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13. ABSTRACT *(Maximum 200 Words)*

Telomerase is an enzyme needed to maintain telomeres, and is therefore needed for the indefinite growth of cancer cells. An anti-telomerase drug might therefore be a good anti-tumor agent. To help in finding such drugs, two-hybrid and three-hybrid screens have been done in an attempt to identify new components of the telomerase complex. The two-hybrid screen yielded no new components. The three-hybrid screen yielded multiple interactors, but it is still not clear whether these are genuine components of telomerase. The most interesting of these is poly (ADP-ribose) polymerase (PARP), which is known to be involved in DNA repair, and is a homolog of a protein known to function at telomeres. In addition, telomerase activity has been reconstituted in vitro. Finally, the role of a yeast telomerase component, Est1, was defined. Est1 is an RNA binding protein that binds the telomerase RNA, and helps bring it to the telomere. RNA binding proteins are also found in the telomerase complexes from other organisms, including mammals, and so the understanding of this yeast component may improve our understanding of human telomerase.

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FOREWORD

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Bruce Fuchs March 13, 2001

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Introduction:

Chromosome ends are capped by structures called telomeres. Since DNA polymerases synthesis DNA only in the 5' to 3' direction, they cannot completely replicate the ends of a linear molecule (Watson 1972; Olovnikov 1973). The problem is solved by an enzyme called "telomerase", which adds repetitive DNA (TTAGGG, in mammals) to chromosome ends to balance the loss of sequences at replication (Greider and Blackburn 1985; Greider and Blackburn 1987). Normal somatic cells lack telomerase activity, and so the telomeres of somatic cells get shorter and shorter with every cell doubling (Harley et al. 1990). Somatic cells have a limited replicative capacity (Hayflick 1961), and the lack of telomerase seems to be the reason for this, since expression of telomerase in otherwise normal fibroblasts allows them to double indefinitely, escaping the Hayflick limit (Bodnar et al. 1998).

Immortal cells must have a method of maintaining telomeres, and indeed it has been found that immortalized cells lines and all or almost all tumor cells have telomerase (e.g., Kim et al. 1994; Langford et al. 1995; Hiyama et al. 1996; Sommerfeld et al. 1996). Thus, it seems that switching on telomerase is an essential step in tumorigenesis. Telomerase may be the perfect target for anti-tumor drugs--normal somatic cells neither have it nor need it, while tumor cells do have it and do need it. The long term objective of this proposal is to enable the development of anti-telomerase drugs for cancer therapy.

However, since telomerase is a non-abundant and poorly characterized enzyme, our short term objectives have been to identify new components of the telomerase complex, to produce telomerase activity in vitro for biochemical characterization, and to understand in more detail the mechanism by which telomerase works. There are three known components of human telomerase, hTR (the telomerase RNA) (Feng et al. 1995), hTRT (the reverse transcriptase) (Lingner et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Harrington et al. 1997), and a protein called either TR1 or TLP1 (Harrington et al. 1997; Nakayama et al. 1997). hTR and hTRT are necessary and sufficient for *in vitro* activity; the function of TR1/TLP1 is not known. However, it seemed likely that a function as complex as that fulfilled by telomerase might require additional components, and therefore we used several methods to find additional proteins that might be part of the telomerase complex. Candidates for novel telomerase components were sought using a two-hybrid screen (for proteins interacting with the catalytic telomerase component hTRT, and using a three-hybrid screen (for proteins interacting with the telomerase RNA, hTR).

Body of the Report.

Aim 1. Identification of novel telomerase components using a 3-hybrid screen. In Aim 1, we used the three-hybrid screen (SenGupta et al. 1996) to find novel proteins interacting with hTR, the telomerase RNA. We found 36 different proteins in this screen (Table 1).

Table 1. Clones found in three-hybrid screen with hTR.

Clones with no match to database (9 clones): 1a, 231, 313, 319, 321, 329, 343, 397, 459.

Genes in databases with match to one or more clones:

1. Hax-1 (13 clones). Poorly understood.
2. nuclear factor NF90 homolog. (5 clones). RNA binding protein. Poorly understood.
3. FRG1. Poorly understood.
4. DEK. Weak homology to Tetrahymena p95 telomerase component. Proto-oncogene. Poorly understood.
5. HMG1 (2 clones). High mobility group protein.
6. Inosine monophosphate dehydrogenase.
7. Proteosome subunit HC3 (5 clones)
8. Saposin protein A-D. Poorly understood.
9. Hsp90
10. Epstein-Barr virus mRNA (2 clones)
11. ribosomal protein S6 (2 clones)
12. nuclear factor NF 45 homolog
13. NAD(H)-specific iso-citrate dehydrogenase (3 clones)
14. I-plastin mRNA
15. Erp31. Poorly understood.
16. activin beta-A subunit
17. poly (ADP-ribose) polymerase
18. c-myc
19. K1AA0098
20. K1AA0078, Rad21 homolog
21. K1AA0026
22. BAC397c4
23. Thymidine kinase
24. FBKIII 11c tyrosine kinase
25. Human cyclin C, 15 clones
26. Proteasome subunit RC6, 2 clones
27. Beta-hemolysin

Many of these appeared to be uninteresting, and may have been relatively non-specific. There were nine proteins about which absolutely nothing is known, and these are still difficult to evaluate. In addition, there were 5 proteins that appeared to be relatively interesting. These were: (1) hax-1, the protein we found most frequently; (2) DEK, a potential oncogene with slight homology to the Tetrahymena telomerase component p95; (3) poly (ADP-ribose) polymerase (PARP), a protein with a BRCA1 domain, and involved in DNA repair. A homolog of PARP called tankyrase is known to be at telomeres and is directly involved in telomere elongation (Smith et al. 1998; Smith and de Lange 1999; 2000); (4) KIAA0078, the human homolog of *S. pombe* Rad21, which is a cohesin; and (5) c-myc, an oncogene, and a transcription factor known to help control expression of telomerase.

To see if these proteins were truly associated with telomerase, we planned to immunoprecipitate them, and see if telomerase activity or the telomerase RNA co-precipitated. However, we had difficulty obtaining antibodies which successfully immunoprecipitated these proteins with acceptably low non-specific precipitation of other material. To begin to solve this problem, we tagged hax-1 with three tandem copies of the HA epitope, expressed the tagged hax-1 in human cells, and immunoprecipitated the hax-1 protein with monoclonal antibody from the 12CA5 cell line against HA. We labeled proteins with ^{35}S, and in this way showed that our immunoprecipitations were successful, clean, and specific. Unfortunately, we could not detect any telomerase activity or telomerase RNA above background in these immunoprecipitates. Therefore we conclude that hax-1 is probably not (tightly) associated with telomerase *in vitro*.

We would still like to test the association of PARP with telomerase, pending identification of a suitable antibody. Possibly we will tag PARP with the HA epitope, as we did with hax-1.

We also tested the expression of many of the three-hybrid positives in various transformed or primary cell lines. We did not see a convincing correlation between expression and telomerase activity for any of the novel candidates tested. c-myc was not tested, since it was already clear from previous work that there would be a positive correlation.

Aim 2.

The original Aim 2 was to clone the reverse transcriptase subunit by an expression strategy. However, the telomerase subunit from yeast and *Euplotes* was found by other labs using other methods, and the human subunit subsequently appeared in databases as a result of cDNA sequencing projects Harrington et al. 1997;Lingner et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997). Therefore we discontinued Aim 2, and substituted Aims 3, 4, and 5. The rationale for this substitution was discussed in detail in one of the previous annual reports.

Aim 3. Identification of novel telomerase components using a two-hybrid screen.

The two-hybrid screen is a method of finding proteins interacting with a given protein (Fields and Song 1989). Since the human reverse transcriptase subunit of telomerase, hTRT, had just been found, the two-hybrid screen seemed a natural and high-

priority method to find novel telomerase components.

The hTRT gene was amplified by PCR from plasmid pCI-Nco-hEST2-HA (a gift of Dr. R. Weinberg's lab) and subcloned into each of two two-hybrid vectors, pGBD-C1 (TRP1 marker), and pGBDU-C1 (URA3 marker). These two vectors were then transformed into yeast strain PJ69-4a (James et al. 1996), which has three reporter genes for positive two-hybrid interactions: ADE2 (extremely stringent), HIS3 (moderately stringent), and lacZ (low stringency). Because the subcloned hTRT was tagged with HA, we were able to show using Westerns and the 12CA5 antibody that the human enzyme was indeed expressed in the yeast transformants.

Strain pJ69-4a bearing pGBD-C1 (TRP1) – hTRT was transformed with a HeLa cDNA library (Hannon et al. 1993) in pGAD-GH (LEU2 marker). A total of 7 x 10^6 transformants were obtained. In addition, train pJ69-4a bearing pGBDU-C1 (URA3) – hTRT was transformed with the HeLa cDNA library and a total of 3.6 x 10^6 transformants were obtained. Summing the two, just over 10^7 transformants were examined.

Interactors were selected on –his plates (either –his –leu –trp or –his –leu –ura). A total of 62 colonies appeared within 3 days, and an additional 449 appeared by 7 days. These were tested for their expression of *ADE2* (i.e., their ability to grow on –ade plates) and their expression of B-galactosidase. 20 were Ade+, and 10 expressed B-galactosidase, but there was no overlap between the two groups (i.e., none of the 20 Ade+ colonies also expressed B-galactosidase).

All the Ade+ colonies were tested for dependence of the Ade+ phenotype on the hTRT plasmid (*TRP1* or *URA3*). The 20 clones were grown non-selectively, and replica-plated to –trp or to –ura medium, as appropriate. For all 20 clones, we were able to obtain Leu+ Trp- or Leu+ Ura- segregants. Unfortunately, all 20 were still Ade+, showing that the Ade+ phenotype did not require the hTRT plasmid, and so was not due to any interaction of any protein with hTRT.

Similarly the B-galactosidase phenotype was not dependent on the hTRT plasmid, and in fact the B-galactosidase phenotype was difficult to reproduce. Difficulties with the B-galactosidase phenotype have also been encountered by other investigators using this strain.

Finally, we checked all of the remaining colonies of the 62 that appeared within 3 days of transformation for dependence of the His+ phenotype on the hTRT plasmid. The clones were grown non-selectively, and replica-plated to –trp or to –ura medium, as appropriate. We obtained Leu+ Trp- or Leu+ Ura- segregants. Unfortunately, all of these were still His+, showing that the His+ phenotype did not require the hTRT plasmid, and so was not due to any interaction of any protein with hTRT. In summary, no proteins interacting with hTRT were obtained.

The three-hybrid screen (SenGupta et al. 1996) had yielded putative telomerase RNA interactors, and these interactors were obtained from a two-hybrid activation domain library. A protein interacting with the telomerase RNA might also interact with the reverse transcriptase, and this was easy to test since we already had hTRT in a two-hybrid DNA binding construct. Therefore, we tested some of the positives from the three-hybrid screen against hTRT for a two-hybrid (i.e., protein-protein) interaction. So far, we have tested clones #70 (FRG1), #339 (DEK), #20 (NF90), #266 (hax-1), #60

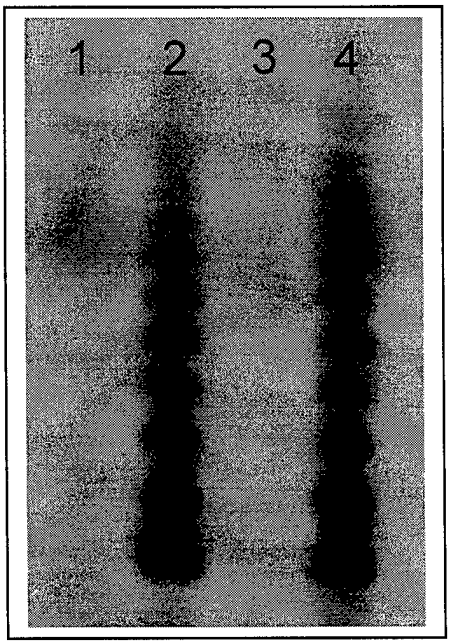
(hax-1), #62 (cyclin G), #693, #315, #124, and #331. None of these gave a His+ phenotype, so there was no evidence for a two-hybrid interaction.

All these experiments were done with an HA-tagged hTRT clone obtained from Dr. R. Weinberg. We had used the HA-tagged form so that we could use Western analysis to demonstrate that the protein was actually being expressed in yeast before we did a large-scale two-hybrid screen. Recently, Dr. Weinberg informed us that this HA-tagged hTRT does not behave exactly like wild-type, untagged hTRT. Both the tagged and the untagged forms give telomerase activity *in vitro*. However, unlike wild-type hTRT, the tagged hTRT fails to maintain telomeres in vivo in human cells. It is possible that this defect of the HA-tagged hTRT also prevents some wild-type two-hybrid interaction. Thus, it might be worth re-doing the two-hybrid screen with a new clone of hTRT that does not include an HA tag.

Aim 4. Reconstitution of telomerase activity in vitro.

We planned to reconstitute telomerase activity on a large scale. However, as a preliminary experiment, we have reconstituted telomerase activity on a small scale using in vitro transcription/translation. The telomerase RNA and the reverse transcriptase (hTRT) were both cloned behind a T7 polymerase promoter. These were then transcribed and translated *in vitro* using the TNT T7 coupled reticulocyte lysate system from Promega Corporation. After transcription/translation, telomerase activity was assayed using the TRAP assay. Results are shown in Fig. 1. Reconstitution of telomerase activity in vitro has also been accomplished by other workers (Beattie et al. 1998; Weinrich et al. 1997)

Fig. 1. Reconstitution of Telomerase activity *in vitro*.



A TRAP assay on *in vitro* transcribed/translated hTR and hTRT. The ladder of bands in lanes 2 and 4 shows a positive result. Lane 1, negative control, no hTR or hTRT. Lane 2, 1 µl of the hTR/hTRT mixure. Lane 3, 2 µl of the hTR/hTRT mixture. Lane 4, 5 µl of the hTR/hTRT mixture. It is not known why a negative result was obtained in Lane 3.

Despite the appearance of telomerase activity in the TRAP assay, little or no activity could be detected in a classical primer elongation assay that did not involve PCR amplification of product. We did rough quantitation of the amounts of hTR and hTRT made, which were significant. It appears that only a vanishingly small proportion of the hTR and hTRT reconstitute active telomerase.

We discussed these findings with Dr. C. Harley of Geron (pers. comm.) and discovered that Geron had made hTR and hTRT on a large scale, but also had a continuing problem with efficient reconstitution. Nevertheless, with protein and RNA made on a large scale, even inefficient reconstitution made substantial amounts of telomerase available for biochemical characterization at Geron.

Aim 5. Define the molecular role of Est1.

The only absolutely necessary components for telomerase activity are hTR and hTRT. Presumably the hTR RNA can bind to the hTRT reverse transcriptase. However, characterized telomerase complexes have other components as well. A third component of human telomerase is called TR1 or TLP1 (Harrington et al. 1997; Nakayama et al. 1997). This large protein has RNA binding motifs, and so could participate in binding to hTR. Strikingly, the Tetrahymena telomerase complex also contains a protein (p80) believed to bind to RNA. We noticed that a third known component of yeast telomerase, Est1, also contains very weak RNA binding motifs. As an indirect route towards determining the role of TR1/TLP1 in humans, we characterized the molecular role of Est1 in yeast, with the specific idea of determining the role of RNA binding.

First of all, we showed by co-immunoprecipitation that the telomerase RNA , TLC1, co-precipitates with Est1, and this is true whether the reverse transcriptase, Est2, is present or not, suggesting a direct physical interaction between TLC1 and Est1 (Zhou et al., 2000).

Next, we made a series of mutations in the RNA binding motifs of Est1. These mutations had three simultaneous effects: they abolished the co-precipitation of TLC1 with Est1; they caused telomere shortening; and they gave versions of Est1 that could not support viability (Zhou et al. 2000). The effects of some mutations were more severe than others, and the severity of each phenotype was correlated (i.e., a severe loss of Tlc1-Est1 binding in the co-immunoprecipitation assay was correlated with severe telomere shortening). Thus, it appears that TLC1 binds directly to Est1 via the RNA binding motifs of Est1, and that this binding is necessary for telomerase function in vivo (Zhou et al. 2000).

We also provided additional support for a previous observation that Est1 can bind to telomeric DNA (Zhou et al. 2000). Thus, Est1 can bind both to telomeric DNA, and also to telomerase RNA. This strongly suggests a model in which the role of Est1 is to tether telomerase to the telomere. Perhaps TR1/TLP1 and *Tetrahymena* p80 have a similar role.

Research Accomplishments:

- Identified 36 proteins possibly binding to human telomerase RNA
- Tested hax-1 for association with telomerase

- Screened over 10^7 HeLa cDNA clones for interaction with the human telomerase reverse transcriptase
- Screened 10 positive clones from the three-hybrid screen for a two-hybrid interaction with human telomerase reverse transcriptase
- Reconstituted human telomerase in vitro.
- Demonstrated a physical interaction between Est1 and telomerase RNA
- Binding of telomerase to Est1 depends on the RNA-binding motifs of Est1
- Binding of telomeric DNA to Est1 does not depend on the RNA-binding motifs

Reportable Outcomes:

Zhou, J., Hidaka, K., and Futcher, B (2000). The Est1 Subunit of Yeast Telomerase Binds the Tlc1 Telomerase RNA. Mol. Cell Biol. *20*, 1947-1955.

Conclusions:

By a three-hybrid screen, 36 candidate proteins were found that may interact with the human telomerase RNA. Although most of these are probably not real interactors, there are three tantalizing proteins that may be interesting. These are DEK (a weak homolog of Tetrahymena p95), poly (ADP ribose) polymerase (PARP), which, together with its homolog tankyrase, may have an important role at telomeres, and c-myc, already known to be an important transcription factor for control of expression of telomerase. Further investigation of these three proteins could reveal new mechanisms for regulating telomerase via interactions with the telomerase RNA.

In vitro reconstitution was extremely inefficient. This suggests that additional assembly components (chaperones, etc.) may be important, and this will be an interesting area for future work.

Finally, we found that Est1 has the role of binding to telomerase RNA via RNA binding motifs, and bringing it to the telomere. This may shed light on the role of RNA binding proteins in other telomerases, and also help define the interaction between the telomerase complex and the telomere.

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Zhou, J., Hidaka, K., and Futcher, B. (2000) The Est1 Subnuit of Yeast Telomerase Binds the Tlc1 Telomerase RNA. Mol. Cell. Biol. 20, 1947-1955.

Appendix: The Est1 Subunit of Yeast Telomerase Binds the Tlc1 Telomerase RNA.

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The Est1 Subunit of Yeast Telomerase Binds the Tlc1 Telomerase RNA

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Est1 is a component of yeast telomerase, and *est1* mutants have senescence and telomere loss phenotypes. The exact function of Est1 is not known, and it is not homologous to components of other telomerases. We previously showed that Est1 protein coimmunoprecipitates with Tlc1 (the telomerase RNA) as well as with telomerase activity. Est1 has homology to Ebs1, an uncharacterized yeast open reading frame product, including homology to a putative RNA recognition motif (RRM) of Ebs1. Deletion of *EBS1* results in short telomeres. We created point mutations in a putative RRM of Est1. One mutant was unable to complement either the senescence or the telomere loss phenotype of *est1* mutants. Furthermore, the mutant protein no longer coprecipitated with the Tlc1 telomerase RNA. Mutants defective in the binding of Tlc1 RNA were nevertheless capable of binding single-stranded TG-rich DNA. Our data suggest that an important role of Est1 in the telomerase complex is to bind to the Tlc1 telomerase RNA via an RRM. Since Est1 can also bind telomeric DNA, Est1 may tether telomerase to the telomere.

Telomeres are the natural ends of linear chromosomes. Telomeres are maintained at a characteristic length by a balance between two forces, loss of telomeres during DNA replication and synthesis of telomeres by an enzyme called telomerase. Telomerase is a special reverse transcriptase which contains not only a reverse transcriptase catalytic subunit but also an RNA molecule which serves as the template for telomere elongation (11). In yeast, the catalytic subunit is called Est2 (7, 20, 25, 26), and the RNA template is called Tlc1 (39).

Telomerases from several organisms have been partially characterized (3, 7, 12, 13, 24, 26, 29, 30). In general, these complexes contain components in addition to the catalytic subunit and the RNA template (10, 12, 24, 30, 36). For the yeast *Saccharomyces cerevisiae*, genetic screens have identified five genes (*EST1*, *EST2*, *EST3*, *EST4/CDC13*, and *TLC1*) (20, 27, 39) whose mutations lead to progressive telomere shortening and eventual loss of viability (i.e., senescence). *EST2* and *TLC1* encode the reverse transcriptase (25, 26) and the RNA template (39), respectively. The Cdc13 or Est4 protein can bind the single-stranded G-rich telomeric sequence both in vitro and in vivo (2, 23, 32). This protein apparently caps the telomere, protecting it from nucleolytic digestion. The functions of the other two genes, *EST1* and *EST3*, are less clear. Neither of them is required for in vitro telomerase activity (5, 25), even though mutants exhibit the same senescence phenotype as *TLC1* or *EST2* mutants (20). There is evidence that Est1 is associated with telomerase, since Est1 coprecipitates with Tlc1 and telomerase activity (21, 40). In addition, Est1 may be associated with the telomere since, like Cdc13, Est1 can bind single-stranded G-rich telomeric DNA in vitro (43). However, the affinity of Est1 for such DNA is low, much lower than the affinity of Cdc13. Unlike Cdc13, Est1 requires a free end for binding to DNA (43). We noticed a possible RNA-binding motif in Est1 and have studied the role of this motif with the idea that Est1 might bind Tlc1 directly.

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MATERIALS AND METHODS

Yeast strains, genetic manipulations, and plasmids. All yeast strains were derived from W303 (*MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 ssd1-d/ssd1-d* [*psi*$^+$]) (41). Yeast media and transformation were as described previously (42).

The plasmids for generating *est1*Δ, *ebs1*Δ, *est2*Δ, and *est3*Δ strains were made as follows. The *EST1* open reading frame was amplified by PCR and cloned into pT7Blue (Novagen). The *Nru*I-*Eco*RV fragment of *EST1* was then replaced with *URA3* on a *Sma*I fragment. The *EBS1* gene was cloned into pBSII-SK(+). The *Hinc*II-*Hin*dIII fragment of *EBS1* was then replaced with *URA3* on a *Sma*I-*Hin*dIII fragment. The *est2* and *est3* mutations were generated in diploid W303 by oligomer (oligo)-directed recombination (38) using the oligos shown in Table 1. Haploid *est1*Δ, *ebs1*Δ, *est2*Δ, and *est3*Δ strains were obtained by tetrad dissections of heterozygous diploids. Haploid *tlc1*Δ strains were made by sporulation of a *tlc1*Δ/*TLC1* diploid (40).

Plasmid pVL242 was a gift from V. Lundblad. It contains *GAL-EST1* tagged with a triple hemagglutinin (3HA) sequence and was used for the Est1 localization study shown in Fig. 1. Plasmid pJZ-3HA-*EST1* was constructed as follows. pLexA-*EST1* was constructed by cloning a *Bam*HI-*Pst*I fragment carrying *EST1* into pBTM116 (2μm based, *ADH1* promoter). This 2.1-kb fragment was obtained by amplifying *EST1* using oligos EST1-5Bam and EST1-3Pst (Table 1). A 3HA sequence, produced by PCR using oligos N3HAEST1 and BglII3HA and template pMPY-3×HA (37), was inserted into pLexA-EST1 at the *Bam*HI site between the LexA gene and *EST1* to generate pJZ-3HA-EST1, the 3HA-tagged *EST1* plasmid. Thus, *EST1* is expressed at a high copy number from the *ADH1* promoter. Both pLexA-EST1 and pJZ-3HA-EST1 fully complement the senescence and short-telomere phenotypes of an *est1*Δ strain. *EST1* mutant alleles were made using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). The oligos used for mutagenesis are listed in Table 1. The mutations were confirmed by DNA sequencing.

Yeast cell extracts, immunoprecipitation, total RNA preparation, and Northern blot analysis. For preparation of yeast extracts, yeast cells were grown to mid-log phase (optical density at 600 nm [OD_{600}], about 1) with selection for plasmid-borne markers. Cells were harvested by centrifugation and washed once with ice-cold water and once with high-salt lysis buffer (HSLB) (40). All operations thereafter were carried out at 4°C. The cell pellet from 50 ml of cells at an OD_{600} of 1 was suspended in HSLB containing protease inhibitors and an RNase inhibitor, and about 750 μl of acid-washed glass beads was added. The cells were then either quickly frozen to −70°C and stored or broken by shaking in a Mini-Bead-Beater (Biospec). Breaking was accomplished by 30 s of vibration at the top speed twice, separated by 3 min of cooling on ice. The mixture was centrifuged at 14,000 rpm at 4°C for 5 min in an Eppendorf microcentrifuge, and the resulting supernatant was transferred into a fresh tube. The beads were washed with an additional 300 μl of the same buffer and spun to collect additional protein. The two supernatants were combined and spun again under the same conditions. The supernatant was transferred into a clean tube. The protein concentration of such cell extracts was typically 12 to 20 mg/ml, as determined by the Bradford method (Bio-Rad kit). Such extracts were used for immunoprecipitation.

TABLE 1. Oligos used in this study

Name	Length (nucleotides)	Sequence (5′→3′)	Purpose
EST1-5Bam	43	GAATTCCCGGGGATCCGTATGGATAATGAAGAAGTTAACGAAG	PCR cloning of *EST1*
EST1-3Pst	39	GGGCGTCGACCTGCAGTCAAGTAGGAGTATCTGGCACTT	PCR cloning of *EST1*
N3HAEST1	36	ACCCCAGATCTTTAAATACCCATACGATGTTCCTGA	3HA tagging of *EST1*
BglII3HA	26	CCCCAGATCTGAGCAGCGTAATCTGG	3HA tagging of *EST1*
EST1-RNP1A	63	GAGAAGATATTATTTTCGGGGAATTTTCTTCGCTAACGCTGCACTAACAGATTTTAATGACG	Making RNP1-NC
EST1-RNP1B	63	CGTCATTAAAATCTGTTAGTGCAGCGTTAGCGCAAGAAAATTCCCGAAAATAATATCTTCTC	Making RNP1-NC
EST1-RNP1C	62	GAGAAGATATTATTTTCAAGGAATTCTCTTGCGTTAACTTTGCACTAACAGATTTTAATGAC	Making RNP1-C
EST1-RNP1D	62	GTCATTAAAATCTGTTAGTGCAAAGTTAACGCAAGAAGAATTCCTTGAAAATAATATCTTCTC	Making RNP1-C
EST1-RNP2A	47	GCACTTCATTCGCCGGGTTGCTGAACGACGCGATAAATAGTCCACTG	Making RNP2-ga
EST1-RNP2B	47	CAGTGGACTATTTATCGCGTCGTTCAGCAACCCGGCGAATGAAGTGC	Making RNP2-ga
EST1-RNP2C	52	GGAAGTTTTGCACTTCATTCGCCGGATCCCTGAACGACTTGATAAATAGTCC	Making RNP2-gs
EST1-RNP2D	52	GGACTATTTATCAAGTCGTTCAGGGATCCGGCGAATGAAGTGCAAAACTTCC	Making RNP2-gs
EST1F511SA	48	CATTAACTTTGCACTAACAGATTCTAATGACGATTATGTGTATGATTC	Making F511S
EST1F511SB	48	GAATCATACACATAATCGTCATTAGAATCTGTTAGTGCAAAGTTAATG	Making F511S
EST1D513IA	45	CTTTGCACTAACAGATTTTAATATCGATTATGTGTATGATTCTCC	Making D513I
EST1D513IB	45	GGAGAATCATACACATAATCGATATTAAAATCTGTTAGTGCAAAG	Making D513I
EST2KOUP	70	ATGAAAATCTTATTCGAGTTCATTCAAGACAAGCTTGACATTGATCTACAGACCAGGGAACAAAAGCTGG	Making *est2Δ*
EST2KODW	70	CAGCATCATAAGCTGTCAGTATTTCATGTATTATTAGTACTAATTAACTATATGCTATAGGGCGAATTGG	Making *est2Δ*
EST2KODGUP	19	CCATAACTAACACGCCCTC	Diagnosis of *est2Δ*
EST2KODGDW	21	GGCTTATTACAAAGTTTGCGG	Diagnosis of *est2Δ*
EST3KOUP	79	GGGATAACAAGTAAACAATGCCGAAAGTAATTCTGGAGTCTCATTCAAAGCCAACAGACTCAGAGGGAACAAAAGCTGG	Making *est3Δ*
EST3KODW	75	GCCTGCAGAAGGTCATAAATATTTATATACAAATGGGAAAGTACTTAACGATCCGACTTCTATAGGGCGAATTGG	Making *est3Δ*
EST3KODGUP	20	GGTCAGAATGGGCGCTTGTC	Diagnosis of *est3Δ*
EST3KODGDW	21	CTCTAGAGGAGTACTTATCGG	Diagnosis of *est3Δ*
TELPG	27	GTGTGTGGGTGTGTGTGTGGGTGTGTG	Telomere probe

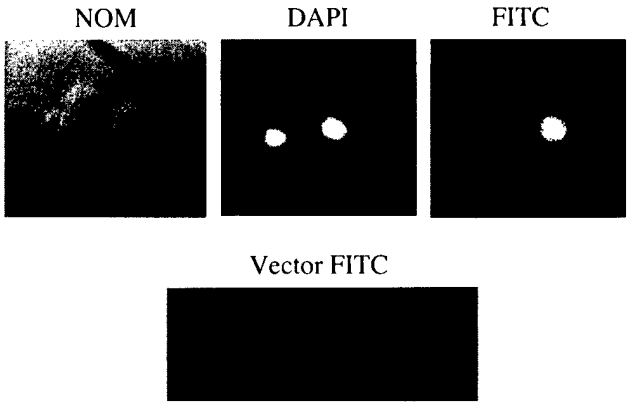

FIG. 1. Est1 is a nuclear protein. (Top panels) Est1 tagged with 3HA was expressed from the *GAL* promoter (plasmid pVL242). Cells were fixed and stained with 4′,6′-diamidino-2-phenylindole (DAPI), 12CA5 (anti-HA antibody), and fluorescein isothiocyanate (FITC)-labeled secondary antibody. Cells were visualized by Nomarski optics (NOM) or by fluorescence for DAPI (DAPI) or fluorescence for FITC (FITC). About one-third of all cells examined showed strong tag- and antibody-dependent nuclear FITC staining. (Bottom panel) Control strain containing an empty vector processed for FITC staining.

Immunoprecipitation was carried out by incubating 500 to 1,000 μl of cell extract with 0.5 to 1.0 μl of 12CA5 ascitic fluid (ascitic fluid contained about 30 mg of total protein per ml) at 4°C for 60 min, followed by the addition of 10 to 20 μl of HSLB-washed protein A-agarose beads. The incubation was continued for a further 60 to 90 min. The beads were collected by a gentle, brief spin and washed four times with the same buffer but containing neither protease inhibitors nor an RNase inhibitor. The beads were suspended in 20 to 50 μl of HSLB and then split for RNA preparation and for Western blot analysis.

For RNA preparation, the immunoprecipitate was diluted fivefold with 10 mM Tris–1 mM EDTA (pH 8) and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were precipitated with 0.3 M sodium acetate and 3 volumes of ethanol. This RNA was called immunoprecipitated RNA (IP-RNA).

For total RNA preparation, 3 to 5 ml of mid-log-phase cells at an OD_{600} of about 1 was pelleted, washed once with ice-cold water, and suspended in 250 μl of LETS buffer (0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris-HCl [pH 7.4], and 0.2% sodium dodecyl sulfate in diethylpyrocarbonate-treated water). Acid-washed glass beads (300 μl) and 300 μl of phenol-chloroform were added to the cell suspension. The mixture was vortexed at the top speed for 15 s twice, with an interval of 3 min on ice. Another 200 μl of LETS buffer was added, and the mixture was vortexed briefly. The organic and aqueous phases were separated by spinning at 14,000 rpm for 5 min. The upper aqueous phase was transferred to a clean tube, and the RNA was precipitated with ethanol.

Northern analysis was carried out as follows. Total RNA (2.5 to 7.5 μg) or one-third of a sample of IP-RNA was fractionated by electrophoresis on a 6.0% formaldehyde–1.0% agarose gel and transferred to NYTRAN PLUS Nylon membranes (Schleicher & Schuell, Inc.). The blots were probed with the ^{32}P-labeled full-length *ACT1* fragment and the labeled full-length *TLC1* fragment.

Genomic DNA preparation and Southern blot analysis. Genomic DNA was prepared after cells were broken with glass beads. For Southern analysis of telomere length, 1.0 μg of genomic DNA was digested with *Xho*I or *Pst*I endonuclease at 37°C for 2 h. In some Southern blots (e.g., see Fig. 3 and Fig. 5C, right panel), 3 ng of DNA from an *Hae*II-*Nde*I digest of plasmid pBST3 was added to the digested genomic DNA. This digestion produced a fragment of 511 bp and a fragment of 1,436 bp that hybridize to yeast telomeric sequences and so serve as molecular size markers on Southern blots. Electrophoresis was carried out at 80 V for about 5 h. The separated DNA was transferred to NYTRAN PLUS Nylon membranes and probed with a ^{32}P-labeled yeast telomere sequence (TELPG; Table 1).

Single-stranded DNA-binding assay. The binding reaction was conducted with a total reaction volume of 20 μl. The binding buffer (43) contained 50 μg of poly(dI-dC) per ml. To this binding buffer was added 5.0 μl of the immunoprecipitate that had been washed once with binding buffer after the standard immunoprecipitation protocol (see above). After incubation at room temperature for 8 min, a mixture of 3.0 ng of ^{32}P-labeled TELPG (27-mer) and 6.6 ng of ^{32}P-labeled oligo EST1-RNP1C (62-mer) was added to the reaction, and incubation was continued for another 15 min at room temperature. The reaction was stopped by gently adding 1,000 μl of binding buffer. The beads were collected immediately, washed gently three times in binding buffer, and eventually suspended in 10 μl of binding buffer. Four microliters of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to the suspension. The mixture was heated in boiling water for 3 min before being loaded onto a 7.6% polyacrylamide–7.0 M urea–1× Tris-borate-EDTA sequencing gel.

After electrophoresis, the gel was placed on Whatman paper and vacuum dried. The signal was quantified using a Molecular Dynamics PhosphorImager.

Telomerase assay. Telomerase was purified and assayed as previously described (34). About 15 μg of each partially purified fraction was used for each telomerase assay. For the RNase A control, 50 ng of RNase A was added to the partially purified telomerase fraction; this mixture was incubated at room temperature for 5 min, and then telomerase activity was assayed.

RESULTS

Est1 is a nuclear protein. If Est1 is a component of active telomerase at telomeres, then it ought to be a nuclear protein. On the other hand, if Est1 is less directly involved in telomerase function, then it might have some other location. We overexpressed a functional, 3HA-tagged version of Est1 and localized it by immunofluorescence. All the Est1-dependent immunofluorescence appeared to be in the nucleus (Fig. 1).

The association of Est1 with Tlc1 does not depend on *EST2* or *EST3*. We have previously shown that the Est1 protein is associated with the Tlc1 RNA (40). To find out whether this interaction depends on other telomerase components, we examined the Est1-Tlc1 association in *est2* and *est3* deletion strains. A functional, 3HA-tagged version of Est1 was expressed in yeast and immunoprecipitated. Immunoprecipitates were processed for Northern blotting, and the presence of Tlc1 and control RNAs was assayed. Figure 2 shows the result from a typical experiment. The Tlc1 RNA was detected in the tagged Est1 immunoprecipitates from wild-type, *est2*, and *est3* cells. This association was specific, since control *ACT1* mRNA was not detected in the 3HA-tagged Est1 immunoprecipitate, and no RNA was detected in the untagged Est1 immunoprecipitate. The Tlc1 RNA signal revealed by Northern analysis is not due to any DNA contamination, since RNase A treatment completely eliminates the signal (see Fig. 6, lanes 26 and 27). These results indicate that the association of Est1 and Tlc1 does not require Est2 or Est3, the only other known components of the telomerase complex. Thus, the interaction of Est1 with Tlc1 may be direct.

The coprecipitation of Tlc1 with Est1 was consistently less efficient in *est2* or *est3* mutant cells than in wild-type cells (Fig. 2). This result could be a sign that Tlc1-Est1 complex formation in vivo is aided by Est2 and Est3, although they are not required. Alternatively, the relatively poor coprecipitation of Tlc1 in the *est* mutants could be due to the sickness of the *est* cells, which in our experiments led to somewhat lower protein concentrations in cell lysates.

Ebs1 is a homolog of Est1. Database searches show that the Est1 protein has 48% similarity (27% identity) to the protein encoded by the poorly characterized gene *EBS1* (open reading frame YDR206w). This similarity is spread throughout the two proteins but is most pronounced in a 100-amino-acid region centered on a putative RNA recognition motif (RRM) in Ebs1 (see below). Interestingly, disruption of *EBS1* resulted in slightly shortened telomeres (Fig. 3), although there was no senescence phenotype. Furthermore, there was an indication that *est1 ebs1* double mutants had a slightly stronger senescence phenotype than *est1* single mutants alone. *est1* single-mutant spore clones show a variable time to senescence, but *est1 ebs1* double mutants always senesce as fast as the fastest-senescing *est1* single mutants. These results suggest that *EBS1* is slightly redundant with *EST1* for telomere maintenance.

It has previously been shown that protein extracts from *est1* mutants have telomerase activity in vitro (5). Since we now believe that *EBS1* may be a homolog of *EST1*, we wondered whether this in vitro telomerase activity in *est1* strains might have required *EBS1*. Therefore, we made extracts from an *est1 ebs1* double mutant. These extracts also contained telomerase activity (Fig. 4), supporting the previous conclusion that *EST1* function is not essential for in vitro telomerase activity.

Est1 has a functionally important RRM. Computer analysis of the Ebs1 protein showed that it contained a good match to an RRM (Fig. 5). The RRM in Ebs1 led us to a putative RRM in Est1 (Fig. 5). Although this putative Est1 RRM has only a poor match to a consensus RRM, it is also true that RRMs of this class can share structural similarity without highly conserved sequence similarity, and it is the structural similarity that is important (1, 4, 17). An RRM consists of 70 to 90 amino acid residues (1, 4, 17). RNP1 and RNP2 are two important and more highly conserved segments within an RRM. The RRM crystal structures of human hnRNP C and U1 snRNP A show that the RRM forms a module of four antiparallel beta sheets on the surface and two alpha helices underneath (a βαββαβ secondary structure). RNP1 and RNP2 form the two central antiparallel beta sheets and are directly involved in single-stranded RNA interactions.

On the basis of sequence and structural information, we created two mutations in the putative Est1 RNP1 (Fig. 5A). The first was called RNP1-NC; this is a triple substitution which replaces three structurally important residues with three dissimilar amino acids. Thus, these are nonconservative (NC)

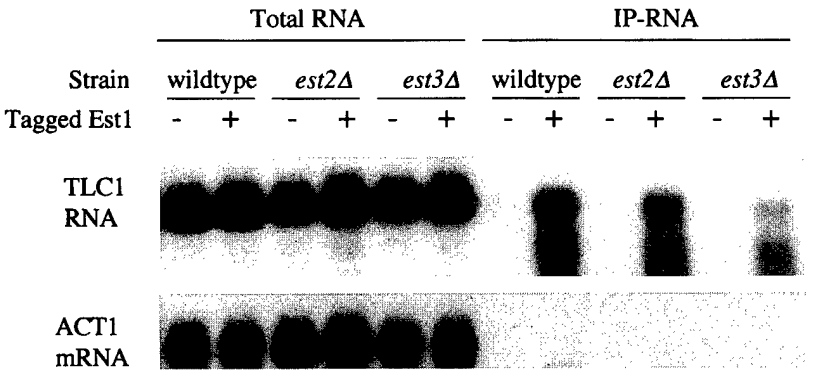

FIG. 2. The association of Tlc1 RNA with Est1 does not depend on *EST2* or *EST3*. The amount of Tlc1 RNA (top panel) or *ACT1* mRNA (bottom panel) in various experiments was assayed by Northern blotting. "Total RNA" shows the total RNA from wild-type, *est2*, or *est3* strains, each of which either carries (+) or does not carry (−) tagged Est1 expressed from the *ADH1* promoter (pJZ-3HA-EST1). "IP-RNA" shows the RNA associated with 3HA-tagged Est1 after the latter was immunoprecipitated from tagged (+) or untagged control (−) strains with monoclonal antibody 12CA5. The amount of Tlc1 precipitated was somewhat smaller in the *est2* and *est3* strains than in the wild-type strain in all three of three experiments.

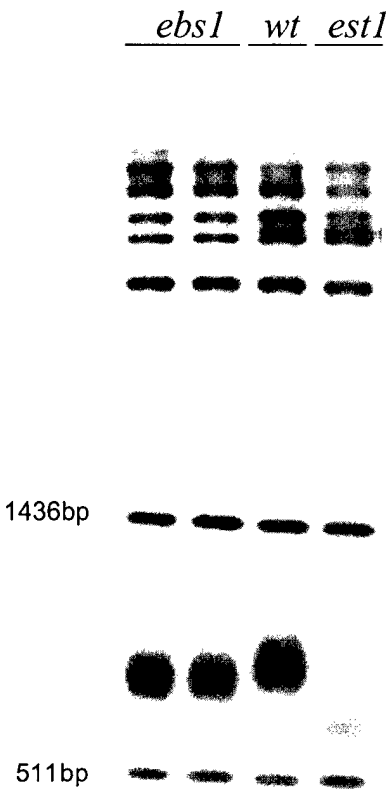

FIG. 3. The *ebs1* mutant has short telomeres. Genomic DNA was isolated from two *ebs1* null mutants, a wild-type (*wt*) strain, and an *est1* null mutant. DNA was digested with *Pst*I, mixed with 511- and 1,436-bp size markers, and fractionated by agarose gel electrophoresis. After Southern blotting, DNA was hybridized with a ^{32}P-labeled telomeric sequence.

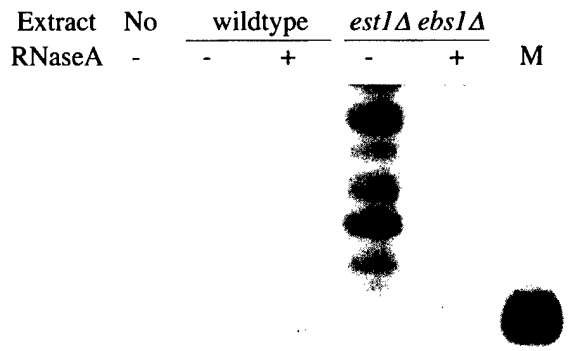

FIG. 4. The *EBS1* gene is not essential for telomerase activity in vitro. Telomerase was partially purified (34) from a wild-type strain and from an *est1 ebs1* double mutant. Telomerase activity was assayed (34) in the absence (−) or presence (+) of 50 ng of RNase A. Two to three times as much protein was used in the *est1 ebs1* assay as in the wild-type assay, accounting for the apparently higher level of telomerase activity. Lane M, end-labeled 28-mer which serves as a marker for the input telomeric primer oligo.

changes, and the RNP1-NC mutation would be expected to reduce or eliminate RRM function. The second mutation was called RNP1-C; this is a double substitution which replaces two structurally important residues with similar amino acids found in the RNP1 motifs of some proteins. Thus, these are conservative (C) changes, and if this region of Est1 is truly an RRM, then RNP1-C might not reduce RRM function. These mutant forms of Est1 were tagged with a 3HA epitope.

The *est1-rnp1-nc* mutant failed to complement either the senescence or the telomere shortening of *est1*Δ strains (Fig. 5B and C). Furthermore, when Est1-RNP1-NC was immunoprecipitated, no Tlc1 RNA could be detected in the immunoprecipitate (Fig. 6, lanes 15 and 19; two individual transformants). This was not because of a lack of Est1-RNP1-NC protein, because Western analysis showed that equivalent amounts of wild-type Est1 and Est1-RNP1-NC were immunoprecipitated (data not shown). These results suggest that the *est1-rnp1-nc* mutation eliminates or severely impairs the ability of Est1 to bind to Tlc1, and this change correlates with a loss of *EST1* function.

In contrast, the *est1-rnp1-c* mutant, in which two amino acids are substituted with conserved residues expected to function in the context of an RNP1 motif (Fig. 5), complements the *est1*Δ phenotype for viability and gives telomeres only slightly shorter than the wild type (Fig. 5). Est1-RNP1-C coimmunoprecipitates with Tlc1 only slightly less well than it does with wild-type Est1 (Fig. 6). Again, the ability to coimmunoprecipitate with Tlc1 is correlated with genetic function.

We also made two mutations in the RNP2 region of Est1. Few mutagenesis studies have been done with the RNP2 region of other RRMs, so it was not clear what phenotypes our RNP2 mutations should create. The RNP2-ga mutation makes two nonconservative changes; the first change is at the beginning of the beta strand, and the second is after the end of the beta strand, in the following loop. The RNP2-ga mutant is wild type for the three phenotypes assayed (cell viability, telomere length, and the Tlc1 RNA association) (Fig. 5 and 6). The RNP2-gs mutation makes two nonconservative changes; the first, again, is at the beginning of the beta strand, and the second (L to S) is in the beta strand at a well-conserved hydrophobic residue that appears to be important for the interaction with RNA, at least in some RRMs (17). The RNP2-gs mutant has significantly shortened telomeres, but it rescues the senescence of *est1*Δ strains (Fig. 5). This mutant protein has a reduced ability to associate with Tlc1 (Fig. 6).

In all these experiments, the ability of mutant proteins to maintain telomere length was highly correlated with their ability to coimmunoprecipitate with Tlc1 RNA (Table 2).

The EST1-RNP1-NC mutant has dominant negative phenotypes. The failure of the triple substitution, *est1-rnp1-nc*, to complement an *est1* mutation raised the question of whether the mutations might have grossly disturbed protein structure (although the fact that Est1 and Est1-RNP1-NC proteins were found equally abundant by Western analysis argues against this notion). However, we found that wild-type *EST1* cells transformed with overexpressed *est1-rnp1-nc* had at least three phe-

TABLE 2. Summary of *EST1* RRM mutants

EST1 allele	Phenotype[a]		
	Viability	Tlc1 RNA binding	Telomere length
Wild type	+++	+++	+++
RNP1-NC	−−−	−−−	−−−
RNP1-C	+++	++	++
RNP2-ga	+++	+++	+++
RNP2-gs	+++	+	+

[a] +++, wild-type behavior; ++ or +, *EST1* activity slightly or considerably less than wild-type activity, respectively; −−−, no *EST1* activity (i.e., no complementation of an *est1* mutant).

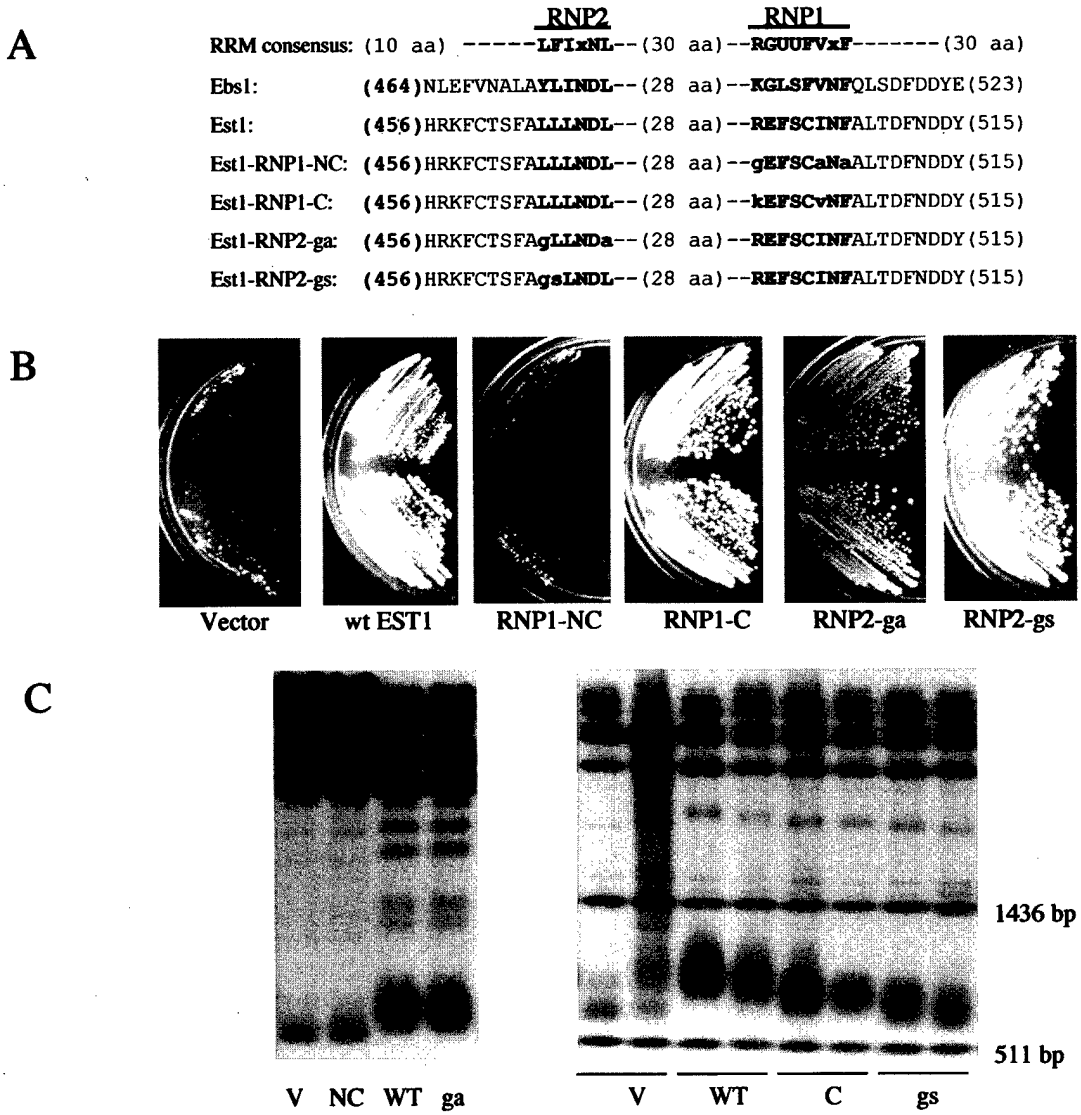

FIG. 5. Phenotypes caused by altering the RRM region of Est1. (A) The RRM consensus sequence (1, 17) is compared to RRM-like regions in Ebs1 and Est1. The most highly conserved regions (RNP1 and RNP2) are shown in bold lettering. U, hydrophobic amino acid (aa). The amino acid changes made in the four *est1* mutants are indicated by lowercase letters. To the right of RNP1 in Est1 is the sequence FNDDY; F is F511, and the first D is D513. These amino acids are altered in two previously characterized alleles of *est1* (43) (see the Discussion). (B) A presenescent *est1*Δ strain (about 35 generations removed from the original spore clone) was transformed with plasmids based on pJZ-3HA-EST1 harboring the empty vector, wild-type (wt) *EST1*, or the NC, C, ga, or gs mutant allele of *EST1*. Two independent colonies from each transformation were streaked on YEPD plates and photographed after 60 h of growth. (C) Genomic DNA was extracted from each transformant, digested with *Xho*I (left panel) or *Pst*I (right panel), and fractionated by agarose gel electrophoresis. After Southern blotting, telomeric DNA was detected with ^{32}P-labeled oligo TELPG. V, transformants containing an empty vector; WT, wild type. In the right panel, the two sharp bands just below and above the fuzzy telomere bands are molecular size markers of 511 and 1,436 bp, respectively (see Materials and Methods).

notypes. First, they grew slowly (data not shown). Second, as assayed with a Coulter Channelyzer, average cell size was abnormally large, and microscopic examination showed that some cells were large dumbbells, typical of DNA damage arrest (data not shown). Third, these cells had relatively short telomeres (Fig. 7). These dominant phenotypes suggest that the mutant Est1-RNP1-NC protein retains some functions of wild-type Est1 and consequently disturbs normal telomere maintenance. This notion in turn suggests that the disruption of the putative RRM causes a fairly specific defect in the protein as opposed to a general defect in protein folding.

The Est1-RNP1-NC mutant can bind single-stranded telomeric DNA. Virta-Pearlman et al. reported that Est1 protein can bind to yeast single-stranded TG-rich telomeric DNA, albeit with low affinity (43). In some proteins, RRMs can bind to single-stranded DNA (8, 14, 16, 31, 35). In fact, single-stranded TG-rich telomeric DNA may be particularly good at interacting with RRMs (8, 16, 31). Thus, we wished to know whether the RRM of Est1 was responsible for the DNA-binding activity detected by Virta-Pearlman et al. (43). We developed a single-stranded DNA-binding assay (see Materials and Methods). This assay compares the ability of immunoprecipitated 3HA-tagged Est1 protein to bind two different single-stranded DNAs, one with a telomeric sequence and one with an irrelevant sequence. The assay has a high background (i.e., there is significant binding of telomeric sequences even in the absence of Est1); nevertheless, immunoprecipitates from tagged Est1 strains reproducibly bind more telomeric sequence

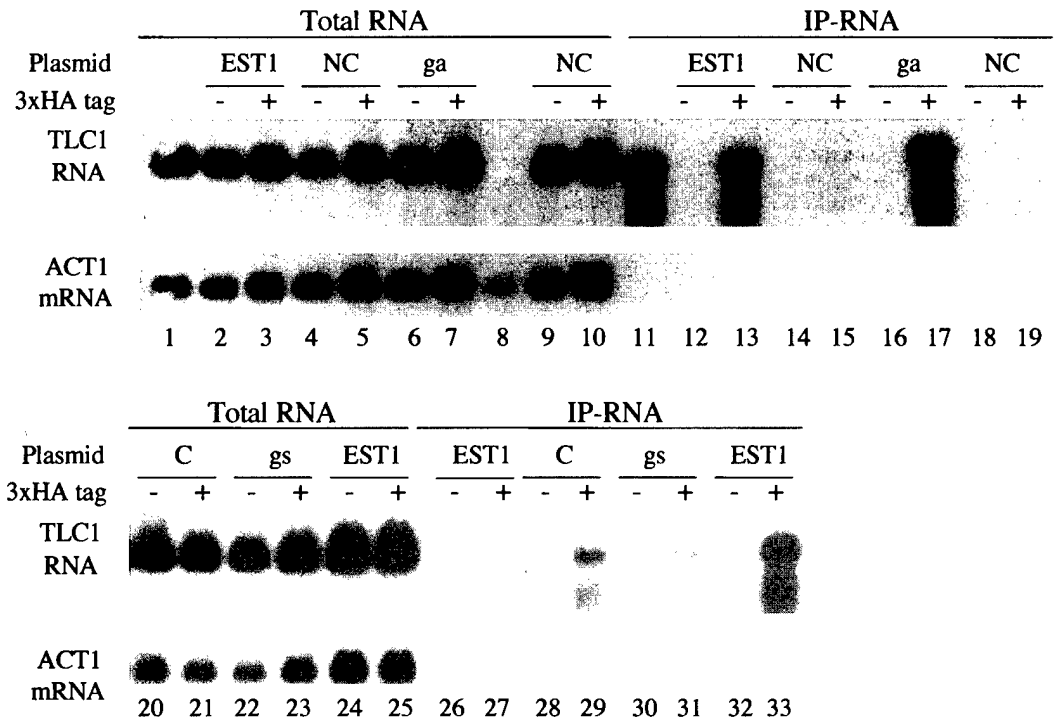

FIG. 6. Association of Tlc1 RNA with Est1 RRM mutant proteins in vivo. Total RNA and immunoprecipitated RNA (IP-RNA) were made from *est1* cells expressing wild-type or mutant forms of either untagged (−) or 3HA-tagged (+) Est1 from the *ADH1* promoter (plasmid pJZ-3HA-EST1 and derivatives). The amounts of Tlc1 RNA and *ACT1* mRNA in total RNA or coimmunoprecipitated with Est1 were assayed by Northern blotting. Two independent transformants of Est1-RNP1-NC were assayed. Controls included RNA from a *tlc1* deletion mutant (lane 8), RNA from a strain carrying 3HA-tagged Est1 expressed from the genomic *EST1* locus (lanes 1 and 11), and IP-RNA treated with RNase A before loading (lanes 26 and 27).

than do immunoprecipitates from untagged control strains. Figure 8 shows the results of one typical experiment. Immunoprecipitates from the tagged Est1 strain retained two- to threefold more telomeric sequence (oligomer TELPG; 27-mer) than immunoprecipitates from the untagged control strain (Fig. 8). Seven independent experiments (Table 3) were evaluated by analysis of variance (ANOVA), and the difference between the tagged and untagged strains was significant at the 0.05 level. The immunoprecipitated Est1 protein from *tlc1*Δ cells gave similar results (data not shown), indicating that the Tlc1 RNA bound to Est1 in vivo does not significantly interfere with the DNA-binding capacity of Est1.

Figure 8 also shows the result from one typical experiment with the immunoprecipitated Est1-RNP1-NC mutant protein. Est1-RNP1-NC binds single-stranded TG-rich telomeric DNA just as well as does wild-type Est1. Again, the difference between tagged Est1-RNP-NC and untagged Est1-RNP-NC in multiple experiments (Table 3) was found statistically significant by an ANOVA. This result suggests that the RNA-binding motif is not needed for the single-stranded DNA-binding activity of Est1. We note, however, that this result does not directly address the issue of whether the wild-type RRM of Est1 is capable of binding single-stranded G-rich DNA. Because our experiments have been done with Est1 immunoprecipitated from yeast cells, the RRM-independent interaction between single-stranded telomeric oligos and immunoprecipitated Est1 could be due to some protein associated with Est1 and not necessarily to direct binding of single-stranded DNA to Est1. It is also possible that mutant RRM retains the ability to bind single-stranded DNA, even though it binds RNA very poorly.

Two other alleles of *est1* separate the single-stranded DNA-binding function from the RNA-binding function. There are two previously known nonfunctional alleles of *est1* that happen to fall within the RRM. These are $est1_{F511S}$ and $est1_{D513I}$ (F511 and D513 are shown in Fig. 5A) (43). These alleles are phenotypically similar to *est1-mp1-nc* in that they fail to complement an *est1* deletion, and they cause telomere shortening when overexpressed. However, both mutant proteins, when purified from *Escherichia coli*, have wild-type ability to bind single-stranded DNA in vitro (43). We have tested both of these mutant proteins for RNA binding by immunoprecipita-

TABLE 3. Summary of yeast single-stranded telomeric DNA binding[a]

Est1 protein	Ratio of 27-mer to 62-mer							
	Data from expt:							
	1	2	3	4	5	6	7	Avg
Est1	1.7	1.8	2.3	2.3	2.4	2.1	1.4	2.0
3HA-tagged Est1	4.4	5.1	5.4	4.8	2.7	4.4	2.2	4.1
Est1-RNP1-NC				1.6	1.6	2.0	1.2	1.6
3HA-tagged Est1-RNP1-NC				2.9	4.9	4.4	1.7	3.5

[a] Data from 11 independent pairs of experiments are presented as the ratio of 27-mer (telomeric oligo) to 62-mer (nontelomeric oligo). Seven of these pairs of experiments compared the single-stranded DNA-binding activity of 12CA5 immunoprecipitates from untagged and tagged wild-type Est1 strains. The average ratio for untagged Est1 was 2.0; the average ratio for tagged Est1 was 4.1. By ANOVA, this difference was found statistically significant at the 0.05 level. The other four pairs of experiments compared the single-stranded DNA-binding activity of 12CA5 immunoprecipitates from untagged and tagged Est1-RNP1-NC strains. Again, ANOVA showed that the ratios (1.6 versus 3.5) were significantly different. In addition to the experiments shown here, there were one pair of experiments with wild-type Est1 and one pair of experiments with Est1-RNP1-NC (both of these pairs were done on the same day); they were excluded from the analysis because of abnormally high ratios in all parts of the experiments, including controls.

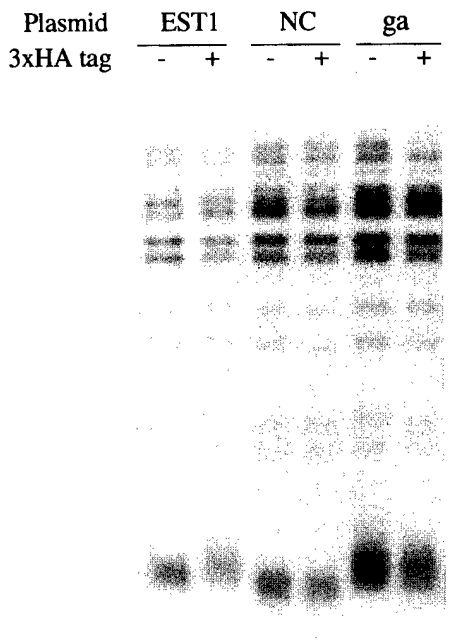

FIG. 7. The *est1 rnp1 nc* mutant has a dominant short-telomere phenotype. Genomic DNA from an *EST1* W303α strain also expressing plasmid-borne *EST1* or *est1-rnp1-nc* or *est1-rnp2-ga* from the *ADH1* promoter (pJZ-3HA-EST1 and derivatives) was digested with *Xho*I and analyzed by Southern blotting with ^{32}P-labeled TELPG as a probe.

tion. Comparable amounts of wild-type and mutant proteins were immunoprecipitated (data not shown), but only the wild-type protein caused coimmunoprecipitation of substantial amounts of Tlc1 (Fig. 9). Thus, these alleles, like *est1-rnp-nc*, separate the RNA-binding function of Est1 from the single-stranded DNA-binding function.

DISCUSSION

We have shown previously that Est1 is in a complex with active telomerase. Here we report that Est1 binds to Tlc1 RNA. This binding is very likely direct, as it requires neither *EST2* nor *EST3*. When mutations are made in the RRM of Est1, Est1 partially or wholly loses its ability to associate with Tlc1. Furthermore, the severity of this association defect is correlated with the severity of the telomere shortening and senescence phenotypes of each mutant.

Previously, it has been shown that Est1 is a single-stranded DNA-binding protein in vitro with specificity for TG-rich telomeric sequences (43). In many cases, RRMs can bind both single-stranded RNA and single-stranded DNA. In particular, it seems quite common to find RRMs capable of binding TG-rich single-stranded DNA (8, 15, 16, 18, 19, 22, 28, 31, 33, 35). This fact raises the issue of whether the previously observed binding to TG-rich single-stranded DNA was due to the RRM of Est1. One argument that this might be the case is that the deletion of amino acids 435 to 565 of Est1 greatly diminished single-stranded DNA binding (43) and removed the RRM.

However, there are a number of strong arguments that single-stranded DNA binding probably is not dependent on the RRM. First, Est1 binds d(TGTGTGGG)$_3$ but does not bind the corresponding polyribonucleotide, r(UGUGUGGG)$_3$ (43), contrary to the expectation if an RRM were responsible for binding single-stranded DNA (8, 16). Second, a 1,000- to 10,000-fold molar excess of Tlc1 RNA fails to prevent single-stranded telomeric DNA from binding to Est1 (43). Third, two missense alleles that fall within the RRM of *EST1*, *est1*$_{F511S}$ and *est1*$_{D513I}$, lack the ability to bind Tlc1 RNA (Fig. 9) but retain the ability to bind single-stranded DNA (43). These mutations thus separate the single-stranded DNA-binding function from the Tlc1 RNA-binding function. These two alleles have phenotypes strikingly similar to that of *est1-rnp1-nc*, as expected if their defect were specific for binding Tlc1. Fourth, Est1-RNP1-NC immunoprecipitated from yeast cells (possibly together with associated proteins) can specifically bind single-stranded telomeric DNA (Fig. 8 and Table 3), even though this protein is defective for Tlc1 binding (Fig. 6).

Thus, it appears that Est1 binds to the yeast telomerase RNA, Tlc1, via an RRM, and binds to single-stranded telomeric DNA via some other nearby motif. Therefore, as suggested by Virta-Pearlman et al. (43), Est1 could serve as a bridge between telomerase and telomere ends. That is, by simultaneously binding telomerase RNA through the RRM and binding telomeric DNA through a second motif, it could help anchor telomerase at the telomere. Recently, strong evidence for this bridge model has been provided by Evans and Lundblad (9), who showed that a Cdc13-Est2 fusion could entirely bypass the need for Est1.

The bridge model is consistent with the observation that *est1* mutants have telomerase activity in vitro (where substrate telomeric oligos are provided at high concentrations) but nevertheless suffer telomere shortening in vivo (where telomere concentrations are low). That is, the need for Est1 may be apparent only at low substrate concentrations; the effect of Est1 may be to reduce the K_m of the telomerase reaction. The affinity of Est1 for telomeric DNA is, however, quite low (43); perhaps this interaction is regulated by cell cycle position, telomere length, or other proteins (such as Cdc13) to help regulate steady-state telomere length.

Homologs of Est1 have not been found in other organisms. However, telomerases from other organisms do have protein

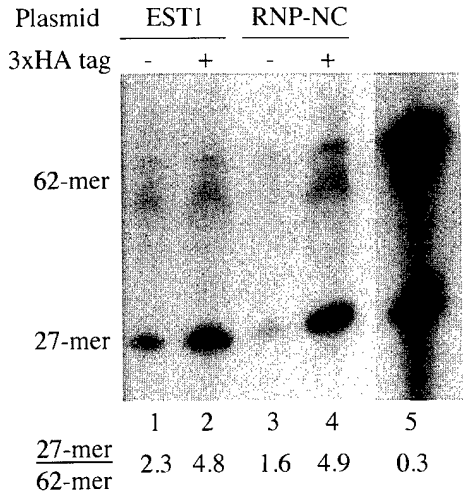

FIG. 8. The Est1-RNP1-NC mutant protein can bind single-stranded telomeric DNA. Monoclonal antibody 12CA5 and protein A beads were mixed with extract from tagged (+) or untagged (−) Est1 strains (lanes 1 to 4). After washing was done, immunoprecipitates were challenged with a mixture of two end-labeled oligos, a 62-mer (EST1-RNP1C) of irrelevant sequence (Table 1) and a TG-rich, telomeric 27-mer (TELPG) (Table 1). Immunoprecipitates were then washed, and the bound oligos were assayed by phosphorimaging after gel electrophoresis. Lane 5 shows 1/20 the amount of the 27-mer–62-mer mixture that was added to the immunoprecipitates in the other lanes. The ratio of 27-mer to 62-mer is shown beneath the lanes.

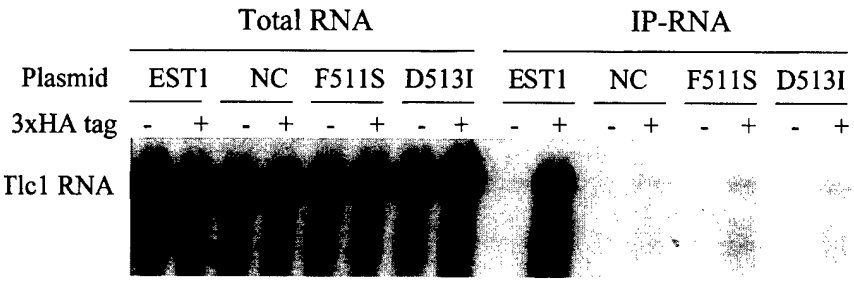

FIG. 9. The Est1 F511S and D513I mutant proteins are defective in binding Tlc1 RNA. Total RNA (left) and IP-RNA (right) were made from *est1* cells expressing wild-type or mutant forms of either untagged (−) or 3HA-tagged (+) Est1 from the *ADH1* promoter (plasmid pJZ-3HA-EST1 and derivatives). The amount of Tlc1 RNA in total RNA or coimmunoprecipitated with Est1 was assayed by Northern blotting. The amount of immunoprecipitated protein was assayed by Western blotting (data not shown).

components in addition to the reverse transcriptase subunit. In *Tetrahymena*, proteins p80 and p95 are associated with telomerase, and p80 can be cross-linked to the RNA component in vivo (6). A human protein called TP1 (also called TLP1) is associated with human telomerase and has significant homology to *Tetrahymena* p80 (12, 30). Furthermore TP1 can interact with the RNA component of human telomerase in the yeast-based three-hybrid assay, suggesting that TP1 binds to RNA directly. Thus, *Tetrahymena*, human, and perhaps other telomerases may contain RNA-binding proteins with functions analogous to the function of Est1. Since RNA-binding motifs are often short and are not all of the RNP1 or RNP2 type, functional homologs of Est1 will not necessarily have any protein sequence similarity to Est1. Moreover, it is not clear that tethering of telomerase to telomeres must occur through the RNA template, as it apparently does in *S. cerevisiae*. Instead, in some organisms, tethering might occur through the reverse transcriptase catalytic subunit.

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