain RNA recognition motifs [RRMs], but such motifs are unlikely to be present given the hEST1C structure [15].) To determine if comparable residues of *Kl*Est1 are required for telomere maintenance, we created a series of substitution mutants based on sequence alignment (Table 1). Each cluster of mutations was introduced into the $EST1_{NTD}$ allele, and the resulting mutant was tested in the *est1*Δ background. As shown in Fig. 3B, all of the mutant strains exhibited various degrees of telomere shortening. Both the RR and QKR mutations within the TPR subdomain of *Kl*Est1 caused significant telomere shortening, as did the KF mutations in the DSH subdomain. Several mutants that exhibited substantial telomere shortening also showed evidence of senescence upon repeated passages (e.g., the RR-KF and RR-QKR mutants) (see Fig. S2 in the supplemental material; data not shown). In general, combining different clusters of mutations resulted in a more severe defect, suggesting that these groups of residues contribute independently to telomere maintenance (Fig. 3B, compare the RR-KF mutant to the RR and KF mutants). However, it was difficult to assess quantitatively the relative severity of the telomere shortening phenotype because of clonal heterogeneity (even though the mutants were all analyzed ~50 generations after the

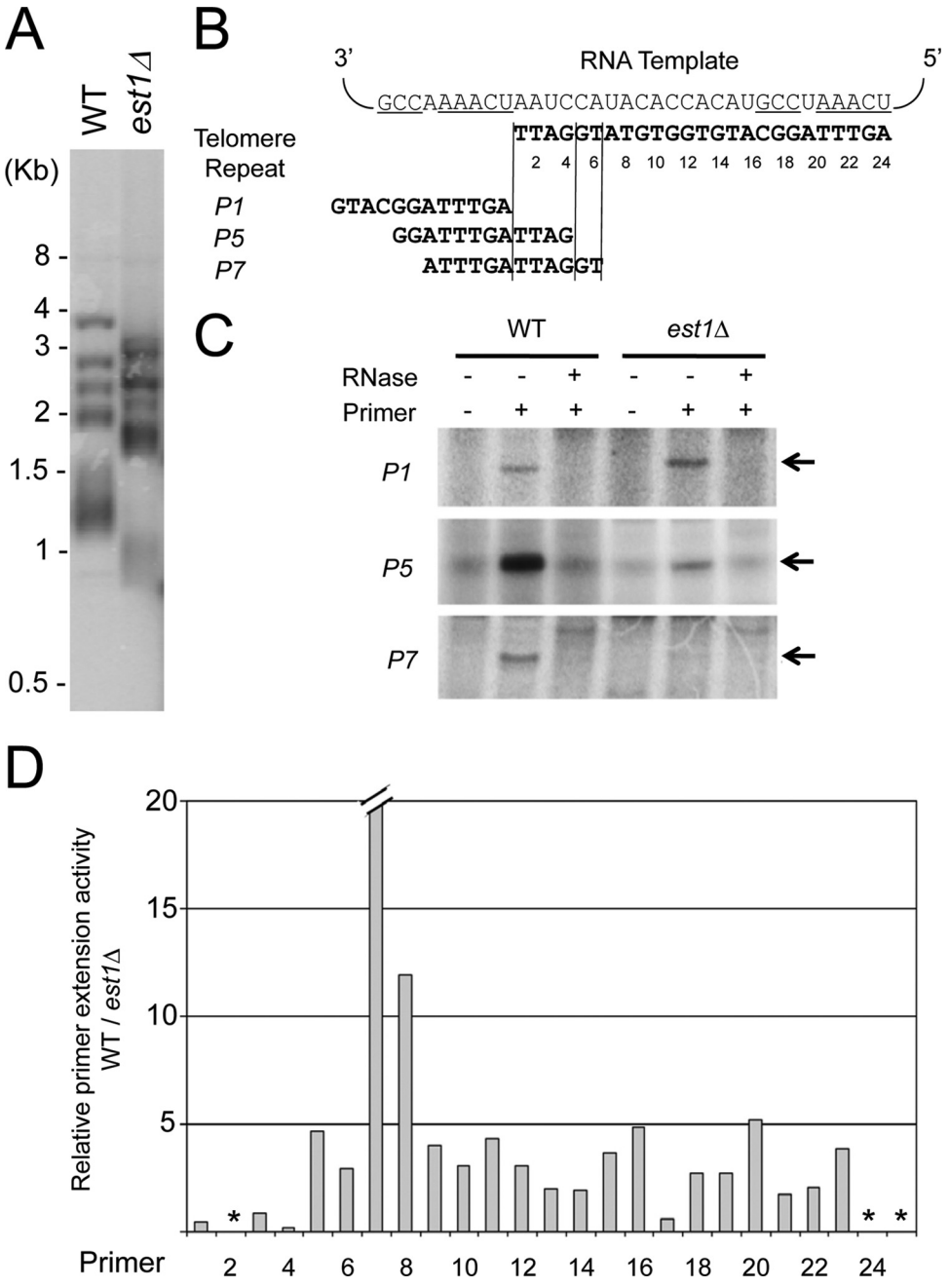

FIG 2 Deletion of *KlEST1* impairs telomere maintenance *in vivo* and telomerase activity *in vitro*. (A) Chromosomal DNAs were isolated from the wild-type (WT) strain and the *est1*Δ mutant (~50 generations after strain construction) and subjected to Southern analysis using an oligonucleotide probe that corresponds to two copies of the *K. lactis* telomere repeats (see Materials and Methods). (B) The template region of telomerase RNA is displayed; RNA residues presumably involved in alignment are underlined (46). A series of 12-nt primers that represent different regions and permutations of the *K. lactis* telomere repeat unit were designated by numbers. The precise sequences of three primers (P1, P5, and P7) are shown as examples. (C) Telomerase from the wild-type and *est1*Δ strains were isolated by DEAE chromatography and tested by primer extension assays using the P1, P5, and P7 primers in the presence of ^{32}P-labeled dTTP, dGTP, and dATP, respectively. In other assays, we consistently observed comigration of the major RNase-sensitive product (indicated by arrows) with the primer + 1 size standard prepared by labeling of the primer with terminal transferase. (D) The levels of primer extension products were quantified by using a PhosphorImager. The ratios of the wild-type telomerase activity to *est1*Δ activity on all the primers were calculated and plotted. *, the levels of primer extension products generated by telomerase on primers P2, P24, and P25 were too low to yield reliable quantifications. For primer P7, the DEAE fraction derived from the *est1*Δ mutant was essentially inactive, resulting in a very high WT-to-*est1*Δ activity ratio.

introduction of the mutant alleles). Notably, all mutant proteins were detected at similar levels in cell extracts, indicating that the mutations did not grossly impair protein expression or stability (Fig. 3C). Taken together, these results suggest that residues in both the TPR and DSH subdomains play a conserved function in telomere maintenance *in vivo*.

Next, we characterized the association of Est1 mutants with telomerase RNA (Ter1) in cell extracts using an IgG-Sepharose

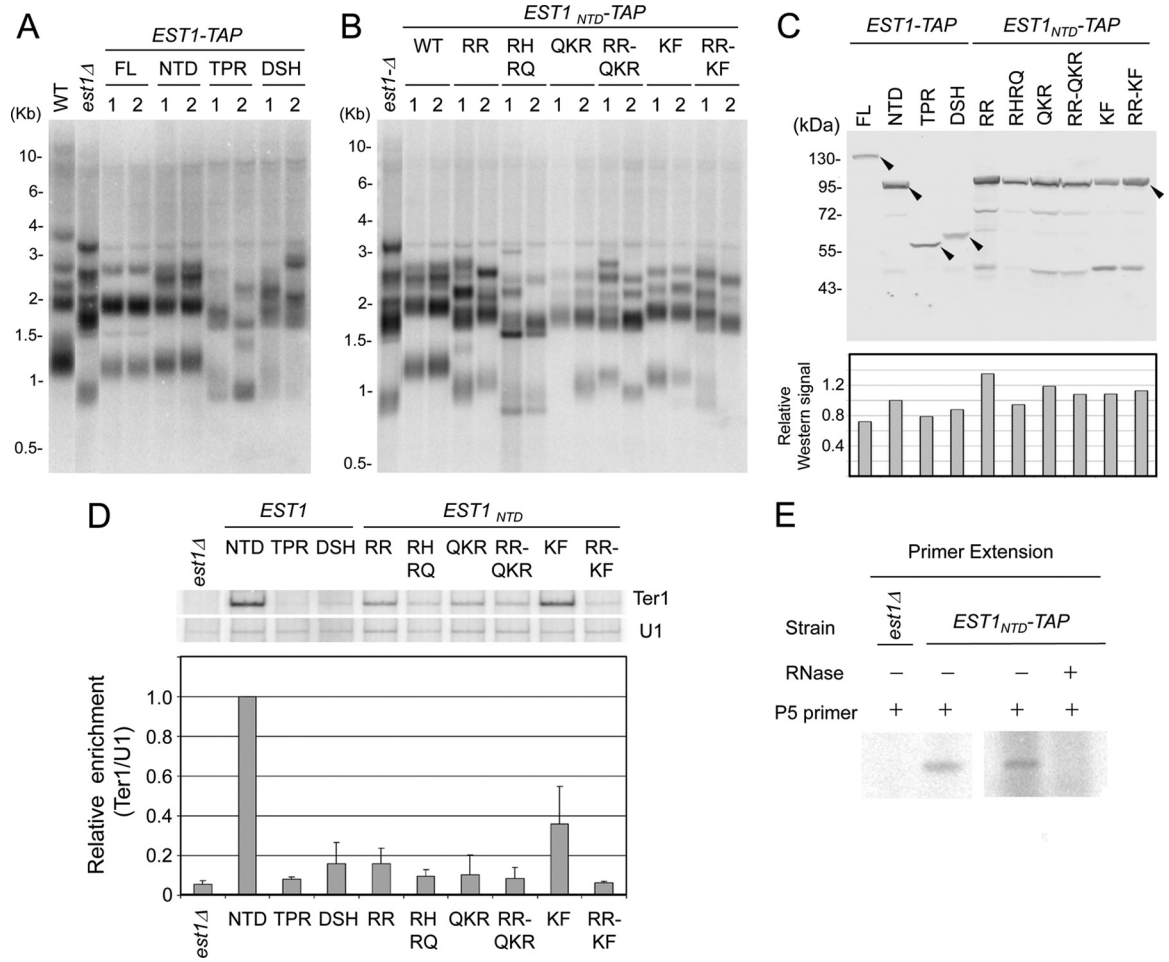

FIG 3 Effects of *EST1* mutations on telomere maintenance and Est1-Ter1 association. (A) Chromosomal DNAs were isolated from the wild-type, *est1*Δ, and two independently derived strains bearing full-length or truncated *EST1* alleles (~50 generations after the introduction of the *EST1* alleles into the null strain) and subjected to Southern analysis of telomere restriction fragments. (B) Same as for panel A, except the chromosomal DNAs were isolated from strains reconstituted with $EST1_{NTD}$ and various point mutants of $EST1_{NTD}$. (C) (Top) The TAP-tagged Est1 proteins in cell extracts were analyzed by Western blotting using antibodies directed against protein A. (Bottom) Signals were quantified, normalized to that for $EST1_{NTD}$-TAP, and plotted. (D) (Top) Extracts were prepared from the *est1*Δ mutant as well as strains reconstituted with various *EST1* alleles and subjected to the IgG-Sepharose pulldown assay. The levels of Ter1 and U1 RNA in the pulldown samples were measured by RT-PCR. P^{32}-labeled dCTP was included in the reaction mixtures to allow identification of the PCR products by PhosphorImager scanning of gels. (Bottom) The radioactive signals for the Ter1 and U1 RT-PCR products were quantified by PhosphorImager analysis, and the ratios of Ter1 to U1 products were calculated and plotted. Data are derived from two independent experiments. (E) The IgG-Sepharose pulldown samples from the indicated strains were subjected to primer extension assays using the P5 primer and [α-^{32}P]dGTP.

pulldown assay. Consistent with the dispensability of CTD for telomere maintenance, we found that $Est1_{NTD}$ associated strongly with *K. lactis* Ter1 in cell extracts (Fig. 3D). Indeed, we consistently detected twice as much Ter1 in the $Est1_{NTD}$ pulldown sample than in the $Est1_{FL}$ sample (data not shown), possibly because of the higher levels of the $Est1_{NTD}$ protein in cell extracts (Fig. 3C). Primer extension analysis of the $Est1_{NTD}$ pulldown sample indicates that this protein is associated with active telomerase complex (Fig. 3E). In contrast to $Est1_{NTD}$, all of the other mutants exhibited reduced association with Ter1 (3- to 16-fold reduction), suggesting that the regions deleted or the residues mutated (in the TPR and DSH subdomains) are all required for optimal Ter1 association. As in the case of telomere maintenance, combining different clusters of point mutations resulted in a more severe Ter1 association defect. For example, the RR and KF mutations separately reduced Ter1 association 6- and 3-fold, respectively, whereas the combined mutant suffered an ~15-fold reduction in association (Fig. 3D). We conclude that the association between $Est1_{NTD}$ and Ter1 plays an important role in telomere maintenance in *K. lactis*.

The consistently detrimental effects of our point mutations on Ter1 association are not unexpected given the consequences of

TABLE 1 Amino acid substitutions in the *EST1* mutants characterized in this and two previous studies

S. cerevisiae		*K. lactis*	
Allele name	Amino acid substitutions	Allele name	Amino acid substitutions
est1-38	R111A, R112A, K113A, R115A	RR	R111A, R118A
est1-39	R118A, K122A, K123A	RHRQ	R111A, H117A, R118A, Q119A
est1-41	E222A, K223A, R226A	QKR	Q219A, K220A, R223A
Est1-RNP1-NC	R499G, I504A, F506A	KF	K522A, F529A

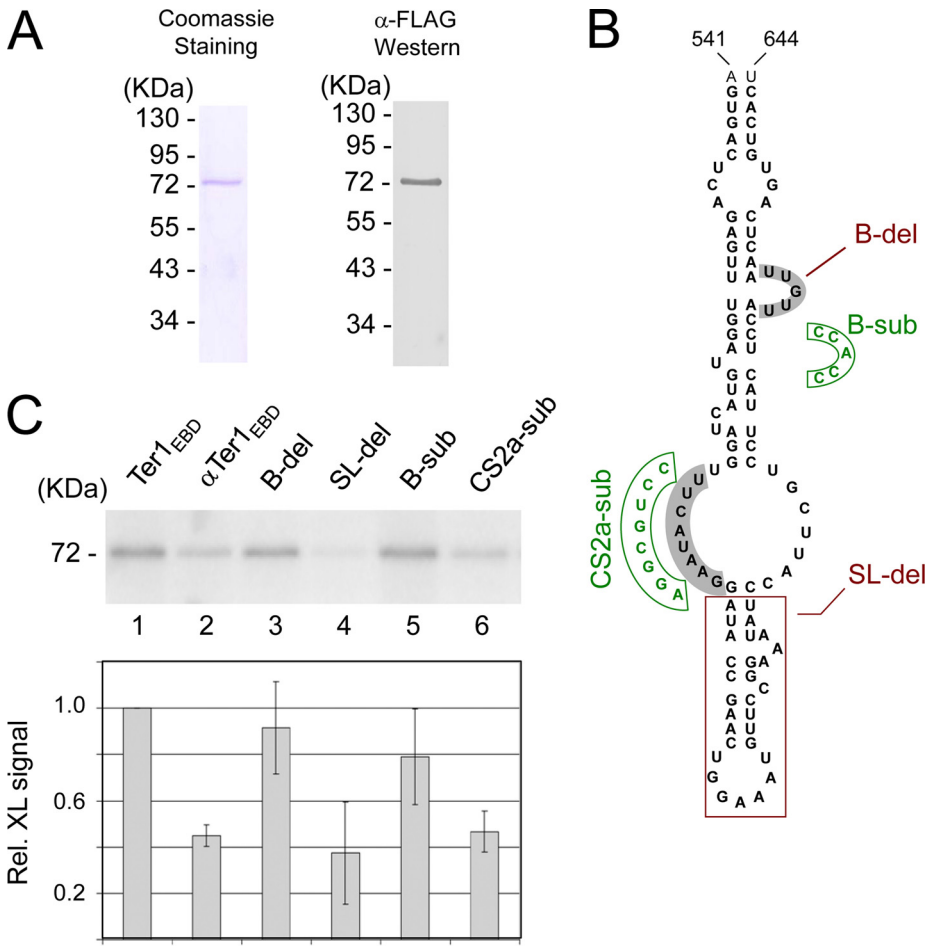

FIG 4 *Kl*Est1 interacts directly with the putative Est1 binding site of telomerase RNA. (A) $Est1_{NTD}$ was expressed in *E. coli* and purified as described in Materials and Methods. The purified protein was analyzed by SDS-PAGE, followed by Coomassie staining (left) or Western blotting using antibodies against the FLAG tag fused to the C terminus of the recombinant protein (right). (B) The secondary structure of $Ter1_{EBD}$ as predicted by RNAfold is displayed. The mutations tested as shown in panel C, including B-del, SL-del, B-sub, and CS2a-sub, are indicated. (C) Purified $Est1_{NTD}$ (12.5 nM) was incubated with ^{32}P-labeled $Ter1_{EBD}$, antisense $Ter1_{EBD}$ (α-$Ter1_{EBD}$), or mutants of $Ter1_{EBD}$ (16 nM) and subjected to UV irradiation and RNase digestion. The products were analyzed by SDS-PAGE and PhosphorImager scanning. Representative assays are shown at the top and the summary data from three independent experiments are shown at the bottom.

comparable mutations on *S. cerevisiae* Est1-Tlc1 interactions. Nevertheless, to minimize the possibility that our system overdiagnoses RNA-binding defects, we characterized an *est1* mutant (K467E) that is analogous to the *est1-60* allele of *S. cerevisiae EST1* (K444E), previously shown to impair telomerase recruitment and activation but not RNA binding (33). As predicted, the *K. lactis* mutant manifested substantial telomere loss but normal Est1-Ter1 association in cell extracts (see Fig. S3 in the supplemental material). This result further suggests that the Cdc13-Est1 interaction previously described in *S. cerevisiae* is at least partially conserved in *K. lactis*.

***Kl*Est1 interacts directly with the Est1 binding domain of Ter1.** *S. cerevisiae* Est1 was initially reported to possess a nonspecific RNA-binding activity (45). However, further studies *in vivo* and *in vitro* implicated a specific region of Tlc1 (the *S. cerevisiae* telomerase RNA) in Est1 association (10, 38). In particular, a bulged stem in this region of Tlc1 appears to be a critical determinant of Est1-Tlc1 interaction. Based on potential sequence and structural similarities between this region of Tlc1 and other yeast telomerase RNAs, a conserved Est1 binding domain (EBD) has been proposed (17). The EBD, which is typically ~100 nt long, consists of several stems and loops located 3′ to the template region of telomerase RNA. The putative EBD in *K. lactis* Ter1 has been shown to be required telomere maintenance *in vivo* (17). However, its ability to interact with Est1 has not been investigated. We therefore sought to determine biochemically if *Kl*Est1 can interact directly with $Ter1_{EBD}$. Because the CTD of *Kl*Est1 is prone to proteolytic degradation in *E. coli* (data not shown) and is dispensable for telomere maintenance (Fig. 3A), we recombinantly expressed and purified $Est1_{NTD}$ with a C-terminal FLAG tag (Fig. 4A). The protein was first subjected to electrophoretic mobility shift assays (EMSAs) using $Ter1_{EBD}$ as the probe. Despite using a variety of published assay conditions, we were unable to detect complex formation between Est1 and Ter1 (data not shown) (10, 45). This observation is in agreement with an earlier study on *Sc*Est1 but at odds with another report (10, 38). However, by employing a UV cross-linking assay, we were able to demonstrate a direct physical interaction between $Est1_{NTD}$ and $Ter1_{EBD}$. To detect cross-linking, purified $Est1_{NTD}$ was incubated with uniformly labeled $Ter1_{EBD}$, and the mixture was subjected to UV irradiation followed by RNase digestion, gel electrophoresis, and PhosphorImager scanning. Consistent with a covalent linkage between

$Est1_{NTD}$ and $Ter1_{EBD}$, an ~75-kDa labeled species can be detected (Fig. 4C, lane 1). $Ter1_{EBD}$ was a preferred binding target because the antisense $Ter1_{EBD}$ ($\alpha Ter1_{EBD}$) exhibited reduced cross-linking efficiency (Fig. 4C, compare lanes 1 and 2). We then tested four $Ter1_{EBD}$ mutants that had been shown previously to cause telomere shortening (17) and found that two of these mutants (SL-del and CS2a-sub) cross-linked to Est1 with reduced efficiency (Fig. 4B and C). The magnitude of reduction was about 60% for each mutant. A similar difference in the cross-linking efficiency of the wild-type and mutant RNAs was observed when we varied the RNA concentrations from 2 to 16 nM (see Fig. S4 in the supplemental material). The correlation between the defects of the Ter1 mutants in Est1 binding and telomere maintenance suggests that the direct RNA-binding activity of Est1 is required for telomere maintenance.

We have demonstrated that mutations in the TPR and DSH subdomains of Est1 caused defects in Ter1 association in cell extracts (Fig. 3C). To investigate whether the defects can be accounted for by deficiencies in the direct binding activity of Est1, we purified the $Est1_{NTD}$ RR and RHRQ mutants and subjected them to the UV cross-linking assay using wild-type $Ter1_{EBD}$, antisense $Ter1_{EBD}$, and the CS2a-sub mutant as the probes. As shown in Fig. 5A, compared to the wild-type protein, the cross-linking efficiencies of both mutant proteins to $Ter1_{EBD}$ were reduced about 3-fold (lanes 1, 4, and 7). The mutant proteins nevertheless prefer $Ter1_{EBD}$ to the antisense control and the CS2a-sub mutant by the same magnitude as the wild-type protein (compare lanes 4 to 6 and lanes 7 to 9 to lanes 1 to 3). Hence, these residues within the TPR domain appear to contribute to the affinity but not the sequence specificity of telomerase RNA binding; if these residues contribute to specificity, then one would expect the mutant proteins to exhibit reduced preference for wild-type RNA.

We next attempted to determine if the TPR or DSH subdomain alone can be cross-linked to $Ter1_{EBD}$. However, we were only able to purify adequate quantities of *Kl*$Est1_{TPR}$ (also with a C-terminal FLAG tag) for the analysis, *Kl*$Est1_{DSH}$ being largely insoluble when expressed in *E. coli*. As shown in Fig. 5B, whereas *Kl*$Est1_{NTD}$ exhibited robust cross-linking to $Ter1_{EBD}$, the same level of *Kl*$Est1_{TPR}$ failed to generate any significant amount of labeled products. Our data thus suggest that the TPR subdomain alone interacts with Ter1 with greatly reduced affinity, if at all. The cross-linking analyses of the substitution mutants and the TPR subdomain both support the notion that the *Kl*Est1-Ter1 association in cell extracts is due at least in part to a direct physical interaction between these two molecules.

*Sc*Est1 has been shown to bind telomere G-strand repeats with moderate affinity (10, 45). We therefore tested purified *Kl*$Est1_{NTD}$ for binding to *K. lactis* telomere repeats using both EMSA and photo-cross-linking. Despite using a variety of target DNA and assay conditions, we were unable to detect significant DNA binding by *Kl*$Est1_{NTD}$, suggesting that this activity is not a conserved property of Est1 orthologues (data not shown).

***Kl*Est1 mediates the functions of *Sc*Ebs1.** As described earlier, *Sc*Ebs1 but not *Sc*Est1 has been shown to render yeast cells rapamycin sensitive, possibly through a role in the NMD pathway (14, 24). We therefore analyzed the rapamycin sensitivity of our *Kl*Est1 mutants. Consistent with a role for *Kl*Est1 in NMD, the *est1*Δ mutant reconstituted with an empty vector exhibited greater resistance to rapamycin than that reconstituted with full-length *Kl*Est1-TAP (Fig. 6A). Interestingly, deleting the CTD abolished

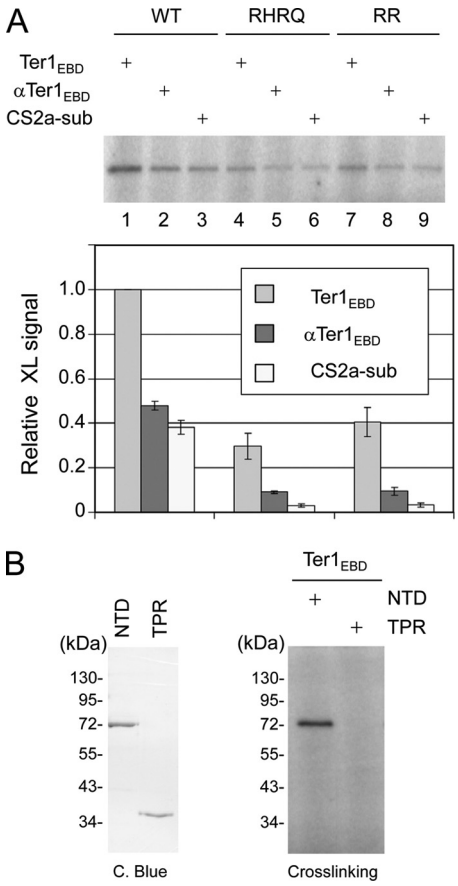

FIG 5 Analysis of $Ter1_{EBD}$ binding by *Kl*Est1 mutants. (A) (Top) Purified wild-type $Est1_{NTD}$ and the RR and RHRQ mutants were subjected to UV irradiation in the presence of the indicated RNAs. The products were subjected to RNase digestion and analyzed by SDS-PAGE and PhosphorImager scanning. (Bottom) The relative signals from three independent experiments were calculated and plotted. (B) Equal molar amounts of purified $Est1_{NTD}$ and $Est1_{TPR}$ were analyzed by SDS-PAGE and Coomassie staining (left) and then subjected to the UV cross-linking assay with ^{32}P-labeled $Ter1_{EBD}$.

almost completely the sensitivity of the reconstituted strain. In addition, neither the TPR nor the DSH subdomain alone was capable of conferring rapamycin sensitivity to the reconstituted strain. We conclude that even though the CTD of *Kl*Est1 is dispensable for telomere maintenance, it is crucial for the role of this protein in the rapamycin-responsive pathway(s). The contribution of *Kl*Est1 to NMD is further supported by measurements of the levels of CYH2 pre-mRNA, a putative NMD target (24). Consistent with an NMD defect, the amount of the CYH2 pre-mRNA in the *est1*Δ mutant was approximately three times that in the wild-type strain (Fig. 6B).

DISCUSSION

Our analysis of *Kl*Est1 has provided insights on the evolution and mechanisms of Est1 family members, including the demonstration of (i) a direct physical interaction between Est1 and telomerase RNA, (ii) a conserved role for the TPR and DSH domain in this interaction, and (iii) a widespread function of Est1 family members in non-sense-mediated decay. The implications of these findings are discussed below.

Activity conservation between *Sc*Est1 and *Kl*Est1. The initial

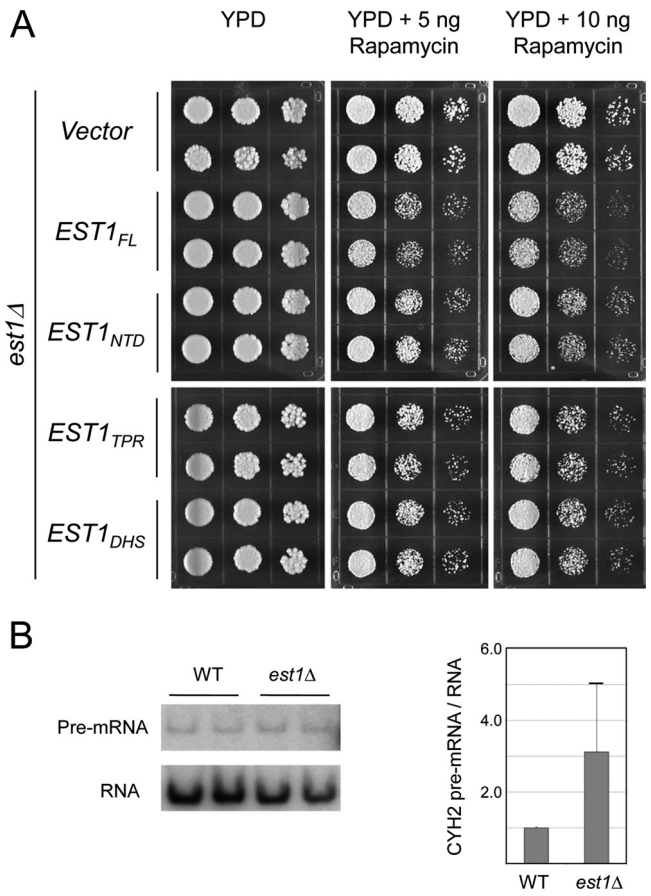

FIG 6 Est1 confers rapamycin sensitivity to the cells. (A) The *est1*Δ null strain reconstituted with wild-type and mutant *EST1*s was spotted on YPD plates without rapamycin and grown at 30°C for 2 days or with the indicated concentrations of rapamycin and grown for 4 days. (B) The levels of CYH2 pre-mRNA and RNA in the wild-type and *est1*Δ strains were measured by RT-PCR. A representative set of assays is displayed (left). The results from two experiments were quantified and plotted (right).

investigation of *Sc*Est1 suggests that the protein possesses a high-affinity but nonspecific RNA-binding activity, as well as a G-strand DNA-binding activity (45). Subsequent studies using pulldown of RNA by Sepharose-bound Est1 and RNA EMSA indicate at least some preference of *Sc*Est1 for its presumed target site in Tlc1 (*S. cerevisiae* telomerase RNA) (10, 38). The authenticity of the DNA-binding activity is less clear. No mutations that selectively abolish the DNA-binding activity are available to assess the physiologic relevance of this activity. In addition, one study suggests that excess RNA can inhibit the formation of an Est1-DNA complex, hinting at a single nucleic acid binding site on this protein (10). Our analysis of *Kl*Est1 clearly establishes RNA binding as a conserved and functionally important property of Est1 homologues. Conversely, our failure to detect a DNA-binding activity in *Kl*Est1 casts some doubts on the significance of this activity.

The RNA-binding activity of *Kl*Est1. Our data argue that the direct binding of *Kl*Est1$_{NTD}$ to Ter1$_{EBD}$ is a key function of this protein. But surprisingly, this interaction appears to be relatively weak and non-sequence specific. In contrast to a previous report on *Sc*Est1, we were unable to detect a *Kl*Est1$_{NTD}$-Ter1$_{EBD}$ EMSA complex using a variety of assay conditions. Moreover, the cross-linking efficiency of Est1$_{NTD}$ to the wild-type EBD was only 2- to 3-fold higher than a variety of inactive control and mutant RNAs. At first glance, such a weak and nonspecific interaction would appear inadequate to underpin the role of *Kl*Est1 in telomere maintenance. However, contacts between *Kl*Est1 and other telomerase components may provide the additional specificity necessary for the assembly of the holoenzyme. There is compelling support, for example, for an interaction between Est1 and Est3 (another telomerase subunit) in both *S. cerevisiae* and *C. albicans* (22, 43). Another potential reason for the low sequence specificity of *Kl*Est1 may be related to its role in NMD, which could involve its binding to other RNA targets with distinct sequences and structures.

As noted before, the structure of *Kl*Est1$_{NTD}$ is likely to be largely alpha-helical and to contain a TPR-like subdomain (Fig. 1B). Because TPR domains are known to mediate diverse cellular functions through interaction with target peptides using its concave surface, the potential involvement of this structural module in RNA binding is unexpected (1, 8). To gain insights on the mechanisms of RNA binding, we generated a homology model of *Kl*Est1$_{NTD}$ and mapped the positions of functionally important residues (see Fig. S5 in the supplemental material). Notably, while the three clusters of residues implicated in RNA binding are scattered in different surface areas, they are all located away from the putative peptide-binding, concave groove. This observation suggests that Est1 could use distinct, nonoverlapping surfaces for binding peptide and RNA targets. One cluster of residues required for RNA binding is located in a loop protruding from the edge of the TPR (RR), another is on the convex surface (QKR), and a third one is near the end of the DSH domain (KF). Assuming that all three clusters contact RNA directly, the rather large spatial separations between them imply a large RNA target site, which is consistent with the proposed Ter1$_{EBD}$.

While this work was under review, Webb and Zakian reported an unbiased search for mutations that reduce *S. pombe* Est1-Ter1 interaction (47). Notably, they also identified residues in the TPR domain as being required for the interaction. Indeed, one of the amino acids implicated in fission yeast Ter1 binding (R194) aligns to K223 of *Sc*Est1 and K220 of *Kl*Est1, which we and others have shown to be involved in the corresponding interactions in budding yeast (reference 11 and this work) (see Fig. S6 in the supplemental material). This striking mechanistic conservation in turn suggests that Ter1 binding was an ancestral function of yeast Est1.

In addition to binding RNA, budding yeast Est1 is likely to interact with multiple protein targets, including Cdc13, Est3, and other less-well-characterized factors (37, 43, 50, 51). Data from mutational analysis suggest that both the TPR and DSH domains are involved in these interactions as well (33, 34, 37, 51). How the protein and RNA interaction functions of Est1 are coordinated is clearly worthy of further investigation.

Evolution of Est1 homologues. As described earlier, *Sc*Est1 and *Sc*Ebs1 are evidently ohnologs that emerged as a consequence of whole-genome duplication and perform distinct functions in telomere maintenance and NMD in *Saccharomyces cerevisiae*. An obvious question, then, is whether their functional divergence represents a case of subfunctionalization or neofunctionalization (7, 18). In other words, did the ancestral protein perform both functions, which were then delegated to different descendants? Or did one of the descendants evolve new activities? Our finding that *Kl*Est1 participates in both telomere maintenance and NMD reg-

ulation supports the notion of subfunctionalization. Specifically, our results suggest that Est1 and Ebs1 together exemplify the duplication, degeneration, and complementation mechanisms of subfunctionalization, which also apply to other telomere-related proteins such as Sir2/Hst1 (13, 19). Indeed, the observation that human Est1 homologues have roles in both pathways argues that these functions are inherent to the ancient Est1 that existed prior to the divergence of yeasts and mammals. The fixation of *EST1* paralogs in evolutionarily distant lineages suggests that the multiplicity of functions performed by the ancestral gene may be difficult to optimize in a single polypeptide.

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