Est1 has the properties of a single-stranded telomere end-binding protein

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In *Saccharomyces cerevisiae*, deletion of the *EST1* gene results in phenotypes identical to those displayed by a deletion of a known component of telomerase (the yeast telomerase RNA), arguing that *EST1* is also critical for telomerase function. In this study, we show that the Est1 protein binds to yeast G-rich telomeric oligonucleotides in vitro. Binding is specific for single-stranded substrates and requires a free 3′ terminus, consistent with the properties expected for a protein bound to the 3′ single-stranded G-rich extension present at the telomere. Assessment of the in vivo function of this single-stranded DNA-binding protein has shown that *EST1* acts in the same pathway of telomere replication as the *TLC1* telomerase RNA, by several different genetic criteria: *est1 tlc1* double mutant strains show no enhancement of phenotype relative to either single mutant strain, and *EST1* dominant mutations have an effect on telomeric silencing similar to that displayed by *TLC1* previously. We propose that Est1 is a telomere end-binding protein that is required to mediate recognition of the end of the chromosome by telomerase.

[*Key Words:* Est1; telomeres; senescence; DNA binding; telomerase]

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The synthesis and maintenance of telomeres requires the enzyme telomerase as well as other telomeric DNA binding proteins (for review, see Blackburn and Greider 1995; Zakian 1995; Greider 1996). Telomerase is a specialized reverse transcriptase that elongates the G-rich strand of chromosomal termini by using a region of its internal RNA component as a template to dictate the sequence of the newly synthesized telomeric DNA (Greider and Blackburn 1989; Yu et al. 1990; Singer and Gottschling 1994; Feng et al. 1995; McEachern and Blackburn 1995). After the G-rich strand is elongated by telomerase, the complementary C-rich strand is thought to be replicated by conventional DNA polymerases, although telomere-specific proteins may regulate this process (Zahler and Prescott 1989; Vermeesch and Price 1994).

The end of the chromosome has been shown in a number of species to terminate with a single-stranded extension of the G-rich strand, which is complexed with proteins specific for this structure (for review, see Fang and Cech 1995; Henderson 1995). The most well characterized telomere end-binding proteins are from the ciliates *Oxytricha* and *Euplotes*, which specifically bind the 3′ single-stranded extension of the T_4G_4-containing strand, thereby protecting the telomeric DNA from *Bal*31-mediated nuclease digestion and chemical modification (Gottschling and Zakian 1986; Price and Cech 1987; Price 1990). Similar factors with the properties expected of a terminus-specific binding activity have also been identified in extracts from *Xenopus* and *Tetrahymena* (Cardenas et al. 1993; Sheng et al. 1995). These proteins potentially could play multiple roles in vivo at the telomere: They have been proposed to provide a cap against degradation of the telomere and they may regulate telomerase, either positively or negatively (Gray et al. 1991; Price 1992; Shippen et al. 1994; Vermeesch and Price 1994). However, the absence of reverse genetic techniques in these organisms has prevented a direct test of either of these hypotheses. Yeast has also been predicted to have telomere end-binding proteins, based primarily on genetic criteria (Stavenhaven and Zakian 1994; McEachern and Blackburn 1995; Wiley and Zakian 1995), but efforts to identify proteins that display the expected features of an end-binding activity and an in vivo role in telomere maintenance have not been successful (Lin and Zakian 1994; Konkel et al. 1995).

In addition to telomere end-binding proteins, the enzyme telomerase must also bind the 3′ terminus of the extended G-rich strand to replicate and maintain the telomere. This enzyme has been studied most extensively in *Tetrahymena*; only in this species have both the RNA component as well as two protein subunits of the core enzyme been cloned (Greider and Blackburn 1989; Collins et al. 1995). In *Saccharomyces cerevisiae*, efforts to identify protein subunits of telomerase, like the attempts to identify a terminus-specific factor, have so far been unsuccessful. The best-characterized candidate for a telomerase protein component is the *EST1*

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gene of *S. cerevisiae*. This hypothesis has been based on the genetic phenotypes exhibited by *est1*$^-$ strains: Deletion of *EST1* results in progressive telomere shortening and a senescence growth phenotype, characteristics originally predicted for a defect in telomerase (Lundblad and Szostak 1989). These phenotypes are indistinguishable from those displayed by a strain with a deletion of the yeast telomerase RNA (Singer and Gottschling 1994; this work), arguing that *EST1* is also essential, either directly or indirectly, for telomerase function. However, analysis of whether extracts prepared from *est1*$^-$ strains are defective for telomerase activity has produced conflicting results. One report indicated that Est1 was essential for enzymatic activity (Lin and Zakian 1995), whereas another study found that telomerase activity was still present at roughly wild-type levels in fractionated extracts prepared from a strain in which the *EST1* gene was deleted (Cohn and Blackburn 1995). Although this contradiction could be the consequence of different assay conditions, the latter data argue that Est1p is not an essential part of the catalytic core of the enzyme (but do not exclude the possibility that Est1 is a noncatalytic component of telomerase). A second set of studies has shown that the Est1 protein can be coimmunoprecipitated with the yeast telomerase RNA (Lin and Zakian 1995; Steiner et al. 1996). Because these experiments did not monitor the stoichiometry of the Est1–RNA interaction, these studies could not be used to definitively argue whether Est1 was an integral component of the telomerase complex. Therefore, these studies have left open the precise role of Est1 at the telomere.

We have analyzed purified Est1 protein to determine whether it exhibits biochemical properties consistent with a direct role at the telomere. This approach has revealed that Est1 has the properties of a single-stranded telomere DNA binding protein, with a specific requirement for a free 3′ terminus. Genetic analysis comparing *EST1* and *TLC1* has also demonstrated that *EST1* is required for the telomerase-mediated pathway for maintaining the telomere. These results indicate that *EST1* mediates the interaction of telomerase with the chromosomal terminus via its single-stranded end-binding activity.

Results

Est1 protein binds to single-stranded yeast telomeric DNA

To determine whether Est1 acts directly at the telomere, we asked whether Est1 protein could bind yeast telomeric substrates in vitro. Recombinant Est1 protein containing a $(His)_6$ tag at the amino terminus was purified as a soluble protein from *Escherichia coli* by Ni-NTA–agarose affinity purification. The resulting eluate exhibited a single prominent band (Fig. 1A), which was not present in mock preparations from a strain carrying a vector with no insert (data not shown). Immunological detection of the recombinant Est1 protein (with anti-gene 10 antisera to detect a T7 gene 10 epitope fused in-frame at the Est1 amino terminus) demonstrated that the purified protein

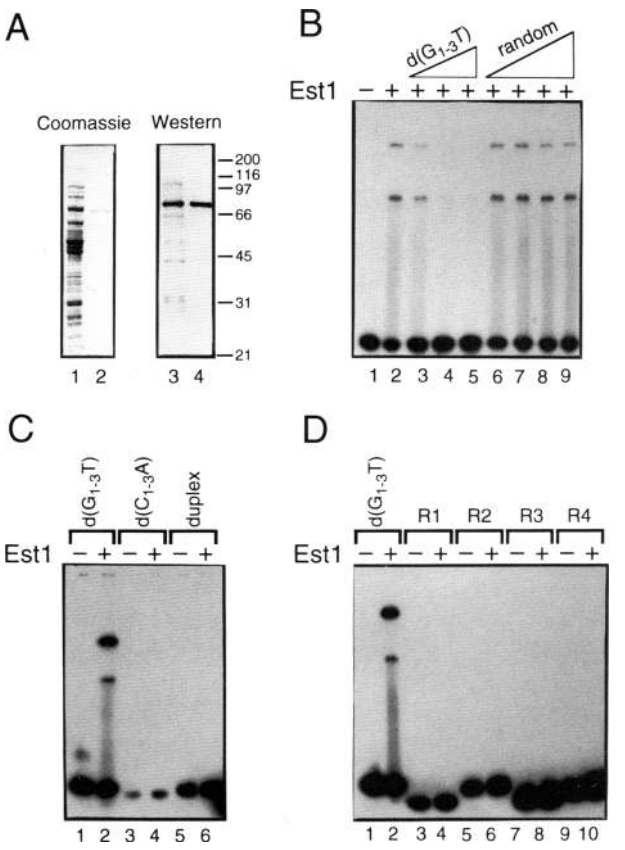

Figure 1. Est1 protein binds yeast single-stranded telomeric substrates. (*A*) Amino-terminal $(His)_6$ tagged-Est1 protein was affinity purified as described in Materials and Methods. Extract supernatant (lanes *1*,*3*) and the resulting Est1-containing eluate (lanes *2*,*4*) separated by SDS–PAGE are shown after Coomassie blue staining and Western detection. (*B*) Gel mobility shift assays with 200 nM purified Est1 protein and 50 pM end-labeled $d(TGTGTGGG)_3$; unlabeled $d(G_{1-3}T)$ competitor was present at 0.2 nM (4×), 1.0 nM (20×), and 5.0 nM (100×), and random sequence competitor (R1) at 0.2 nM, 1.0 nM, 5.0 nM, and 10.0 nM. (*C*,*D*) Gel mobility shifts using the same reaction conditions as above, but with a different preparation of Est1p. The sequences of the substrates (all at 50 pM) are as follows: $d(G_{1-3}T) = d(TGTGTGGG)_3$; $d(C_{1-3}A) = d(CCCACACA)_3$; duplex = $d(TGTGTGGG)_3/d(CCCACACA)_3$; R1 = d(CACTATCGACTACGCGATCA); R2 = d(AGCGGATAACAATTTCACACAGGA); R3 = d(TAATACGACTCACTATAGGGAGA); R4 = d(GGTCGTCAGACTGTCGATGAAGCC). Although the signal in lanes *3* and *4* in *C* is under-represented, darker exposures of this and similar experiments showed no binding to the $d(C_{1-3}A)$ substrate.

was in fact Est1 (Fig. 1A). The apparent molecular weight of the purified protein was ~70 kD, somewhat smaller than the 86 kD predicted for the recombinant protein. However, Est1 protein preparations of identical observed molecular weight were obtained when the $(His)_6$ tag and gene 10 epitope were at the carboxyl terminus (data not shown), indicating that the discrepancy in size was not attributable to premature translation stops. The amino

terminal- and carboxy terminal-tagged Est1 proteins showed equivalent binding and competition behavior with telomeric and nontelomeric substrates (Fig. 5, below, and data not shown).

Figure 1B shows that Est1 protein formed specific complexes with a single-stranded yeast telomeric oligonucleotide d(TGTGTGGG)$_3$, corresponding to a portion of a *S. cerevisiae* chromosomal telomere sequence (Shampay et al. 1984; Walmsley et al. 1984). Complex formation was specific for the Est1 protein, as no binding was observed with eluate from a mock extract (Fig. 5, below). Est1p was capable of binding to several different variations of the yeast d(G$_{1-3}$T) telomeric sequence (Fig. 4, below, and data not shown) but did not interact with the complementary C-rich telomeric strand or with a telomeric DNA substrate that was fully duplex (Fig. 1C). Binding was specific for the yeast G-rich oligomer, in that addition of excess cold yeast telomeric oligo competed effectively for formation of the Est1–telomeric DNA complex (Fig. 1B and 6B, below). In contrast, addition of ≤200-fold molar excess of a single-stranded random sequence oligomer did not compete for binding (Fig. 1B), and no binding was observed with four random sequence oligomers similar in size to the telomeric oligo, with G-content ranging from 15% to 30% (Fig. 1D).

The mobility shift experiments in Figure 1 showed two different complexes forming with Est1 protein. To investigate this in more detail, we examined the pattern of complex formation with increasing amounts of Est1 protein, while holding the d(TGTGTGGG)$_3$ oligomer concentration constant. Figure 2 shows that at low concentrations of Est1 relative to substrate, only the faster migrating complex was formed, but increased protein concentration resulted in the appearance of a slower migrating complex, with a reduction in the faster migrating complex. This observation is consistent with a transition to a multimeric Est1 complex at higher protein concentrations, although these data do not address the oligomerization state of either complex. A second observation pertinent to the two complexes was the presence of a continuous diffuse signal present below the lower band. The signal was diffuse rather than a series of discrete bands and continued to the position of unbound labeled oligo, which is not consistent with a set of breakdown products of Est1 binding to the telomeric oligomer. Instead, it is most likely attributable to dissociation of the faster migrating complex during migration through the acrylamide gel and subsequent release of the labeled oligo. This indicates that the faster migrating complex is less stable than the slower migrating complex under these electrophoresis conditions. From quantitation of the titration experiment, we determined that the observed dissociation constant for Est1 binding to the telomeric DNA oligomer is 250 nM, a relatively weak affinity for DNA. However, it is comparable to that of the *Oxytricha* α-subunit by itself for single-stranded telomeric DNA, whereas the complete *Oxytricha* telomere-binding heterodimeric complex binds telomeric DNA much tighter (Fang et al. 1993); Est1p may also have a binding partner that increases its affinity for DNA.

Binding requires a free 3′ terminus

In vivo, the end of the chromosome terminates with the G-rich strand protruding as a 3′ single-stranded extension (Klobutcher et al. 1981; Pluta et al. 1982; Henderson and Blackburn 1989; Wellinger et al. 1993, 1996). If the Est1 binding activity described above reflects an in vivo

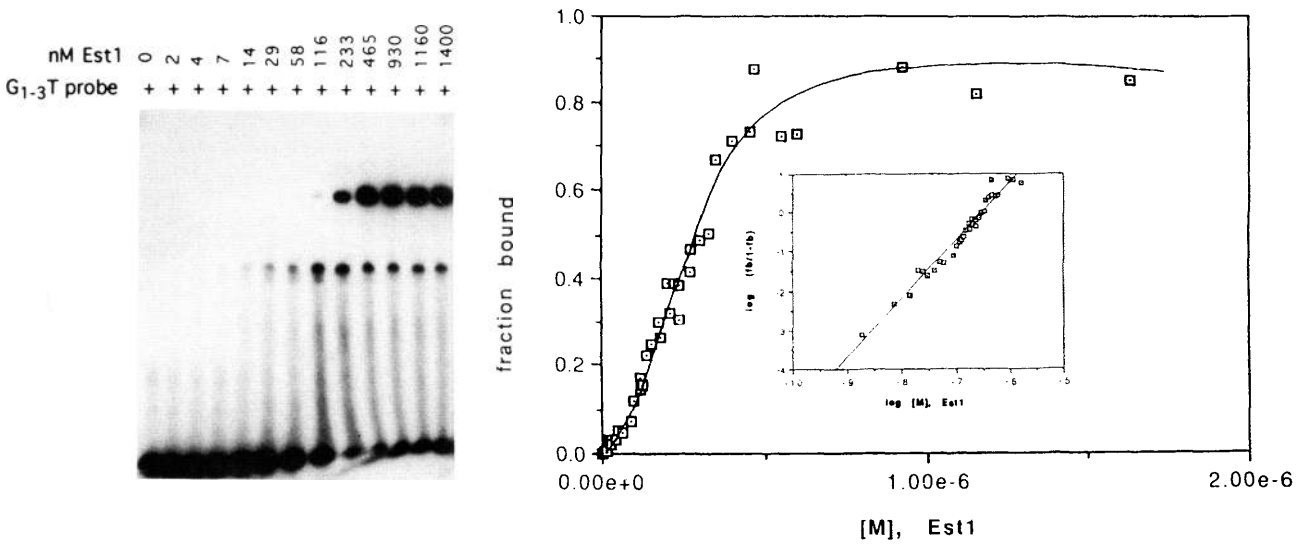

Figure 2. Est1 protein forms two complexes with yeast telomeric oligomers. Telomeric DNA gel shifts were performed using a fixed concentration (50 pM) of the d(TGTGTGGG)$_3$ telomeric DNA oligomer and varying the concentration of Est1 from 0 to 1400 nM. Quantitative data from PhosphorImager analysis of more than four independent gel mobility shifts were used to determine equilibrium binding curve (plotted as the fraction of DNA bound vs. the EST1 concentration) and the resulting Hill plot (insert); a representative gel shift is shown.

role at the 3′ end of the chromosome, one prediction is that this activity would require a free 3′ single-stranded terminus. To test this, completely single-stranded substrates were compared with partially duplex substrates in the gel shift binding assay. (5′R)-Ye and Ye-(3′R) are two single-stranded yeast telomeric oligonucleotides with 15 nucleotides of nontelomeric sequence added to the 5′ and 3′ termini of the d(TGTGTGGG)$_3$ oligonucleotide, respectively. Both bound to Est1 protein in a direct binding test (Fig. 3), although Ye-(3′R) was not capable of forming the faster migrating complex with Est1p (discussed below). Conversion of (5′R)-Ye to a molecule with 15 bp of duplex at the 5′ end reduced binding only slightly relative to the fully single-stranded (5′R)-Ye oligo (Fig. 3, cf. lanes 4 and 8). In contrast, masking the single-stranded 3′ terminus of Ye-(3′R) with 15 bp of duplex DNA completely eliminated binding of Est1 to this substrate (Fig. 3, cf. lanes 12 and 16). This demonstrates that Est1 requires a free single-stranded 3′ end for binding to telomeric substrates, consistent with a role for Est1 as a telomere end-binding protein in vivo.

Est1 binds other G-rich telomeric substrates to variable extents

As telomerase is capable of elongating telomeric primers from different organisms (Greider and Blackburn 1985; Morin 1989; Prowse et al. 1993; Mantell and Greider 1994), we examined whether Est1 was capable of binding to nonyeast G-rich telomeric sequences. Single-stranded oligonucleotides corresponding to the *Tetrahymena*, human, and *Oxytricha* telomeric sequences were recognized to varying extents by Est1 (Fig. 4A,B). Little or no complex formation was observed with both *Tetrahymena* and human telomeric oligos, whereas the *Oxytricha* d(G$_4$T$_4$)$_3$ telomeric sequence [assayed at a fourfold higher oligo concentration, relative to the d(G$_{1-3}$T) oligomer] appeared to bind Est1 to roughly the same degree as did the *S. cerevisiae* sequence (Fig. 4B and data not shown). The efficiencies of these particular telomeric sequences to seed new telomere formation in vivo have not been tested relative to each other in the same experiment, although all three types of telomeric sequences provide a substrate at some level for the addition of yeast telomeric repeats (Pluta et al. 1984; Brown 1989; Lustig 1992). However, in an in vivo assay that semiquantitatively measures telomere healing onto yeast plasmids terminating with synthetic sequences, a d(G$_{1-3}$TA) oligo (related to the human repeat sequence tested here), and *Tetrahymena* telomeric sequences have been shown to be poor substrates relative to d(G$_{1-3}$T) (Lustig 1992).

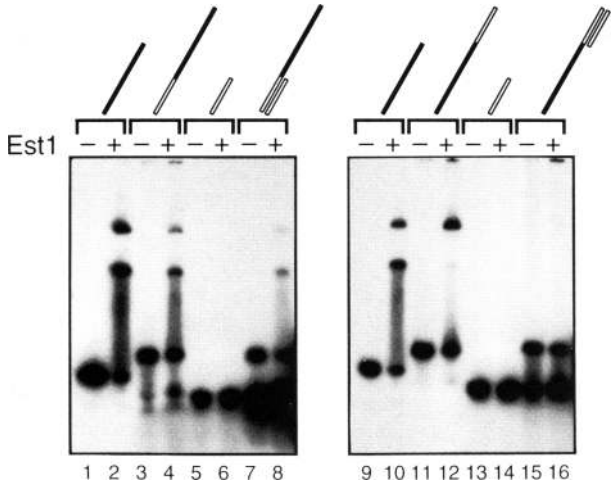

Figure 3. Binding is specific for a free 3′ terminus. Est1-telomeric DNA gel shifts of single-stranded and partially duplex substrates; see Materials and Methods for the details of the labeling of the partially duplex substrates. (Lanes *1,2,9,10*) d(TGTGTGGG)$_3$; (lanes *3,4*) (5′R)-Ye = d[CCGGTAGTGTCCTGG(TGTGTGGG)$_3$]; (lanes *5,6*) 5′R = d(CCAGGACACTACC); (lanes *7,8*) (5′R)-Ye annealed to 5′R; (lanes *11,12*) Ye-(3′R) = d[(GGGTGTGT)$_3$GGTCCTGTGATGGCC]; (lanes *13,14*) 3′R = d(GGCCATCACAGGACC); (lanes *15,16*) Ye-(3′R) annealed to 3′R. Although all oligos are shown here at 50 pM, no complex formation was observed with Ye-(3′R) annealed to 3′R even at 200 pM. (Solid bar) G-rich telomeric DNA; (open bar) nontelomeric DNA.

To further explore the sequence specificity of Est1p telomeric binding, several variants of the yeast telomeric oligo, d(TGTGTGGG)$_3$, were also tested. These oligos were assayed at 200 pM, fourfold higher than the concentration used with the yeast d(G$_{1-3}$T) substrate, to help visualize the formation of weak complexes. The substrate d[GTG(TGTGGG)$_3$TGT], another variant of the d(G$_{1-3}$T) sequence found at yeast telomeres, was equally competent at Est1 complex formation (Fig. 4C and data not shown). However, the d(TG$_{1-5}$) and d(TG)$_n$ oligos showed reduced binding to Est1, and binding was almost completely abolished with a d(G$_{1-3}$T$_{1-3}$) oligo (Fig. 4C). This shows that altering the sequence even slightly from that found in vivo at a yeast telomere, although still maintaining the same number of G and T residues as found in d(TGTGTGGG)$_3$, has substantial effects on the ability to bind Est1 protein.

For several of the non-d(G$_{1-3}$T) oligomers that were capable of binding Est1, only the slower migrating complex was observed (lanes 6 and 10 in Fig. 4B; lanes 6,8, and 10 in 4C, and lane 12 in Fig. 3). The absence of the faster migrating complex suggests that formation of this less stable complex is further destabilized with less optimal substrates. In the case of the Ye-(3′R) substrate, which only differs from d(TGTGTGGG)$_3$ by the addition of nontelomeric sequences at the 3′ terminus, this argues that the interaction of Est1 with 3′ d(G$_{1-3}$T) sequence contributes to the stability of this complex. Although this hypothesis may also account for the absence of the faster migrating complex with some of the non-d(G$_{1-3}$T) substrates in Figure 4, we do not yet fully understand the substrate-dependent formation of these two complexes.

Est1 DNA binding maps to a carboxy-terminal region of the protein

To determine whether telomeric DNA binding activity

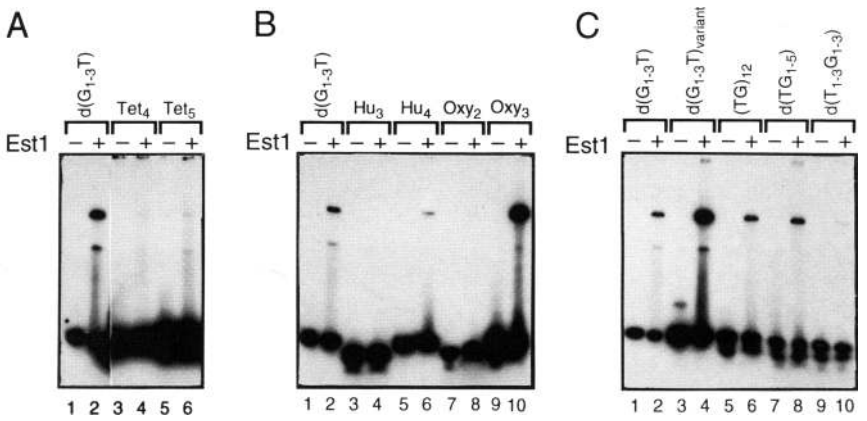

Figure 4. Est1p binds to nonyeast telomeric oligonucleotides. DNA gel shift analyses used 200 nM Est1 protein and end-labeled single-stranded oligos. All oligos were used at 200 pM, to help visualize weak complex formation, with the exception of the d(G$_{1-3}$T) oligo, which was at 50 pM; the same Est1 preparation (also used in Fig. 1C and 1D) was used for *A–C*. The substrates were d(G$_{1-3}$T) = d(TGTGTGGG)$_3$; Tet$_4$ or Tet$_5$ = 4 or 5 repeats of the *Tetrahymena* telomeric repeat sequence d(T$_2$G$_4$); Oxy$_2$ and Oxy$_3$ = 2 or 3 repeats of the *Oxytricha* telomeric repeat sequence d(T$_4$G$_4$); Hu$_3$ or Hu$_4$ = 3 or 4 repeats of the human telomeric repeat sequence d(T$_2$-AG$_3$); d(G$_{1-3}$T) variant = d[GTGl(TGTG$_3$)$_3$TGT]; d(TG)$_{12}$; d(TG$_{1-5}$) = d(TGTG$_4$TGTGTG$_5$TGTGTGT); d(T$_{1-3}$G$_{1-3}$) = d(G$_3$TGT$_2$G$_3$TGTG$_3$T$_3$GTG$_3$).

could be mapped to a specific region of the Est1 protein, we constructed a panel of six deletion mutations that each removed ~110 amino acids. The in-frame deletions were constructed in an *EST1*-containing vector with the (His)$_6$ tag at the carboxyl terminus to ensure that the proteins isolated after affinity purification were the desired deletion derivative and not a truncated version resulting from an unstable protein. Five of the six deleted proteins were still capable of binding telomeric DNA to a degree roughly comparable to that of the intact protein (Fig. 5). One deletion derivative, Est1p–Δ14, which removed 130 amino acids (amino acids 435–565), had greatly diminished DNA binding, indicating that the determinants for telomere DNA recognition reside in this region of the protein. The deletion encompassing this region was also defective in vivo for *EST1* activity, as assayed genetically by a complementation assay, although this defect was not specific to this deletion; *est1*-Δ12 and *est1*-Δ13 were also defective in vivo, whereas *est1*-Δ15 retained partial function (data not shown). The region defined by Δ14 may also contain additional properties essential for *EST1* activity, as two missense mutations in this region that were capable of DNA binding in vitro were defective for *EST1* function in vivo; these two mutations are discussed in more detail below. The 130-amino-acid region defined by this deletion analysis does not contain any previously identified DNA binding motifs; in particular, no sequence similarity has been observed between Est1p and previously identified proteins that bind to the single-stranded termini of ciliate telomeres (Fang and Cech 1991; Wang et al. 1992).

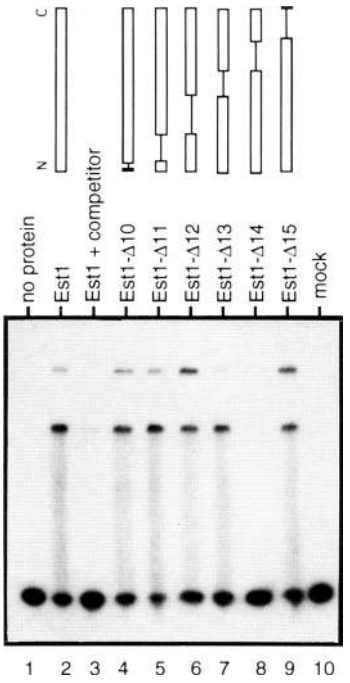

Figure 5. Est1 DNA binding maps to a 130-amino-acid domain. Gel mobility shifts were conducted with 200 nM Est1 protein and 50 pM end-labeled d(TGTGTGGG)$_3$; all Est1 constructs have the (His)$_6$ tag at the carboxyl terminus. (Lane *1*) No Est1 protein added; (lane *2*) 200 nM full-length Est1 protein; (lane 3) 200 nM full-length Est1 protein plus a 50-fold molar excess of cold d(TGTGTGGG)$_3$ competitor; (lanes *4–9*) Est1-Δ10 protein to Est1-Δ15 protein, deleted for amino acids 9–32, 41–156, 159–323, 323–431, 435–565, and 569–699, respectively; (lane *10*) eluate from a mock-prepared extract. Although the proteins purified from these six deletion derivatives showed the expected reduction in size on SDS–PAGE gels, the complexes formed with telomeric DNA exhibited the same mobility as complexes formed with the intact protein. However, more substantial truncations in the size of Est1p did alter the mobility of the protein–DNA complex (data not shown).

Est1 protein exhibits a nonspecific RNA binding activity

Several vertebrate proteins have been identified on the basis of their ability to bind single-stranded telomeric DNA and subsequently shown to be members of the het-

erogeneous nuclear ribonucleoprotein (hnRNP) family (McKay and Cooke 1992a,b; Ishikawa et al. 1993). These proteins bind even more tightly to similarly sized single-stranded RNA oligomers corresponding in sequence to telomeric repeats. To test whether Est1p behaved in a similar manner, a r(G_{1-3}U) oligonucleotide, corresponding in sequence to the d(TGTGTGGG)$_3$ telomeric DNA oligonucleotide used in the above experiments, was synthesized in vitro and tested for binding to Est1. In contrast to the vertebrate hnRNP proteins, Est1p showed no ability to form complexes with this ribo-oligonucleotide (Fig. 6A). However, Est1 protein did exhibit a general RNA binding activity with a variety of larger RNA substrates; complex formation with 197 and 220 nucleotide RNAs are shown in Figure 6A and C. The ability of Est1p to bind seven RNAs of unrelated sequence ranging in size from ~200 nucleotides to 1.3 kb (Fig. 6 and data not shown) argued that binding was nonspecific with regard to sequence. In addition, binding was independent of whether the RNA transcript encoded a telomerase RNA or not. Figure 6A and C show binding to the 197 nucleotide *Euplotes* telomerase RNA and a 220 nucleotide transcript of unrelated sequence; competition experiments revealed no preference between these two substrates. Furthermore, no enhanced binding to the 1300 nucleotide yeast telomerase *TLC1* RNA was observed, relative to the six other RNA substrates tested (data not shown). Although we cannot rule out the possibility that lack of specific binding to the yeast telomerase RNA was attributable to improper folding of this RNA in these experiments, these data indicate that Est1p interacts with RNA in a nonspecific manner in vitro.

To determine the region of Est1p required for RNA complex formation, the same deletion panel used to localize telomeric DNA binding was also assayed for RNA binding. Est1p-Δ14, which was defective for DNA binding (Fig. 5), also completely abolished RNA gel-shift activity (data not shown). As RNA and DNA binding mapped to the same general region of the protein, this suggested that RNA binding might be simply a nonspecific artifact of DNA binding. To investigate this, we asked whether the binding sites for these two nucleic acids were the same by assaying whether RNA was capable of competing for telomeric DNA binding. Figure 6B shows that a 1000- to 10,000-fold molar excess of cold RNA had little effect on telomeric DNA binding. Two different RNA species that bind Est1p were used as competitors, one of which was the *TLC1* yeast telomerase RNA. The lack of competition was not attributable to reduced ability of Est1p to bind RNA; in fact, as shown in Figure 6C, the affinity of Est1p for RNA was 50 nM, fivefold higher than the affinity for telomeric DNA. Therefore, although RNA and DNA binding map to the same region of the Est1 protein, the two nucleic acid binding activities appear to be functionally distinct.

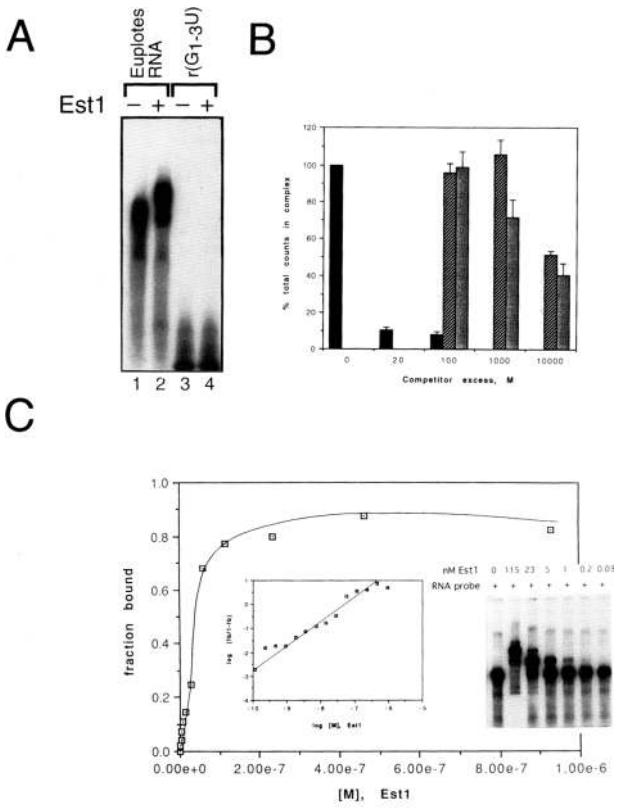

Figure 6. Est1 protein binds RNA nonspecifically. RNA gel shifts were performed as described in Materials and Methods; Est1 protein was at 20 nM and all RNA substrates at 500 pM. (*A*) Binding to the 197 nucleotide *Euplotes* telomerase RNA is compared with the absence of binding to a 27 nucleotide r(G_{1-3}U) substrate. (*B*) Quantitation by PhosphorImager analysis of telomeric DNA gel shifts, with 50 pM of d(TGTGTGGG)$_3$ telomeric oligo and 200 nM Est1 protein, in the presence of cold competitors added from 20- to 10,000-fold molar excess; four independent gel-shift assays were quantitated for each competition experiment. d(G_{1-3}T) = d(TGTGTGGG)$_3$, RNA no. 1 = 1100 nucleotide transcript synthesized from linearized pBluescript, and RNA no. 2 = 1300 nucleotide *TLC1* yeast telomerase RNA. (Solid bars) d(G_{1-3}T); (hatched bars) RNA no. 1; (shaded bars) RNA no. 2 (TLC1). (*C*) The dissociation constant for RNA binding was determined from PhosphorImager analysis of four independent gel mobility shifts using an adenovirus 220 nucleotide RNA transcript at a fixed concentration of 50 pM, with Est1 concentration varied from 0 to 115 nM. The equilibrium curve is plotted as the fraction of RNA bound vs. EST1 concentration; the Hill plot and a representative RNA gel shift are shown as inserts.

EST1 functions in the same pathway for telomere replication as TLC1

The above telomeric DNA binding data, combined with the telomere-specific phenotype of *est1* mutations, suggests that Est1p binds to the end of the chromosome in vivo. Another activity in yeast that has also been shown to bind to single-stranded telomeric oligos is yeast telomerase (Cohn and Blackburn 1995; Lin and Zakian 1995; Lue and Wang 1995). Null mutations of either *TLC1* (encoding the yeast telomerase RNA) or *EST1* have been observed previously to have very similar effects on telomere length and cell viability (Lundblad and Szostak

1989; Singer and Gottschling 1994; Lendvay et al. 1996). To test whether this reflects a requirement for these two genes in the same pathway of telomere replication, we examined the phenotype of a strain carrying deletions of both *EST1* and *TLC1*. If these two genes function in two separate pathways, each necessary for telomere function, a double mutant strain would be expected to show an enhancement of phenotype, relative to either single mutant. Figure 7A shows that the decline in cell viability seen in *est1*-Δ and *tlc1*-Δ strains was indistinguishable from that displayed by the *est1*-Δ *tlc1*-Δ double mutant. Similarly, telomere length in these three strains showed the same reduction over time (data not shown). Therefore, elimination of either *EST1* or *TLC1* gene function has identical and nonadditive effects on telomere replication.

Overexpression of certain mutant derivatives of either *EST1* or *TLC1* also have similar effects on telomeric silencing and telomere length maintenance. *TLC1* was uncovered unexpectedly in a genetic screen for cDNAs that, when overexpressed in a wild-type strain, relieved telomeric silencing (Singer and Gottschling 1994); overexpression of the *TLC1* clones also moderately decreased telomere length. Because none of the *TLC1* isolates were full length, Singer and Gottschling proposed that these partial RNA molecules were acting as dominant-negative suppressors, possibly by titrating limiting components of either telomerase or the silencing machinery. Figure 7 shows that increased expression of mutant derivatives of *EST1* had a similar dominant-negative effect on both telomeric silencing and telomere length maintenance. The *est1* mutations used in this experiment were two missense mutations ($est1_{F511S}$ and $est1_{D513I}$) that were defective for *EST* activity when either of these mutant alleles were the only copy of *EST1* present in the cell (data not shown). Overexpression of either of these two *est1* mutant proteins in a wild-type strain strongly suppressed telomeric silencing (Fig. 7B), and the level of suppression correlated with the activity that each allele displayed in vivo in a complementation assay (data not shown).

In addition to the silencing phenotype, overexpression of these two Est1 mutant proteins shortened telomeres by ~80–150 bp (Fig. 7C), similar to that reported for overexpression of *TLC1* derivatives in wild-type yeast (Singer and Gottschling 1994). Because overexpression of wild-type *EST1* does not affect either telomere length or telomeric silencing substantially, one explanation for the behavior of these *est1* missense mutations could be attributable to titration of a limiting component. However, this hypothesized factor does not appear to be the yeast telomerase RNA alone, as increased expression of *TLC1* does not suppress the dominant negative effects of either *est1* missense mutation on telomere length (data not shown).

Discussion

Although *EST1* was shown seven years ago to have a critical role in telomere maintenance (Lundblad and Szostak 1989), its precise function at the telomere has remained unclear. In this study, we have addressed this by studying the Est1 protein in isolation. This approach has demonstrated that purified Est1p has the properties expected of a terminus-binding protein and thereby provides evidence for a specific role for Est1 protein at the telomere. The identification of a single-stranded telomere-binding protein in a genetically tractable system such as yeast has now allowed an assessment of the in vivo role of this class of proteins. We have shown that a genetic comparison between *EST1* and *TLC1* places both of these genes in the same genetically defined pathway for telomere replication. Furthermore, as the phenotypes of an *est1*-Δ strain are as severe as a *tlc1*-Δ strain (Lundblad and Szostak 1989; Singer and Gottschling 1994), this indicates that Est1p activity is as essential for telomerase function in vivo as is the core enzyme itself.

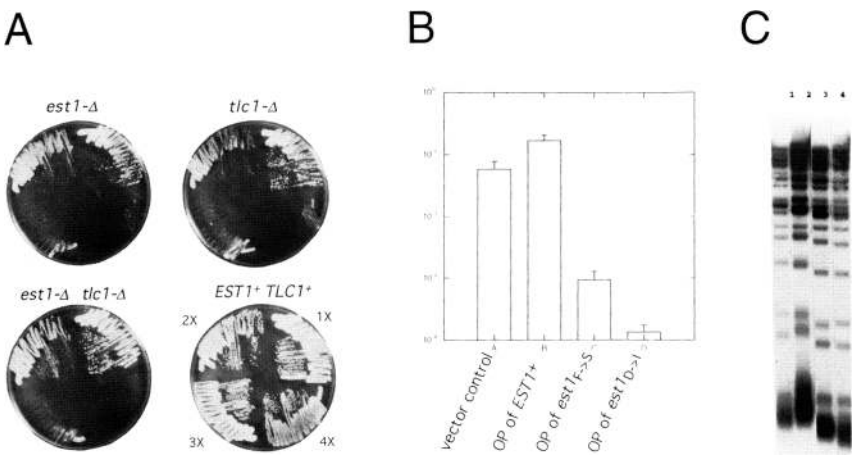

Figure 7. *EST1* and *TLC1* function in the same pathway for telomere replication. (*A*) Viability of *est1*-Δ3::*HIS3*, *tlc1*-Δ::*LEU2*, *est1*-Δ3::*HIS3* *tlc1*-Δ::*LEU2*, and *EST1*$^+$ *TLC1*$^+$ haploid strains, shown as four successive streak-outs (indicated as 1×–4×) on YPD plates, differing from each other by ~25 generations of growth. (*B*) Overexpression of either of two *est1* mutant proteins strongly relieves telomeric silencing of a *URA3* gene placed at telomere VII-L, as indicated by the fraction of cells able to grow in the presence of 5-FOA, which is toxic to cells expressing *URA3*$^+$. Three single colonies for each were assayed; (OP) overproduction. (*C*) Southern blot of genomic yeast DNA, probed with a telomere-specific probe. *EST1*$^+$*TLC1*$^+$ strain with either (Lane *1*) the parental pADH expression vector; (lane *2*) wild-type *EST1*$^+$ expressed from a pADH promoter; (lane *3*) *est1*-6 ($est1_{F511S}$) expressed from a pADH promoter; (lane *4*) *est1*-7 ($est1_{D513I}$) expressed from a pADH promoter.

However, the fact that telomerase activity is present in extracts prepared from an *est1*-Δ strain (Cohn and Blackburn 1995) argues that Est1p is not essential for enzymatic activity. Therefore, we propose that Est1p functions, via its single-stranded DNA binding activity, to direct telomerase to the chromosomal terminus, an activity that is essential in vivo but dispensable in vitro.

There are a number of functional similarities between Est1p and other single-stranded telomere binding proteins, suggesting that Est1p mediates telomerase access as a component of telomeric chromatin. Like these other proteins, Est1p binding is dictated by both the structure and sequence of the 3′ terminus of the DNA substrate. Conversion to a molecule that is partially duplex at the 3′ end eliminates complex formation for both Est1p and terminus-binding factors identified in *Xenopus* egg extracts and *Tetrahymena* extracts (Cardenas et al. 1993; Sheng et al. 1995). Similarly, the *Oxytricha* and *Euplotes* proteins require a single-stranded extension to form a telomeric complex (Gottschling and Zakian 1986; Price and Cech 1987; Price 1990). In addition, all of these telomere binding proteins show sequence-specific binding, with the highest affinity for the telomeric repeat sequence of the species from which the binding protein was identified. Est1p similarly shows relatively high sequence specificity; although it binds *Oxytricha* telomeric sequences roughly as well as a yeast telomeric substrate, it shows little or no binding to human or *Tetrahymena* telomeric substrates. This contrasts sharply with how telomerase recognizes and elongates telomeric primers, as this enzyme, when isolated from a number of different sources, does not differentiate between telomeric primers from different species (Greider and Blackburn 1985; Morin 1989; Shippen-Lentz and Blackburn 1990; Harrington et al. 1995).

An alternative possibility is that Est1p is a noncatalytic component of the telomerase complex. Support for this proposal has come from experiments that monitored the association of this protein with the *TLC1* telomerase RNA, although these experiments did not assess whether Est1p was present in a 1:1 ratio with the telomerase RNA (Lin and Zakian 1995; Steiner et al. 1996). Our results have demonstrated that Est1p has a strong RNA binding activity in vitro, with no preference for the yeast telomerase RNA. This raises questions about the specificity of the in vivo interaction between Est1p and the *TLC1* RNA, which is underscored by the observation that Est1p immunoprecipitates prepared from a strain deleted for *TLC1* still exhibit an RNase-sensitive DNA polymerizing activity (Steiner et al. 1996). One model that could reconcile these observations is to propose that Est1p has a functional but nonspecific RNA binding activity in vivo, with specificity conferred by its location at the telomere. Resolution of a possible in vivo role for Est1 RNA binding will require additional investigation, such as the identification of missense mutants of Est1p that fail to bind RNA in vitro; such mutants can be used to ask whether they exhibit an in vivo telomere maintenance defect and/or altered association with the enzyme.

It is likely that the yeast Est1 protein interacts with other factors as part of its telomere end-binding activity. One potential candidate is the Cdc13 protein, previously shown to have a critical role in telomere metabolism (Garvik et al. 1995). We have shown recently that Cdc13p is also a G-rich single-stranded telomere binding protein with a role in mediating, either directly or indirectly, access of telomerase to the chromosomal terminus (Nugent et al. 1996). However, although both Est1p and Cdc13p specifically bind yeast single-stranded telomeric substrates, only Est1p requires a free 3′ end. This suggests that although both proteins participate in mediating the access of telomerase to the telomere, Est1p may be more critical in bringing the enzyme to the actual 3′ terminus. One potential long-range consequence of this model may be relevant to cancer treatment; because reactivation of telomerase has been proposed to be critical for tumor progression (de Lange 1994; Morin 1995; Shay and Wright 1996), blocking access of the enzyme to the telomere may provide an alternative target for therapeutic intervention, as opposed to inhibition of enzyme activity.

Regulation of both telomerase and other enzymatic activities that act at the telomere is poorly understood at the molecular level. Therefore, identification and characterization of components in a genetic system such as the yeast *S. cerevisiae* may help elucidate this process. Recently, this approach has led to the identification of three additional *EST* genes that function in the same pathway for telomere replication as *TLC1* and *EST1* (Lendvay et al. 1996). Characterization of these components in both lower and higher eukaryotes, in addition to analysis of the enzyme telomerase, may be necessary to fully understand the relationship between telomere length and growth control, and the consequences for cellular aging and cancer.

Materials and methods

Strains and plasmids

The yeast strain, DVL32 (*MAT***a**/*MAT*α *est1*-Δ1::*HIS3*/*EST1*$^+$ *tlc1*-Δ::*LEU2*/*TLC1*$^+$ *ura3*-52/*ura3*-52 *lys2*-801/*lys2*-801 *ade2*-101/*ade2*-101 *trp1*-Δ1/*trp1*-Δ1 *his3*-Δ200/*his3*-Δ200 *leu2*-Δ1/*leu2*-Δ1) used for phenotype analysis of *est1*-Δ and *tlc1*-Δ was constructed from TVL120 (Lundblad and Szostak 1989), by introduction of *tlc1*-Δ::*LEU2*, which removes sequences between nucleotides 192 and 909 of the *TLC1* gene. Yeast strain UCC41 (*MAT*α *lys2 his4 trp1*Δ *ade2 leu2*-3,112 *ura3*-52 with *URA3* and *ADE2* at telomere VII-L; Gottschling et al. 1990) was generously provided by Dan Gottschling.

Vectors for expression of *EST1* in *E. coli* were derived from pRSETA (Invitrogen). pVL244 contains the *EST1* coding sequence ligated into the *Bam*HI and *Pst*I sites of the pRSETA polylinker, to generate a recombinant protein with 40 amino acids at the amino terminus, including six histidine residues and a 12-amino-acid gene 10 epitope. Although we have not tested this recombinant version of Est1 in vivo, we have shown that a number of very similar amino-terminal tagged versions of Est1 (including versions with HIS_6 tags) are fully functional in

yeast. pVL247 was constructed by inserting *EST1* into the *Nde*I site of pRSETA for a carboxy-terminal tag containing 50 amino acids. In-frame deletions of *EST1* (pVL280 to pVL285, containing *est1*-Δ10 to *est1*-Δ15) were initially constructed in pVL247 and subsequently transferred into pVL244. Deletions were constructed using available restriction sites and linker oligos to maintain the coding frame; *est1*-Δ10 was deleted between *Hpa*I and *Nsi*I, removing amino acids 9–32; *est1*-Δ11 was deleted between *Nsi*I and *Nru*I, removing amino acids 41–156; *est1*-Δ12 was deleted between *Nru*I and *Cla*I, removing amino acids 159–323; *est1*-Δ13 was deleted between *Cla*I and *Eco*RV, removing amino acids 323–431; *est1*-Δ14 was deleted between sites *Eco*RV and *Bsm*I, removing amino acids 435–565; and *est1*-Δ15 was deleted between *Bsm*I and the UGA termination codon, removing amino acids 569–699. For in vivo complementation assays (see Genetic Analysis, below), *est1* deletions were transferred into pVL145 (a YCp-*TRP1-EST1* vector). The two missense mutations, *est1*-6 ($est1_{F511S}$) and *est1*-7 ($est1_{D513I}$), were created by oligo-directed single-stranded mutagenesis (V.L. and E.H. Blackburn, unpubl.) in pVL198 (a pUC118 derivative containing *CEN3, TRP1ARS1*, and the 2.58 kb *EST1* gene). For the overexpression studies, $EST1^+$, *est1*-6, and *est1*-7 were cloned into pVL248 [derived from the 2μ vector YEplac112 (Gietz and Sugino 1988), with an inserted pADH promoter and terminator] to generate pVL249, pVL305, and pVL306, respectively.

Genetic analysis

Analysis of telomere length and the senescence phenotype were monitored as described previously (Lundblad and Szostak 1989; Lendvay et al. 1996). All haploid strains were generated by tetrad dissection of DVL132 and grown in parallel. The *est1*-6, *est1*-7, *est1*-Δ12, *est1*-Δ13, *est1*-Δ14, and *est1*-Δ15 mutations were assayed for in vivo activity in the absence of $EST1^+$ gene function by introduction of each mutant version on a YCp vector into an *est1*-Δ3 haploid yeast strain, in parallel with YCp and YCp–$EST1^+$ control vectors. Two to three transformants for each plasmid were assayed for the ability to complement the growth phenotypes of the *est1*-Δ strain. To assay for dominant effects of *est1*-6 and *est1*-7, each mutant derivative, when expressed from the pADH promoter, was introduced into a wild-type $EST1^+$ strain, along with parental control vectors. Three to four transformants were assayed for telomere length and for relief of transcriptional repression of a telomere-located *URA3* gene, as described previously (Gottschling et al. 1990).

Purification of Est1 protein

Expression in *E. coli* of *EST1* from a T7-regulated expression vector (pVL244 or pVL247) was induced by infection with a recombinant M13 phage carrying a cloned copy of the T7 RNA polymerase (Stratagene). Est1p was purified as a soluble recombinant protein using the $(His)_6$ tag present at either the amino or carboxyl terminus, using immobilized metal affinity chromatography purification with Ni-NTA–Agarose (Qiagen) and elution with 250 mM imidizole (pH 7.0). The column was washed with a step-gradient of imidizole (10 mM, 20 mM, and 40 mM imidizole) prior to the elution to remove any bound *E. coli* proteins, which resulted in an enhancement of purity as determined by Coomassie staining. The eluted purified protein was concentrated and washed using Amicon 30 ultrafiltration microconcentrators. Mock protein preps were prepared from a strain carrying a vector with no insert. Protein concentrations were determined using Bradford analysis (Bio-Rad), and immunological detection of the recombinant Est1 protein used antigene 10 antisera (Novagen) to detect a T7 gene 10 epitope fused in frame at either the amino or carboxyl terminus, with visualization with ECL reagents (Amersham) and autoradiography.

Electrophoretic gel shift assays

Telomeric DNA gel shifts were performed in 10 mM HEPES (pH 7.8), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 3% ficoll, and 200 μg/ml poly[d(I-C)] (Pharmacia). Incubations were performed in 10 μl reaction volumes with 200 nM of Est1 protein, end-labeled single-stranded oligonucleotides (boiled at 95°C for 5 min and snap cooled on ice just prior to use) and competitors, when added. To assay the effect of competitors, Est1 protein and cold competitors in the above gel shift buffer were incubated at 25°C for 5–10 min; after addition of labeled oligo, reactions were further incubated for 15–20 min prior to loading. Reactions were electrophoresed through a 5% nondenaturing polyacrylamide gel in 1× TBE at 250 V. The RNA-binding reactions were performed in the same gel shift buffer as above, but heparin (Sigma) was used as the nonspecific competitor at a final concentration of 1 mg/ml, in place of poly [d(I-C)].

DNA/RNA substrates and competitors

DNA oligonucleotides were synthesized and purified by denaturing gel electrophoresis by Genosys Biotechnologies, Inc. For duplex substrates, oligonucleotides were diluted to 10 mM and annealed in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA by heating to 100°C for 1 min followed by slow cooling to room temperature. Single-stranded oligos were 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The duplex substrate used in Figure 1C was prepared by annealing end-labeled d(C_{1-3}A) to cold d(G_{1-3}T). The partially duplex 5′ molecule used in Figure 3 (lanes 7 and 8) was prepared by annealing two oligos [(5′R)-Ye and 5′R] and introducing two [α-^{32}P]dGTP nucleotides with Klenow; the labeled product was subsequently checked on a sequencing gel for the expected two-nucleotide increase in size of the 5′R oligo. The partially duplex 3′ molecule used in Figure 3 (lanes 15 and 16) was prepared by annealing 5′-end-labeled 3′R oligo to cold Ye-(3′R). Labeled DNAs were isolated using NucTrap probe purification columns (Stratagene).

RNA transcripts were synthesized using the MAXIscript in vitro transcription kit (Ambion) using T7, T3, or SP6 polymerase, either in the presence of [α-^{32}P]UTP or with unlabeled ribonucleotides for use as a competitor. Transcripts were purified by NucTrap probe purification columns (Stratagene) or denaturing acrylamide gel electrophoresis and diluted to 500 pM or 0.005 pmole/reaction, heat denatured and snap cooled prior to use in gel shift assays. The 197-nucleotide *Euplotes* telomerase RNA (Shippen-Lentz and Blackburn 1990) and the *S. cerevisiae TLC1* telomerase RNA (Singer and Gottschling 1994) were synthesized from PCR products with incorporated T7 promoter sites, and r(G_{1-3}U) was synthesized from two DNA oligo primers annealed together with an incorporated T7 promoter. The adenovirus construct was generously provided by Sue Berget; adenovirus RNA was synthesized from a SP6 promoter as a run off transcript of *Bam*HI-digested DNA to generate a 220 nucleotide RNA product.

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References

Blackburn, E.H. and C.W. Greider. 1995. *Telomeres.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Brown, W.R.A. 1989. Molecular cloning of human telomeres in yeast. *Nature* **338:** 774–776.

Cardenas, M.E., A. Bianchi, and T. de Lange. 1993. A *Xenopus* egg factor with DNA-binding properties characteristic of terminus-specific telomeric proteins. *Genes & Dev.* **7:** 883–894.

Cohn, M. and E.H. Blackburn. 1995. Telomerase in yeast. *Science* **269:** 396–400.

Collins, K., R. Kobayashi, and C.W. Greider. 1995. Purification of *Tetrahymena* telomerase and cloning of genes encoding two protein components of the enzyme. *Cell* **81:** 677–686.

de Lange, T. 1994. Activation of telomerase in a human tumor. *Proc. Natl. Acad. Sci.* **91:** 2900–2904.

Fang, G. and T.R. Cech. 1991. Molecular cloning of telomere-binding proteins from *Stylonychia mytilis. Nucleic Acids Res.* **19:** 5515–5518.

———. 1995. Telomere proteins. In *Telomeres* (ed. E.H. Blackburn and C.W. Greider), pp. 69–105. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fang, G., J.T. Gray, and T.R. Cech. 1993. *Oxytricha* telomere-binding protein: Separable DNA-binding and dimerization domains of the α subunit. *Genes & Dev.* **7:** 870–882.

Feng, J., W.D. Funk, S.-S. Wang, S.L. Weinrich, A.A. Avilion, C.-P. Chiu, R.R. Adams, E. Chang, R.C. Alsopp, J. Yu, et al. 1995. The RNA component of human telomerase. *Science* **269:** 1236–1241.

Garvik, B., M. Carson, and L. Hartwell. 1995. Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the *RAD9* checkpoint. *Mol. Cell. Biol.* **15:** 6128–6138.

Gietz, R.D. and A. Sugino. 1988. New yeast–*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:** 527–534.

Gottschling, D.E. and V.A. Zakian. 1986. Telomere proteins: Specific recognition and protection of the natural termini of *Oxytricha* macronuclear DNA. *Cell* **47:** 195–205.

Gottschling, D.E., O.M. Aparicio, B.L. Billington, and V.A. Zakian. 1990. Position effect at *S. cerevisiae* telomeres: Reversible repression of Pol II transcription. *Cell* **63:** 751–762.

Gray, J.T., D.W. Celander, C.M. Price, and T.R. Cech. 1991. Cloning and expression of genes for the *Oxytricha* telomere-binding protein: Specific subunit interactions in the telomeric complex. *Cell* **67:** 807–814.

Greider, C.W. 1996. Telomere length regulation. *Annu. Rev. Biochem.* **65:** 337–365.

Greider C.W. and E.H. Blackburn. 1985. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* **43:** 405–413.

———. 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* **337:** 331–337.

Harrington, L., C. Hull, J. Crittenden, and C. Greider. 1995. Gel shift and UV cross-linking analysis of *Tetrahymena* telomerase. *J. Biol. Chem.* **270:** 8893–8901.

Henderson, E. 1995. Telomere DNA structure. In *Telomeres* (ed. E.H. Blackburn and C.W. Greider), pp. 11–34. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Henderson, E.R. and E.H. Blackburn. 1989. An overhanging 3′ terminus is a conserved feature of telomeres. *Mol. Cell. Biol.* **9:** 345–348.

Ishikawa, F., M.J. Matunis, G. Dreyfuss, and T.R. Cech. 1993. Nuclear proteins that bind the pre-mRNA 3′ splice site sequence r(UUAG/G) and the human telomeric DNA sequence d(TTAGGG)$_n$. *Mol. Cell. Biol.* **13:** 4301–4310.

Klobutcher, L.A., M.T. Swanton, P. Donini, and D.M. Prescott. 1981. All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3′ terminus. *Proc. Natl. Acad. Sci.* **78:** 3015–3019.

Konkel, L.M., S. Enomoto, E.M. Chamberlain, P. McCune-Zierath, S.J.P. Iyadurai, and J. Berman. 1995. A class of single-stranded telomeric DNA-binding proteins required for Rap1p localization in yeast nuclei. *Proc. Natl. Acad. Sci.* **92:** 5558–5562.

Lendvay, T., D.K. Morris, J. Sah, B. Balasubramanian, and V. Lundblad. 1996. Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional *EST* genes. *Genetics* **144:** 1399–1412.

Lin, J.-J. and V.A. Zakian. 1994. Isolation and characterization of two *Saccharoymces cerevisiae* genes that encode proteins that bind to (TG$_{1-3}$)$_n$ single strand telomeric DNA in vitro. *Nucleic Acids Res.* **22:** 4906–4913.

———. 1995. An in vitro assay for *Saccharomyces* telomerase requires *EST1. Cell* **81:** 1127–1135.

Lue, N.F. and J.C. Wang. 1995. ATP-dependent processivity of a telomerase activity from *Saccharomyces cerevisiae. J. Biol. Chem.* **270:** 21453–21456.

Lundblad, V. and J.W. Szostak. 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell* **57:** 633–643.

Lustig, A.J. 1992. Hoogsteen G-G base pairing is dispensable for telomere healing in yeast. *Nucleic Acids Res.* **20:** 3021–3028.

McEachern, M.J. and E.H. Blackburn. 1995. Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature* **376:** 403–409.

McKay, S.J. and H. Cooke. 1992a. A protein which specifically binds to single-stranded TTAGGG$_n$ repeats. *Nucleic Acids Res.* **20:** 1387–1391.

———. 1992b. hnRNP A2/B1 binds specifically to single-stranded vertebrate telomeric repeat TTAGGG$_n$. *Nucleic Acids Res.* **20:** 6461–6464.

Mantell, L.L. and C.W. Greider. 1994. Telomerase activity in germline and embryonic cells of *Xenopus. EMBO J.* **13:** 3211–3217.

Morin, G.B. 1989. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* **59:** 521–529.

———. Is telomerase a univeral cancer target? *J. Natl. Cancer Inst.* **87:** 859–861.

Nugent, C., T.R. Hughes, N.F. Lue, and V. Lundblad. 1996. Cdc13p: A single-strand telomeric DNA binding protein with a dual role in yeast telomere maintenance. *Science* **274:** 249–252.

Pluta, A.F., B.P. Kaine, and B.B. Spear. 1982. The terminal organization of macronuclear DNA in *Oxytricha fallax. Nucleic Acids Res.* **10:** 8145–8154.

Pluta, A.F., G.M. Dani, B.B. Spear, and V.A. Zakian. 1984. Elaboration of telomeres in yeast: Recognition and modification of termini from *Oxytricha* macronuclear DNA. *Proc. Natl.*

Acad. Sci. **81:** 1475–1479.

Price, C.M. 1990. Telomere structure in *Euplotes crassus:* Characterization of DNA–protein interactions and isolation of a telomere-binding protein. *Mol. Cell. Biol.* **10:** 3421–3431.

———. 1992. Centromeres and telomeres. *Curr. Opin. Cell Biol.* **4:** 379–384.

Price, C.M. and T.R. Cech. 1987. Telomeric DNA–protein interactions of *Oxytricha* macronuclear DNA. *Genes & Dev.* **1:** 783–793.

Prowse, K.R., A.A. Avilion, and C.W. Greider. 1993. Identification of a nonprocessive telomerase activity from mouse cells. *Proc. Natl. Acad. Sci.* **90:** 1493–1497.

Shampay, J., J.W. Szostak, and E.H. Blackburn. 1984. DNA sequences of telomeres maintained in yeast. *Nature* **310:** 154–157.

Shay, J.W. and W.E. Wright. 1996. Telomerase activity in human cancer. *Curr. Opin. Oncol.* **8:** 66–71.

Sheng, H., Z. Hou, T. Schierer, D.L. Dobbs, and E. Henderson. 1995. Identification and characterization of a putative telomere end-binding protein from *Tetrahymena thermophila. Mol. Cell. Biol.* **15:** 1144–1153.

Shippen, D.E., E.H. Blackburn, and C.M. Price. 1994. DNA bound by the *Oxytricha* telomere protein is accessible to telomerase and other DNA polymerases. *Proc. Natl. Acad. Sci.* **91:** 405–409.

Shippen-Lentz, D. and E.H. Blackburn. 1990. Functional evidence for an RNA template in telomerase. *Science* **247:** 546–552.

Singer, M.S. and D.E. Gottschling. 1994. *TLC1:* Template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266:** 404–409.

Stavenhagen, J.B. and V.A. Zakian. 1994. Internal tracts of telomeric DNA act as silencers in *Saccharomyces cerevisiae. Genes & Dev.* **8:** 1411–1422.

Steiner, B.R., K. Hidaka, and B. Futcher. 1996. Association of the Est1 protein with telomerase activity in yeast. *Proc. Natl. Acad. Sci.* **93:** 2817–2821.

Vermeesch, J.R. and C.M. Price. 1994. Telomeric DNA sequence and structure following de novo telomere synthesis in *Euplotes crassus. Mol. Cell. Biol.* **14:** 554–566.

Walmsley, R.M., C.S.M. Chan, B.-K. Tye, and T.D. Petes. 1984. Unusual DNA sequences associated with the ends of yeast chromosomes. *Nature* **310:** 157–160.

Wang, W., R. Skipp, M. Scofield, and C.M. Price. 1992. *Euplotes crassus* has genes encoding telomere-binding proteins and telomere-binding protein homologs. *Nucleic Acids Res.* **20:** 6621–6629.

Wellinger, R.J., A.J. Wolf, and V.A. Zakain. 1993. *Saccharomyces* telomeres acquire single-strand TG_{1-3} tails late in S phase. *Cell* **72:** 51–60.

Wellinger, R.J., K. Ethier, P. Labrecque, and V.A. Zakian. 1996. Evidence for a new step in telomere maintenance. *Cell* **85:** 423–433.

Wiley, E.A. and V.A. Zakian. 1995. Extra telomeres, but not internal tracts of telomeric DNA, reduce transcriptional repression at *Saccharomyces* telomeres. *Genetics* **139:** 67–79.

Yu, G.-L., J.D. Bradley, L.D. Attardi, and E.H. Blackburn. 1990. In vivo alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature* **344:** 126–132.

Zahler, AM. and D.M. Prescott. 1989. DNA primase and the replication of the telomeres in *Oxytricha nova. Nucleic Acids Res.* **17:** 6299–6317.

Zakian, V.A. 1995. Telomeres: Beginning to understand the end. *Science* **270:** 1601–1607.

Est1 has the properties of a single-stranded telomere end-binding protein.

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