Senescence Mutants of *Saccharomyces cerevisiae* With a Defect in Telomere Replication Identify Three Additional *EST* Genes

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ABSTRACT

The primary determinant for telomere replication is the enzyme telomerase, responsible for elongating the G-rich strand of the telomere. The only component of this enzyme that has been identified in *Saccharomyces cerevisiae* is the *TLC1* gene, encoding the telomerase RNA subunit. However, a yeast strain defective for the *EST1* gene exhibits the same phenotypes (progressively shorter telomeres and a senescence phenotype) as a strain deleted for *TLC1*, suggesting that *EST1* encodes either a component of telomerase or some other factor essential for telomerase function. We designed a multitiered screen that led to the isolation of 22 mutants that display the same phenotypes as *est1* and *tlc1* mutant strains. These mutations mapped to four complementation groups: the previously identified *EST1* gene and three additional genes, called *EST2*, *EST3* and *EST4*. Cloning of the *EST2* gene demonstrated that it encodes a large, extremely basic novel protein with no motifs that provide clues as to function. Epistasis analysis indicated that the four *EST* genes function in the same pathway for telomere replication as defined by the *TLC1* gene, suggesting that the *EST* genes encode either components of telomerase or factors that positively regulate telomerase activity.

IN all eukaryotes examined to date, with the exception of Drosophila and several other dipterans, telomeres are replicated by a specialized DNA polymerase called telomerase (reviewed in BLACKBURN and GREIDER 1995; KIPLING 1995; ZAKIAN 1995). This enzyme is responsible for adding telomeric repeats onto the 3′ end of the G-rich strand of the telomere and uses an internal template present in an RNA subunit to dictate the sequence of the added telomeric DNA. The gene encoding the RNA component of telomerase has been identified in a number of species (GREIDER and BLACKBURN 1989; SHIPPEN-LENTZ and BLACKBURN 1990; LINGNER *et al.* 1994; BLASCO *et al.* 1995; FENG *et al.* 1995; MCEACHERN and BLACKBURN 1995), including the *TLC1* gene from *Saccharomyces cerevisiae* (SINGER and GOTTSCHLING 1994). However, in contrast, the characterization of the protein components of this enzyme has lagged behind. In addition, little is known at a molecular level about how regulation of telomerase activity is achieved.

There are several approaches to identifying additional components of the telomerase enzyme complex. Biochemical purification of telomerase from the ciliate Tetrahymena has recently led to the identification of two proteins that, in addition to the RNA component, comprise subunits of the enzyme (COLLINS *et al.* 1995); homologues of these genes have not been identified yet in other species, including in the completely sequenced *S. cerevisiae* genome. An alternative to the biochemical route is to identify genes that, when mutated, have a set of phenotypes predicted for a defect in telomerase *in vivo*. These two different protocols presumably could lead to identification of at least some of the same factors. However, a genetic approach has the potential to identify components that may be critical for *in vivo* function (for example, as positive regulators) but which are not required *in vitro* and therefore may not copurify with the enzyme.

Two genes have been previously identified in *S. cerevisiae* that, when deleted, result in a cell with phenotypes expected from the elimination of telomerase activity. The first of these is the *EST1* gene, which encodes a highly basic 82-kD protein hypothesized to be a subunit of telomerase (LUNDBLAD and SZOSTAK 1989; LUNDBLAD and BLACKBURN 1990). This proposal has been based largely on the observation that *est1* null mutant strains display two characteristics predicted for a telomerase deficiency: progressive loss of telomere sequences from chromosomal termini and a senescence phenotype (manifested as a steady decline in cell viability). Strong support that such phenotypes could be diagnostic for a defect in telomerase came from the demonstration that

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a yeast strain deleted for the *TLC1* gene, encoding the yeast telomerase RNA, exhibits the same set of phenotypes (SINGER and GOTTSCHLING 1994). Although telomerase activity is still present in both chromatographic fractions or nuclear extracts prepared from an *est1-Δ* strain, indicating that Est1p is not required *in vitro* for catalytic activity (COHN and BLACKBURN 1995; N. F. LUE and V. LUNDBLAD, unpublished results), this does not exclude the possibility that *EST1* is a non-catalytic component of telomerase (LIN and ZAKIAN 1995). Since the Est1 protein binds single-stranded yeast telomere sequences (V. VIRTA-PEARLMAN, D. K. MORRIS and V. LUNDBLAD, unpublished results), Est1 may function as a telomerase component responsible for telomere recognition. Consistent with this, Est1 protein is associated with the telomerase RNA and/or enzyme activity (LIN and ZAKIAN 1995; STEINER *et al.* 1996), although it was not possible to determine whether the association was quantitative in these experiments. The properties of Est1 binding to telomeric DNA are also consistent with a role as a telomere-end-binding protein (V. VIRTA-PEARLMAN, D. K. MORRIS and V. LUNDBLAD, unpublished results), acting as a positive regulator of telomerase function by directing telomerase onto the chromosomal terminus. In either model, the requirement for Est1 function might be obviated in the *in vitro* experiments in which naked DNA is used as a substrate for telomerase elongation.

In this work, as a first step toward defining additional components and/or regulators of yeast telomerase, we identified 22 new mutants of *S. cerevisiae* with phenotypes similar to those displayed by *est1* and *tlc1* strains. Thirteen of these new mutants mapped to the previously identified *EST1* gene; the remainder defined three additional genes, called *EST2, EST3* and *EST4.* Several genetic observations argue that these four *EST* genes, along with *TLC1,* function in a single genetically defined pathway for telomere replication. First, strains carrying mutations in the *EST2, EST3* or *EST4* genes exhibited a set of phenotypes that are indistinguishable from those displayed by *est1-Δ* and *tlc1-Δ* strains. In addition, an alternative pathway for telomere maintenance previously observed to arise in *est1* and *tlc1* mutant strains (LUNDBLAD and BLACKBURN 1993; SINGER and GOTTSCHLING 1994) also occurred in *est2, est3* and *est4* mutant strains. Finally, combining multiple *est* or *tlc* mutations in the same cell did not result in any enhancement of phenotype, arguing against the possibility that these five genes are required for more than one pathway that functions in parallel to replicate the telomere completely. Collectively, this data is consistent with the proposal that *EST2, EST3* and *EST4* also encode either components of telomerase or *in vivo* regulators of this enzyme.

MATERIALS AND METHODS

Yeast strains and media: *S. cerevisiae* strains used in this study are shown in Table 1; YPH275 was the diploid parent of all strains used in these studies. The mutant derivatives MVL1–MVL26 were isolated, as described below, from the haploid strain TVL227-1A, obtained by transformation of YPH275 with plasmid pVL106 (LUNDBLAD and SZOSTAK 1989) and subsequent sporulation and dissection. TLV120 and TLV140 were described previously (LUNDBLAD and SZOSTAK 1989; LUNDBLAD and BLACKBURN 1993); DLV131 was constructed from YPH275 by introducing a *tlc1-Δ::LEU2* disruption, constructed by PCR-mediated deletion (BAUDIN *et al.* 1993) of sequences between 192 and 909. Strains were grown at 30° and in standard media (GUTHRIE and FINK 1991), except that chromosome loss assays were performed on media with limiting adenine (6 μg/ml) to enhance the detection of red Ade$^-$ sectors in colonies. Canavanine plates and 5-FOA CM-LEU plates were prepared as described in AUSUBEL *et al.* 1987. Yeast transformations were performed using the lithium acetate method (SCHIESTL and GIETZ 1989); sporulation and tetrad dissections were carried out using standard techniques (GUTHRIE and FINK 1991).

EMS mutagenesis: TVL227-1A was grown overnight to saturation in CM-LEU-TRP, diluted 10-fold into YEPD media and grown to 0.8–1.0 OD. Forty milliliters of cells were washed twice in sterile dH_2O and resuspended in 12 ml 0.1 M sodium phosphate, pH 7.0; 1.7-ml portions were incubated with 45 μl of EMS (ethylmethane sulfonate) for 1 hr at 30° with slow aeration. Cells were diluted 10-fold in sterile 5% sodium thiosulfate, washed twice in 5% sodium thiosulfate, twice in sterile dH_2O and resuspended in 2.0 ml dH_2O. Aliquots were plated on canavinine plates to monitor the increase in frequency of canavinine resistance and dilutions plated on YEPD plates to determine the percent of cells that survived the mutagenesis procedure; both values were determined relative to a sample of cells handled in parallel but without added EMS. Because of the large number of colonies screened, the entire screen was performed in three stages, with each stage handling between 80,000 and 150,000 colonies. A total of 12 independent mutageneses were performed; the average survival ranged from 30 to 60% and the increase in canavinine resistance was 50- to 150-fold. Note that since all screening steps were performed at 30°, this mutant screen was not specifically designed to recover conditional lethal mutations.

Mutant screen: *Colony sectoring screen:* To detect mutants that show a phenotypic delay in chromosome loss, mutagenized cells were processed through two rounds of growth as single colonies. After mutagenesis, cultures were plated on CM-LEU-TRP plates at a density of 500–1000 per plate and colonies allowed to grow at 30° to full size, for 3–4 days. Pools of ~7500 colonies were generated by resuspending colonies from CM-LEU-TRP plates in sterile dH_2O and replating at a density of ~350 per plate on CM-LEU plates with limiting adenine. The total number of colonies replated from each pool was approximately fourfold the size of the pool, to increase the odds that each colony in the original pool was represented. Plates were incubated for 7 days at 30° to allow full development of red Ade$^-$ sectors; in addition, overnight incubation at 4° prior to scoring for chromosome loss helped enhance detection of red sectors (F. SPENCER, personal communication). If two *est* mutants were isolated from a single pool and later shown to map to the same complementation group, they were considered to be siblings of each other and only one was subsequently analyzed.

Plasmid linearization assay: Colonies with increased numbers of Ade$^-$ sectors were picked into microtiter dish wells containing 0.2-ml CM-LEU media, to select for retention of pVL106 (LUNDBLAD and SZOSTAK 1989). After 2 days growth at 30°, 5 μl aliquots from each well were transferred to CM-LEU-5-FOA plates, using a multiple-channel pipettor, and plates were subsequently incubated 3 more days at 30° to allow the growth of 5-FOA resistant micro-colonies. A wild-type un-

TABLE 1

Yeast strains

Strain	Genotype[a]	Source
YPH275	*MATa/MATα* CF-*SUP11-TRP1*	P. HIETER
TVL120	*MATa/MATα EST1/est1-Δ3::HIS3* CF-*SUP11-TRP1*	LUNDBLAD and SZOSTAK (1989)
TVL140	*MATa/MATα EST1/est1-Δ3::HIS3 RAD52/rad52::LEU2* CF-*SUP11-TRP1*	LUNDBLAD and BLACKBURN (1993)
TVL228	*MATa/MATα EST1/est1-Δ3::HIS3 RAD52/rad52::LEU2 EST2/est2-Δ1::URA3* CF-*SUP11-TRP1*	This study
DVL131	*MATa/MATα TLC1/tlc1-Δ::LEU2* CF-*SUP11-TRP1*	This study
DVL132	*MATa/MATα TLC1/tlc1-Δ::LEU2 EST1/est1-Δ3::HIS3* CF-*SUP11-TRP1*	This study
TVL227-1A	*MATα* CF-*SUP11-TRP1*/pVL106[b]	This study
MVL6[c]	*MATα est1-19*	This study
MVL9	*MATα est1-20*	This study
MVL11	*MATα est1-21*	This study
MVL12	*MATα est1-22*	This study
MVL16	*MATα est1-23*	This study
MVL17	*MATα est1-24*	This study
MVL18	*MATα est1-25*	This study
MVL19	*MATα est1-26*	This study
MVL20	*MATα est1-27*	This study
MVL21	*MATα est1-28*	This study
MVL22	*MATα est1-29*	This study
MVL23	*MATα est1-30*	This study
MVL24	*MATα est1-31*	This study
MVL7	*MATα est2-1*	This study
MVL8	*MATα est2-2*	This study
MVL13	*MATα est2-3*	This study
MVL14	*MATα est2-4*	This study
MVL10	*MATα est3-1*	This study
MVL15	*MATα est3-2*	This study
MVL25	*MATα est3-3*	This study
MVL1	*MATα est3-4*	This study
MVL26	*MATα est4-1*	This study

[a] All strains were isogenic derivatives of YPH275; the diploids also carry *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* and the haploids carry *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*.

[b] pVL106 is an *ARS1 LEU2 CEN3* plasmid used in the plasmid linearization assay; see LUNDBLAD and SZOSTAK (1989) for more details.

[c] Although MVL1–MVL26 are derived from TVL227-1A, during the course of the screen, many mutant isolates lost either CF or pVL106 (or both); therefore, these two elements are not indicated in the genotypes of MVL1–MVL26.

mutagenized strain gives rise to ~10–15 micro-colonies on the 5-FOA plates in this assay; all mutants that showed more than a threefold reduction were saved for further analysis. Each candidate was recovered from the microtiter dish well and streaked for single colonies on CM-LEU plates, and three single colonies for each were retested in the plasmid linearization assay. Candidates that passed this retest were assayed for alterations in telomere length by Southern analysis. In addition, candidates that reached this stage were transferred to 15% glycerols and stored at 70°, to prevent the loss of rapidly senescing strains.

Telomere length analysis: One to two single colonies from CM-LEU streak-outs for each candidate that passed the plasmid linearization assay were grown to saturation in 8 ml YEPD and genomic DNA prepared as described previously (GUTHRIE and FINK 1991). Samples of ~1 μg of DNA digested with *Xho*I enzyme (New England Biolabs) were separated on 20-cm 0.7% agarose gels, transferred to nylon membranes and probed with poly d(GT/CA), as described previously (LUNDBLAD and SZOSTAK 1989). When telomere length was assayed with increasing numbers of generations of growth (Figures 2 and 4), cultures were grown to saturation in YEPD and subsequently diluted by a factor of 10^{-4} and allowed to regrow to saturation; this process was repeated three to four times.

Cellular senescence assays: All mutants with decreases in telomere length of >250 bp were tested for whether they exhibited an associated senescence phenotype by back-crossing each mutant to a wild-type haploid derivative of YPH275 and sporulating and dissecting the subsequent diploid strain. For each mutant, haploid spore products from four to six tetrads were streaked for single colonies on YEPD, incubated at 30° for 48 hr and scored for growth characteristics in parallel with *est1-Δ3::HIS3* haploid spore products generated from a

dissection of TVL120. Single colonies from these "1X" streak-outs were subsequently restreaked and similarly analyzed; this process was repeated up to four times. For the data presented in Figures 2–5, each of these successive YEPD streak-outs were stored at 23° and used to re-assemble a time course on a single plate.

Genetic manipulations: *Complementation analysis:* Diploids were isolated from unselected matings, to prevent selection for reversion or suppression of the senescence phenotype. Haploid strains of opposite mating type were patched together on YEPD plates and allowed to mate for 4–6 hr. Four zygotes were picked for each cross by micromanipulation with a Zeiss dissecting microscope and allowed to grow at 30° for 2 days. The resulting strains were purified once via single colony isolation and tested for mating type and any nutritional markers that differed between the two haploid parents. To assay for complementation of telomere length, one single colony from two to three diploids was grown up and assayed by Southern analysis along with each of the two haploid parents. The growth characteristics and chromosome stability of multiple diploids for a given cross were similarly analyzed in parallel with the haploid parents.

Linkage analysis and construction of double mutant haploid strains: Haploid *est* spores of opposite mating type were used to generate diploids (also by unselected matings) that were heterozygous for two different *EST* genes. Diploids were sporulated and dissected and spores analyzed for cellular senescence and telomere length. Initially, tetrads with four viable spores as well as tetrads with less than four viable spores were analyzed; when no evidence for synthetic lethality was obtained (see Figure 4), subsequent analysis concentrated on tetrads with four viable spores. The following crosses were performed to demonstrate that the new complementation groups (*EST2, EST3* and *EST4*) were distinct from each other and from *EST1* and *TLC1*: (i) *est2-1* × *est1-Δ::HIS3*; (ii) *est2-1* × *tlc1-Δ::LEU2*; (iii) *est2-1* × *est3-1*; (iv) *est3-1* × *est1-Δ::HIS3*; (v) *est3-1* × *tlc1-Δ::LEU2*; (vi) *est4-1* × *est1-Δ::HIS3*; (vii) *est4-1* × *tlc1-Δ::LEU2*. For each cross, eight or more tetrads were analyzed for telomere length and senescence, with at least four tetratype and nonparental ditype tetrads recovered and analyzed for each. The resulting diploid strains were also used to construct the double and single mutant haploid strains analyzed in Figure 4. The *est2-1, est3-1* and *est4-1* haploids used to generate these diploids were the result of at least three backcrosses to wild type, and the *est1-Δ::HIS3* and *tlc1-Δ::LEU2* haploids were isolated from TLV120 and DVL131, respectively.

Identification of est survivor strains: Each freshly generated *est* haploid strain was grown in liquid YEPD culture, with successive dilutions, for 120–150 generations and subsequently plated for single colonies on YEPD plates. After 2 days growth at 30°, large colonies were examined for both growth phenotype and telomere structure on Southern blots, as described above.

Cloning of the *EST2* gene: Freshly dissected *est2-1* mutant strains were transformed with genomic libraries and screened for complementation of the senescence phenotype, using two approaches: (1) an *est2-1 rad52::LEU2* strain was transformed with three different *URA3 CEN* genomic libraries (ROSE *et al.* 1987), with ~2000 transformants recovered for each transformation (which probably was a substantial under-estimate of the transformation efficiency, due to the severe growth defect of the *est2-1 rad52::LEU2* strain). Complementing clones were not apparent among any of these primary transformants; however, upon scraping and replating, healthy colonies were identified from two of the three library transformations. One single colony from each library pool was saved for future analysis; both were shown to have wild-type telomeres as assayed on Southern blots. (2) An *est2-1* strain at an intermediate stage in the senescence progression was transformed with a 2 μ *LEU2* library (ENGEBRECHT *et al.* 1990) and ~30,000 transformants were carefully screened after 40–45 hr for those transformants with a slight growth advantage. Fifty-seven candidates were selected and streaked for single colonies twice in succession to test for complementation of the senescence phenotype. Ten of the 57 candidates showed a healthy growth phenotype by this test; four also had wild-type telomere length and were saved for further analysis (the remainder demonstrated the recombination-dependent telomere bypass pathway previously observed for *est1* mutants; LUNDBLAD and BLACKBURN 1993).

For the six transformants recovered from the above three library transformations, the healthy growth phenotype and wild-type telomere length were shown in each case to be plasmid-dependent. Plasmids were subsequently recovered from each yeast strain by rescue through *Escherichia coli*. A combination of restriction mapping and Southern analysis of the genomic inserts demonstrated that the four plasmids recovered from the 2 μ *LEU2* library were identical to each other, and had genomic inserts that over-lapped with the inserts present in the two plasmids recovered from the *URA3 CEN* genomic libraries. A 4.4-kb subcloned fragment common to these three unique plasmids capable of complementing *est2-1* was subjected to insertional mutagenesis using the bacterial transposon $\gamma\delta$ (STRATHMANN *et al.* 1991). Insertion mutations were mapped by PCR, using primers against the transposon and polylinker sequences that bounded the cloned insert. The $\gamma\delta$ insertions subsequently provided the basis for sequence analysis, using primers unique to the right and left ends of the $\gamma\delta$ transposon; sequence was confirmed by comparison to that generated by the yeast genome sequencing project. The *est2-Δ1::URA3* disruption mutation was constructed by deleting an internal 2.2-kb *Hind*III fragment, removing aa 13 to aa 686 of the *EST2* ORF and inserting a 1.17-kb *URA3 Hind*III fragment. This disruption was introduced into TVL140 to generate an *EST2/est2-Δ1::URA3* heterozygous diploid (strain TVL228), which was confirmed by Southern analysis. Twelve tetrads were analyzed in detail for senescence and telomere phenotypes, as described above and in RESULTS.

RESULTS

Identification of an expanded collection of *S. cerevisiae* mutants with a telomere replication defect and a senescence phenotype: The original identification of the *est1-1* mutant employed a screen that assayed the behavior of a circular plasmid containing inverted repeats of Tetrahymena telomeric sequences bracketing the yeast *URA3* gene (LUNDBLAD and SZOSTAK 1989). At a low frequency in wild-type cells, this circular plasmid can be converted into a stable linear form. This conversion requires the addition of yeast telomeric sequences onto the Tetrahymena termini in order to form functional telomeres. Since the conversion to a linear molecule results in the loss of the *URA3* gene, mutants defective in the linearization process can be identified by screening for colonies that exhibit decreased frequencies of resistance to the drug 5-FOA (Ura^+ cells convert 5-FOA to a toxic intermediate, whereas the loss of *URA3* prevents this; BOEKE *et al.* 1984). Potentially, one subset of mutants that could be identified by this assay would also be defective for the ability to form functional telomeres, once the plasmid was linearized; such mutants should also exhibit shorter

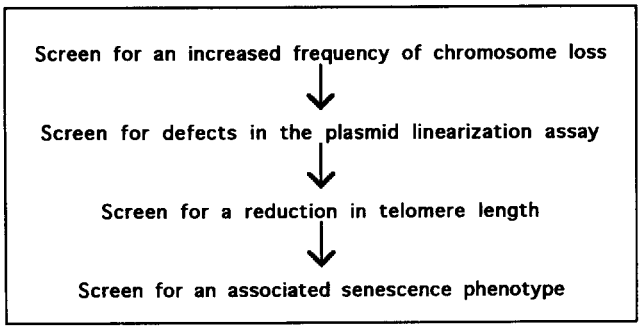


FIGURE 1.—Four-tiered scheme for isolation of new *est* mutants.

chromosomal telomeres. From this original screen, the *est1-1* mutant was the one candidate that fell into this class, with a progressive reduction in telomere length with increasing growth of the culture (LUNDBLAD and SZOSTAK 1989). However, one drawback to the plasmid linearization assay was that the process of testing individual colonies for their frequency of 5-FOA resistance was labor-intensive (see MATERIALS AND METHODS), which limited the original screening to only ~7000 mutagenized colonies.

Once identified, both the *est1-1* mutant and the subsequently constructed *est1*-Δ strain were shown to have two additional associated phenotypes: a senescence phenotype, manifested as a gradual decrease in cell viability and an accompanying progressive increase in the frequency of chromosome loss (LUNDBLAD and SZOSTAK 1989). In order to isolate additional *est*-like mutants from yeast, all four of the phenotypes displayed by *est1* mutants were incorporated into an expanded screening protocol, presented in Figure 1. Due to the difficulty in screening large numbers of colonies in the plasmid linearization assay, this step was first preceded by an enrichment step for colonies that exhibited increased frequencies of chromosome loss. Candidates that showed both an increase in chromosome loss and a defect in the plasmid linearization assay were subsequently screened for alterations in telomere length by Southern blot analysis. Finally, those isolates with short telomeres were tested for an associated senescence phenotype by examining freshly generated haploid mutants for whether they showed the characteristic decline in viability over time previously observed in *est1* and *tlc1* mutants.

Due to one unique aspect of the effect of *est1* mutations on chromosome instability, the standard chromosome loss assay used by others required modification. Detection of alterations in chromosome stability relied on a previously developed color-based visual assay that monitored the presence/absence of a nonessential 150-kb artificial test chromosome (SPENCER *et al.* 1990). A number of screens have been performed that use this assay, or similar variants, to detect mutants that affect chromosome fidelity (MEEKS-WAGNER *et al.* 1986; KOUPRINA *et al.* 1988; SPENCER *et al.* 1990; RUNGE and ZAKIAN 1993). None of these previous screens, which have collectively identified a large number of genes involved in chromosome maintenance, detected mutations in either *EST1* or the new *EST* genes identified in this work. This is presumably due to the fact that the effect of *est1* mutations on chromosome loss shows phenotypic lag: no substantial increase in chromosome instability in an *est1* mutant strain is observed until ~40–60 generations of growth (LUNDBLAD and SZOSTAK 1989). Therefore, to screen for mutants that showed similar delayed effects on chromosome stability required outgrowth after mutagenesis. Since the desired class of mutants was also expected to have a senescence phenotype, growth in liquid would exert a substantial selective disadvantage for such mutants. To prevent this, mutagenized cultures were processed through two rounds of growth as single colonies prior to screening for chromosome instability (see MATERIALS AND METHODS for more details).

A total of 350,000 single colonies from 12 independently mutagenized cultures of yeast were screened by the four-tiered system shown in Figure 1. Individual colonies from cultures mutagenized with EMS to an average survival of ~50% were processed in batches of 80,000–150,000 colonies. The first two steps (chromosome loss and the plasmid linearization assay) resulted in enrichment steps of ~20- and ~50-fold, respectively. Southern blot analysis of telomere length of 375 candidates that passed these two tests led to the identification of 49 mutants with either decreases (ranging from ~50 bp to >300 bp) or increases (ranging from 150 bp to > 2 kb) in telomere length. Of the 36 mutants with shorter telomeres, 19 also had an associated senescence phenotype. These 19 *est* mutants, plus three additional short telomere mutants that had no detectable senescence phenotype but were subsequently shown to map to *EST* genes, are the subject of this report.

The new *est*⁻ mutants map to four genes: Each of the *est* mutants, when crossed to wild type, were recessive for telomere length, chromosome loss and senescence (data not shown). The 19 mutants were also tested for whether they contained mutations in either *EST1* or *TLC1* by crossing each mutant to an *est1*-Δ strain and a *tlc1*-Δ strain; the resulting diploids were examined for both telomere length and viability. All 19 mutant strains fully complemented both phenotypes of the *tlc1*-Δ mutation, indicating that no new alleles of *TLC1* had been identified (data not shown). However, 11 mutants failed to complement both the senescence and short telomere phenotypes of the *est1*-Δ strain. Two additional mutants, with short telomeres but no obvious senescence phenotype, were also subsequently shown by complementation analysis to contain weak *est1* mutations. This complementation data was confirmed for five of these new *est1* alleles by demonstrating that the mutation could be gap-repaired onto an *EST1*-containing plasmid with a gap encompassing the wild type *EST1* gene (C. NUGENT and V. LUNDBLAD, unpublished data). The

identification of 13 *est1* alleles demonstrated that this expanded screen was capable of detecting *est* mutant strains.

The remaining eight *est* strains were crossed against each other in pair-wise combinations, and the resulting diploids were analyzed for telomere length, chromosome loss and senescence. This analysis indicated that these eight mutants defined three new complementation groups: four mutations mapped to *EST2,* three mapped to *EST3* and one isolate mapped to *EST4.* One additional mutant, with short telomeres but an apparently wild-type growth phenotype, was subsequently shown to contain a non-senescent allele of *EST3.* To confirm the complementation analysis, linkage analysis was performed on one to two isolates from each new complementation group to establish that *EST2, EST3* and *EST4* defined three genes that were distinct from each other and from *TLC1* and *EST1* (data not shown; see MATERIALS AND METHODS for more details). In addition, the products of six to eight tetrads with four viable spores of crosses between mutant and wild type were analyzed. In all cases, reduced telomere length and senescence showed 2:2 segregation, with these two phenotypes exhibiting 100% cosegregation, demonstrating that a single recessive nuclear mutation was responsible (data not shown).

The lack of recovery of *tlc1* mutants from this screen was surprising given the fact that a defect in *TLC1* gives rise to the same set of phenotypes as observed in *est* mutant strains (SINGER and GOTTSCHLING 1994) (Figures 2 and 3). One possible explanation is that many EMS-induced mutations in a gene whose product is an RNA would be expected to be phenotypically silent. However, the absence of *tlc1* mutations from our collection, combined with the fact that only one *est4* isolate was recovered, suggests that there may be additional *EST* genes yet to be identified.

***est2, est3* and *est4* mutants are phenotypically indistinguishable from *est1* and *tlc1* strains:** If the three new *EST* genes play a similar role in telomere replication as *EST1* and *TLC1,* mutations in these new genes should display phenotypes similar to those exhibited by *est1*-Δ and *tlc1*-Δ strains. To test this, several mutants from each new complementation group were back-crossed to wild type and sporulated. Freshly generated spore products were examined over time for their growth phenotype and telomere length, in parallel with a freshly generated *est1*-Δ haploid strain. Figure 2A shows the telomere length decline that a representative mutant from each of the new complementation groups displayed over time as compared with that displayed by *est1*-Δ and *tlc1*-Δ strains. Strains were grown for 80 generations, with DNA prepared for Southern analysis every ~15 generations. Telomere length in each mutant declined with time, and did so to the same degree as observed in *est1*-Δ and *tlc1*-Δ strains.

In parallel with the analysis of telomere length, we also analyzed the growth and chromosome stability over time in these new mutants. Freshly dissected mutant haploids strains were streaked to give single colonies three successive times on YEPD plates, in parallel with freshly generated *est1*-Δ and *tlc1*-Δ haploid strains. Each of the new *est* mutants showed a dramatic decline in viability late in their outgrowth. The growth phenotype for each of the new *est2* and *est3* alleles was qualitatively indistinguishable from that displayed by null mutations in *EST1* and *TLC1* (Figure 2B and data not shown). The *est4-1* mutant also showed a clear senescence phenotype but in this case, senescence was delayed in comparison with the other *est* mutants, possibly because the single *est4* allele may be somewhat leaky. In parallel with the senescence phenotype, each new *est* mutant also showed a striking increase in the frequency of chromosome loss (data not shown); the appearance of this increased chromosome instability was delayed, similar to the delay previously exhibited by *est1*-Δ strains (LUNDBLAD and SZOSTAK 1989).

New *est* mutants utilize the same alternative pathway for telomere maintenance as *est1*-Δ or *tlc1*-Δ strains: Previous analysis has shown that late in the outgrowth of *est1* mutant cultures, a small proportion of cells are able to escape the lethal consequences of the absence of *EST1* function. These *est1*-Δ survivors arise as a result of a bypass pathway for telomere maintenance that is activated in late *est1* cultures (LUNDBLAD and BLACKBURN 1993). Activation of this alternative pathway occurs as the result of a global amplification and rearrangement of both telomeric G-rich repeats and subtelomeric regions. This amplification can be quite substantial: in some survivors, the amount of telomeric $d(G_{1-3}T)$ repeats is increased by as much as 40-fold, such that 4% of the genome consists of telomeric DNA (V. LUNDBLAD, unpublished data). As a consequence of this genomic reorganization, telomere function is restored and the survivors have regained a normal or near-normal growth phenotype. This pathway requires the *RAD52* gene, which mediates the majority of homologous recombination events in yeast; in the absence of *RAD52* gene function, *est1 rad52* strains cannot generate late-culture survivors and instead die completely after ~40 to ~60 generations (LUNDBLAD and BLACKBURN 1993). Consistent with the other similarities between *EST1* and *TLC1,* a *tlc1*-Δ strain also exhibits alterations of telomeric and subtelomeric DNA (SINGER and GOTTSCHLING 1994) which is *RAD52*-dependent (Figure 3). This alternative pathway has not been demonstrated to arise in other, non-senescent, telomere replication defective strains of *S. cerevisiae.*

To test whether *est2, est3* and *est4* strains were also capable of participating in this process, two to three isolates for each mutant were grown in culture for 120–150 generations and plated for single colonies. Several single colonies from each culture were analyzed for growth characteristics and telomere structure. Figure 3A shows the Southern blot of one such survivor from

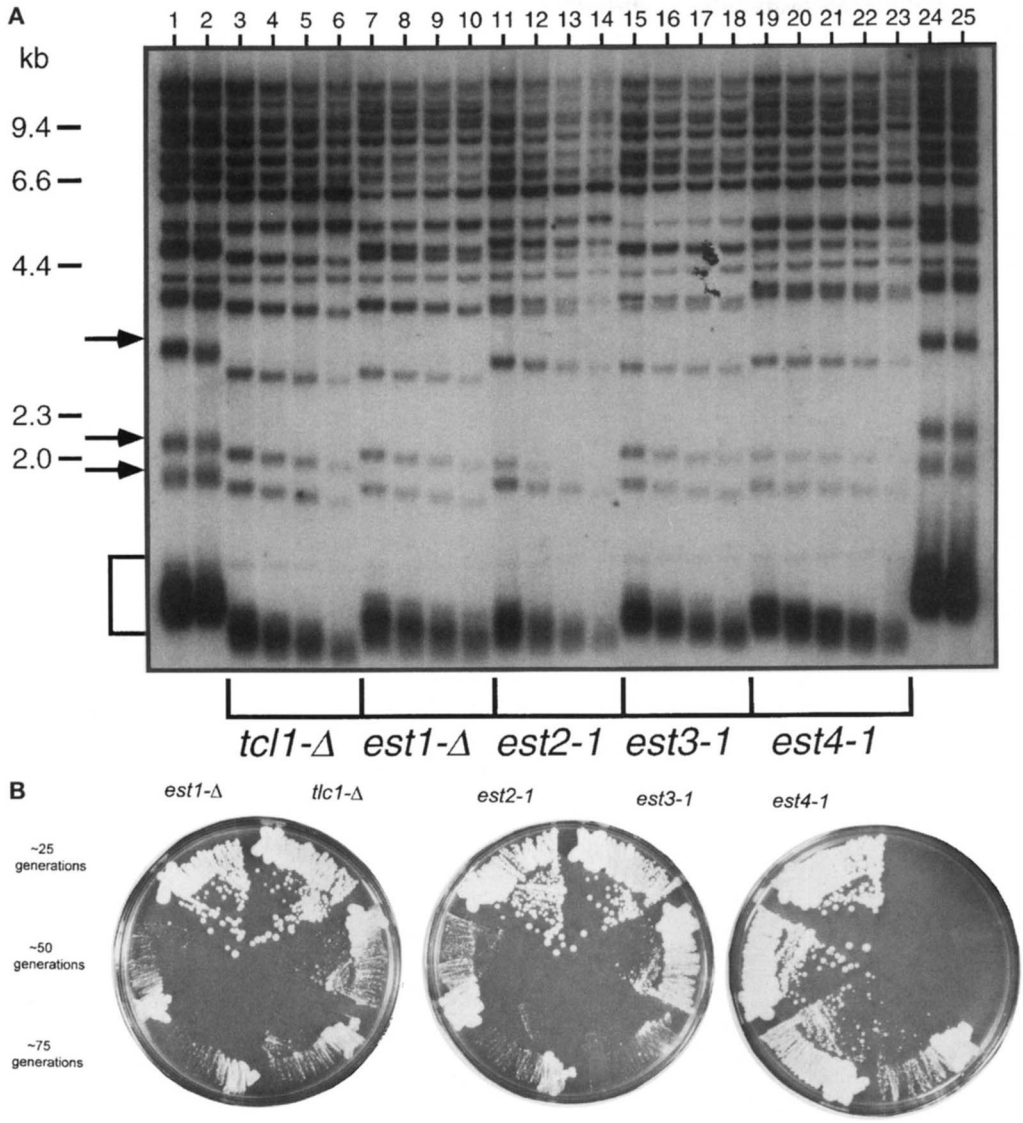


FIGURE 2.—New *est* mutant isolates exhibit the same phenotypes as *est1*-Δ and *tlc1*-Δ strains. (A) Southern blot of genomic yeast DNA grown successively in culture, probed with a telomere-specific probe (poly d[GT/CA]). Lanes 1, 2, 24 and 25 *EST*$^+$ *TLC1*$^+$; lanes 3–6, *tlc1*-Δ::*LEU2*; lanes 7–10, *est1*-Δ*3*::*HIS3*; lanes 11–14, *est2-1*; lanes 15–18, *est3-1*; lanes 19–23, *est4-1*. For the *tlc1, est1, est2* and *est3* strains, four successive subcultures, representing a total of 65–70 generations of growth, are shown; due to the slightly weaker phenotype of *est4-1*, a fifth subculture (corresponding to 13–15 additional generations of growth) is included. Lanes 1 and 2 and lanes 24 and 25 show the first and fourth subculture, respectively, of two *EST*$^+$ *TLC1*$^+$ strains handled in parallel. The bracket indicates a telomeric band that represents approximately 2/3 of the telomeres in this strain (those that are Y′-containing); arrows indicate restriction fragments corresponding to individual non-Y′-containing telomeres. (B) Viability of *est1*-Δ*3*::*HIS3*, *tlc1*-Δ::*LEU2*, *est2-1*, *est3-1* and *est4-1*, shown as three successive streak-outs on YEPD plates, differing from each other by ~25 generations of growth; for *est4-1*, one additional successive streak-out is shown.

each of the five *tlc1* or *est* strains, probed with poly d(GT/CA); all displayed the same type of telomere rearrangement originally observed in *est1* mutants, characterized by extensive amplification of both d(G_{1-3}T) telomeric DNA and Y′ subtelomeric repeats (LUNDBLAD and BLACKBURN 1993). In addition, each of these *est2, est3* and *est4* survivors had now acquired a growth phenotype that closely approximated that of wild type (data not shown), similar to that previously observed for *est1* survivors. The appearance of survivors was not specific to the particular *est* alleles used in Figure 3, as all 19 of the *est* mutants isolated in this mutant screen which displayed a senescence phenotype gave rise to survivors (data not shown).

To test whether the process of generating survivors from *est2, est3* and *est4* mutants was also *RAD52*-dependent, each mutant was crossed to a *rad52*::*LEU2* strain. The resulting diploids were sporulated and dissected, and *est* and *est rad52* haploid strains were analyzed in parallel. Figure 3B shows that the presence of a *rad52* mutation conferred lethality on each strain after ~40–50 generations. In addition, the degree of enhancement of the senescence phenotype in the presence of a *rad52* mutation was the same in *est1, est2, est3* and *tlc1* mutants, providing another point of similarity between mutations in these different genes. Consistent with the somewhat delayed senescence phenotype exhibited by the *est4-1* mutant in a *RAD52*$^+$ strain (Figure 2B), the appearance of lethality in an *est4-1 rad52*::*LEU2* strain was slightly delayed (Figure 3B), relative to the other *est rad52* and *tlc1 rad52* mutant strains.

***TLC1* and the four *EST* genes function in a single pathway for telomere replication:** The above comparison demonstrated that strains carrying mutations in the *EST2, EST3* and *EST4* genes are phenotypically similar to *est1* and *tlc1* strains; in fact, many of the new *est* isolates are indistinguishable from *est1*-Δ and *tlc1*-Δ strains, suggesting that these alleles may also be null mutations. This similarity in phenotype argues that the new *EST* genes function in the same genetic pathway for telomere replication as originally defined by *EST1*. The alternative possibility is that these five genes oper-

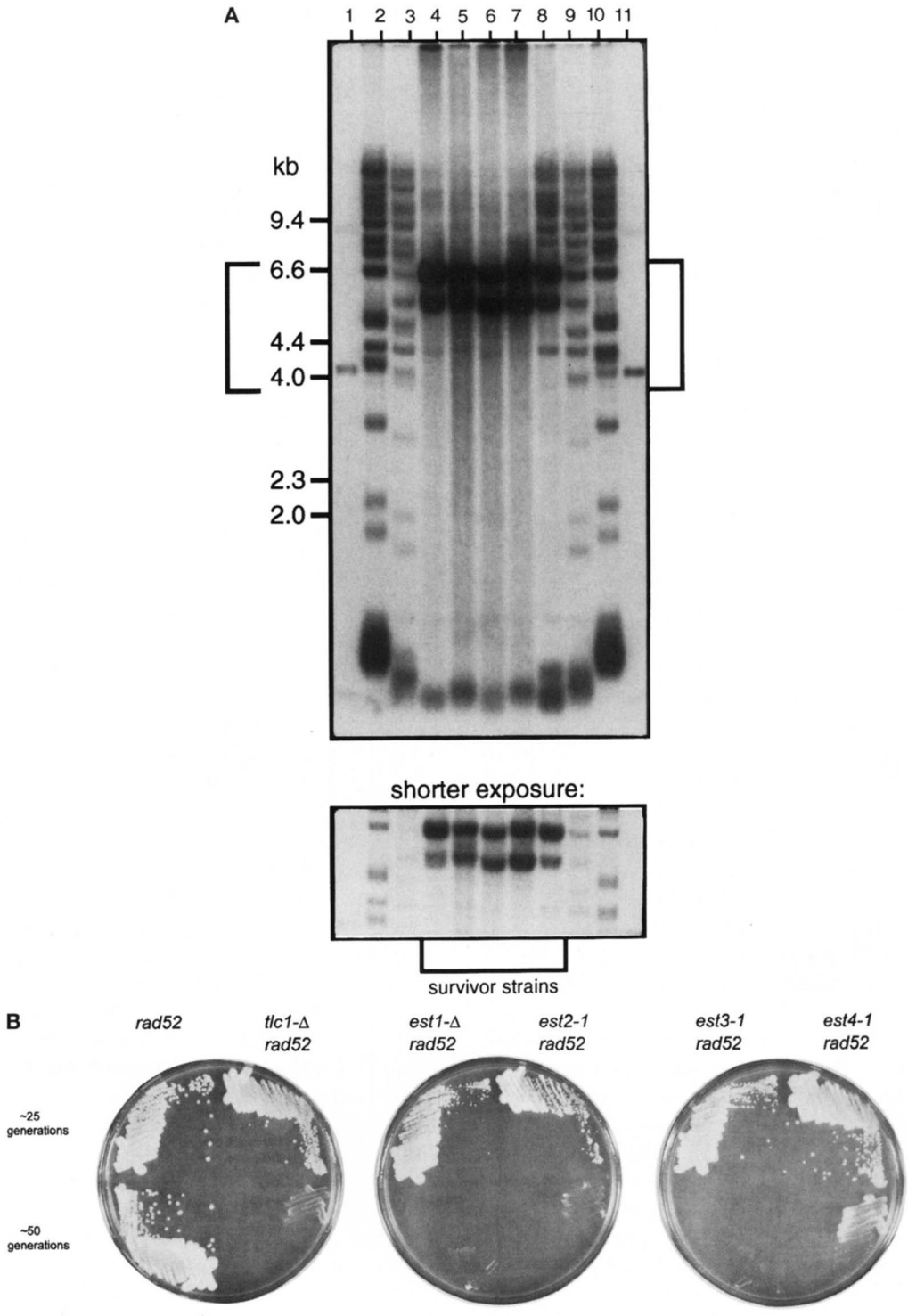


FIGURE 3.—New *est* mutants show the same *RAD52*-dependent alternative pathway for telomere maintenance. (A) Southern blot of genomic yeast DNA probed with poly d(GT/CA). Lanes 1 and 11, a mixture of λ*Hind*III and a single d(G_{1-3}T)-containing 4.0-kb fragment detected by the probe; lanes 2 and 10, *EST*$^+$*TLC1*$^+$; lanes 3 and 9, early *tlc1*-Δ::*LEU2*; lanes 4–8, survivors isolated from *tlc1*-Δ::*LEU2*, *est1*-Δ*3*::*HIS3*, *est2-1*, *est3-1* and *est4-1*, respectively. A shorter exposure of the region indicated in brackets is shown below, to demonstrate the degree of amplification of Y′-containing subtelomeric bands that occurs in survivor strains (LUNDBLAD and BLACKBURN 1993). Note that the probe detects the yeast d(G_{1-3}T) telomeric repeats, indicating that both Y′ and d(G_{1-3}T) sequences are highly amplified in survivors. (B) Viability of *EST*$^+$ *rad52*::*LEU2*; *tlc1*-Δ::*LEU2 rad52*::*LEU2*; *est1*-Δ*3*::*HIS3 rad52*::*LEU2*; *est2-1 rad52*::*LEU2*; *est3-1 rad52*::*LEU2*; and *est4-1 rad52*::*LEU2*, shown as two successive streak-outs.

ate in more than one pathway, each required to form a functional telomere. These two possibilities can be distinguished by examining the behavior of strains that have multiple mutant combinations. The prediction is that if all five genes function in a single pathway necessary to replicate telomeres, multiply mutant strains should show no enhancement of phenotype. This has been demonstrated for *EST1* and *TLC1* (V. VIRTA-

PEARLMAN, D. K. MORRIS and V. LUNDBLAD, unpublished results), showing that *EST1* functions in the same *in vivo* pathway as defined by a known component of telomerase. If, however, one or more genes function in a separate, additional pathway required for telomere replication, certain multiple mutant combinations might be expected to show a more severe phenotype. The substantial enhancement of the phenotype of *est* mutant strains that occurs in the presence of a *rad52* mutation (Figure 3B), which eliminates the back-up bypass pathway for telomere maintenance discussed above, is an example of the latter possibility.

To distinguish between these two formal possibilities, each new *est* mutant was crossed against *est1-*Δ, *tlc1-*Δ or the other *est* mutant strains to generate diploids heterozygous at several *EST/TLC1* loci. The subsequent diploids were sporulated and dissected to generate wild-type, single mutant and double mutant spore products. Each double mutant strain was analyzed for telomere length, senescence and chromosome loss (Figure 4 and data not shown), in parallel with single mutants and wild type. In every case, no enhancement of phenotype was seen for double mutant combinations. Figure 4, A and B, shows telomere length for a representative set of double mutant strains compared with the appropriate single mutant strains; in each strain, telomere length declined over time to the same degree. Similarly, as shown in Figure 4C, there was no alteration in the senescence phenotype in any of the double mutant strains, relative to single mutants. This is in striking contrast to the severe enhancement of senescence that occurred as a result of the introduction of a *rad52* mutation into any of the *est* mutant strains (Figure 3B and 5C). These pairwise combinations argue that each of the new *EST* genes functions in the same pathway for telomere replication as defined by *EST1* and *TLC1*.

The analysis of various double mutant combinations and their haploid counterparts revealed an unexpected observation, which was that the senescence and telomere shortening phenotypes of single mutant strains were somewhat enhanced when these haploids were derived from a diploid parent that was multiply heterozygous for genes in the *EST/TLC1* pathway. For example, the time course of the senescence phenotype of a haploid strain carrying only the *tlc1-*Δ mutation was accelerated when the *tlc1-*Δ strain was derived from DVL132 (*TLC1*$^+$/*tlc1-*Δ *EST1*$^+$/*est1-*Δ), compared with the same isogenic haploid *tlc1-*Δ strain derived from DVL131 (*TLC1*$^+$/*tlc1-*Δ *EST1*$^+$/*EST1*$^+$); (compare the *tlc1-*Δ strains in Figures 2B and 4C). This additive haploinsufficiency was not specific for the *EST1/TLC1* combination and was observed for every possible combination of the five genes in this pathway. Consistent with the accelerated phenotype observed in haploids from these diploids, telomere length was slightly shorter in multiply heterozygous diploids as compared to single heterozygotes (D. K. MORRIS, unpublished data). Note that the conclusions drawn from the data presented in Figure 4, that additive combinations of different mutations do not show enhancement of phenotype, were made from comparisons between sets of double and single mutant strains derived from the same diploid parent; comparisons with the single mutant haploids in Figure 2 are not valid. Although we do not understand the molecular basis for this phenomenon, it suggests that alterations in gene dosage may be disrupting a complex or a set of interacting complexes.

The *EST2* gene encodes a novel, highly basic protein: The wild-type *EST2* gene was cloned by complementation of the senescence phenotype of *est2-1*, from both high and low copy genomic libraries (see MATERIALS AND METHODS for more details). Three independent genomic clones with overlapping inserts were identified that complemented both the growth and telomere length phenotypes of the *est2-1* mutation; no high copy suppressors were identified. Subcloning identified a 4.4-kb genomic segment common to all three inserts which was capable of complementing *est2-1*. This fragment was sequenced and also subjected to mutagenesis with the transposable element γδ (STRATHMANN *et al.* 1991) in order to identify the genetic boundaries of the *EST2* gene. A total of 13 insertion mutations were isolated, mapped by both PCR and sequence analysis, and assayed for their ability to complement *est2-1* (Figure 5A). These data, combined with the sequence of the 4.4-kb fragment, demonstrated that the complementing activity was due to an 884 amino acid ORF (Figure 5B); no additional ORFs >65 amino acids were found within this sequenced insert.

The promoter region of the *EST2* gene exhibited several notable features found in common with that of *EST1*. First, both genes diverge significantly from the consensus sequence found around the initiating AUG of many yeast genes (HINNEBUSCH and LIEBMAN 1991; GALIBERT *et al.* 1996). In addition, for both *EST1* and *EST2*, the region just upstream of the AUG initiation codon has the potential to encode multiple small ORFs, a feature that is not usually observed in the promoter regions of yeast genes (CIGAN and DONAHUE 1987). In the rare genes where such upstream ORFs have been analyzed, they have been shown to be involved in translational control of gene expression (HINNEBUSCH 1992). Within 140 bp of the start of the Est1 protein coding sequence, there are five overlapping ORFs, ranging in size from 3 to 39 codons (LUNDBLAD and SZOSTAK 1989). Similarly, within the first 170 bp of the promoter region of the *EST2* gene, there are five small ORFs, from 4 to 11 codons in length (Figure 5A). Although these small upstream ORFs have not been functionally dissected in detail in either gene, a γδ disruption inserted into the cluster of three upstream ORFs was partially defective for *EST2* activity (Figure 5A and data not shown), suggesting that this region may play an important role in expression of *EST2*. One feature of the *EST2* promoter that was not found in *EST1* was a 32-bp long

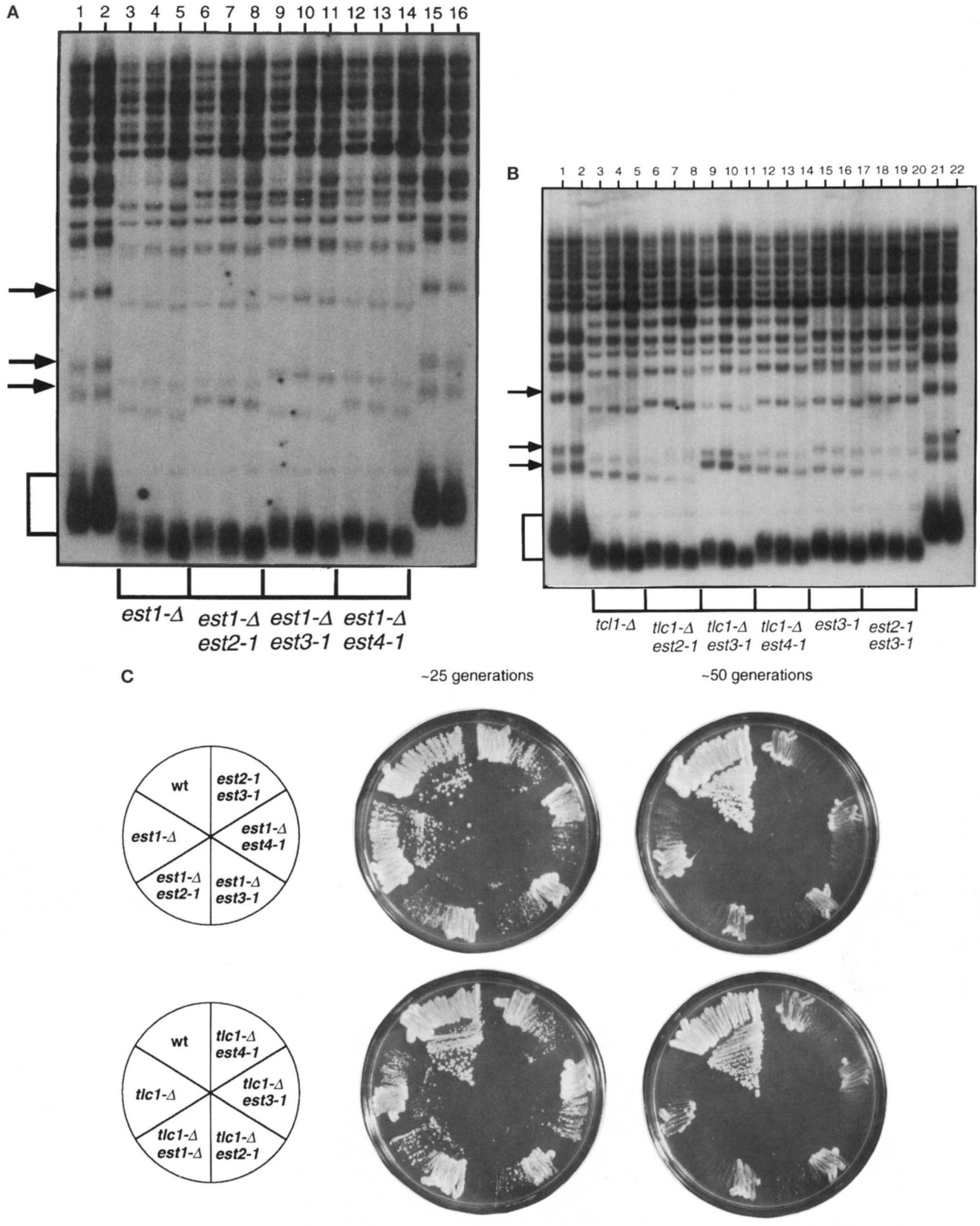
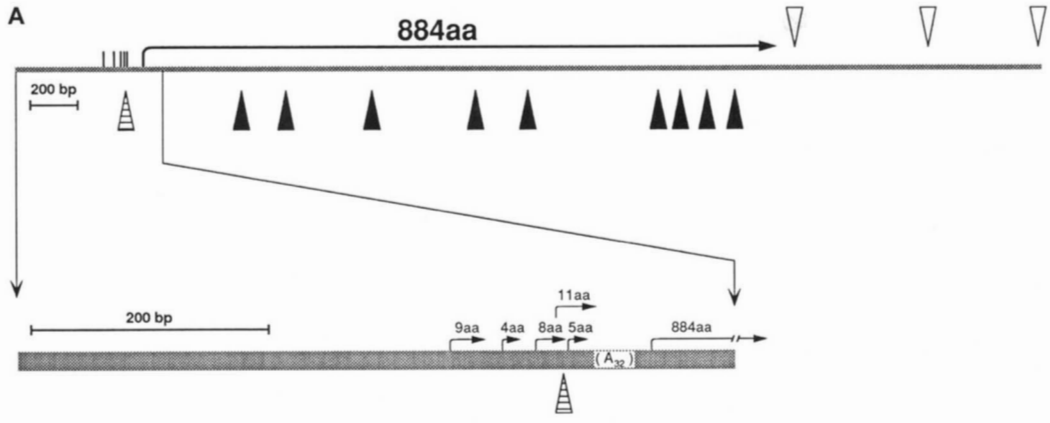


FIGURE 4.—Epistasis analysis of *tlc1* and *est* mutants. (A) Southern blot of genomic yeast DNA. Lanes 1, 2, 15, and 16 *EST*$^+$*TLC1*$^+$ (representing four successive subcultures, handled as in Figure 2A); lanes 3–14, three lanes each (three successive subcultures) of *est1-Δ3::HIS3*; *est1-Δ3::HIS3 est2-1*; *est1-Δ3::HIS3 est3-1*; and *est1-Δ3::HIS3 est4-1*. (B) Southern blot of genomic yeast DNA. Lanes 1, 2, 21, and 22, *EST*$^+$*TLC1*$^+$; lanes 3–20, three lanes each (three successive subcultures) of *tlc1-Δ::LEU2*; *tlc1-Δ::LEU2 est2-1*; *tlc1-Δ::LEU2 est3-1*; *tlc1-Δ::LEU2 est4-1*; *est3-1*; *est2-1 est3-1*. (C) Viability of double mutants shown as two successive streak-outs. Top: (counterclockwise from upper left) wild type; *est1-Δ3::HIS3*; *est1-Δ3::HIS3 est2-1*; *est1-Δ3::HIS3 est3-1*; *est1-Δ3::HIS3 est4-1*; *est2-1 est3-1*; Bottom: wild type; *tlc1-Δ::LEU2*; *tlc1-Δ::LEU2 est1-Δ3::HIS3*; *tlc1-Δ::LEU2 est2-1*; *tlc1-Δ::LEU2 est3-1*; *tlc1-Δ::LEU2 est4-1*



B

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  1  MKILFEFIQD KLDIDLQTNS TYKENLKCGH FNGLDEILTT CFALPNSRKI
 51  ALPCLPGDLS HKAVIDHCII YLLTGELYNN VLTFGYKIAR NEDVNNSLFC
101  HSANVNVTLL KGAAWKMFHS LVGTYAFVDL LINYTVIQFN GQFFTQIVGN
151  RCNEPHLPPK WVQRSSSSSA TAAQIKQLTE PVTNKQFLHK LNINSSSFFP
201  YSKILPSSSS IKKLTDLREA IFPTNLVKIP QRLKVRINLT LQKLLKRHKR
251  LNYVSILNSI CPPLEGTVLD LSHLSRQSPK ERVLKFIIVI LQKLLPQEMF
301  GSKKNKGKII KNLNLLLSLP LNGYLPFDSL LKKLRLKDFR WLFISDIWFT
351  KHNFENLNQL AICFISWLFR QLIPKIIQTF FYCTEISSTV TIVYFRHDTW
401  NKLITPFIVE YFKTYLVENN VCRNHNSYTL SNFNHSKMRI IPKKSNNEFR
451  IIAIPCRGAD EEEFTIYKEN HKNAIQPTQK ILEYLRNKRP TSFTKIYSPT
501  QIADRIKEFK QRLLKKFNNV LPELYFMKFD VKSCYDSIPR MECMRILKDA
551  LKNENGFFVR SQYFFNTNTG VLKLFNVVNA SRVPKPYELY IDNVRTVHLS
601  NQDVINVVEM EIFKTALWVE DKCYIREDGL FQGSSLSAPI VDLVYDDLLE
651  FYSEFKASPS QDTLILKLAD DFLIISTDQQ QVINIKKLAM GGFQKYNAKA
701  NRDKILAVSS QSDDDTVIQF CAMHIFVKEL EVWKHSSTMN NFHIRSKSSK
751  GIFRSLIALF NTRISYKTID TNLNSTNTVL MQIDHVVKNI SECYKSAFKD
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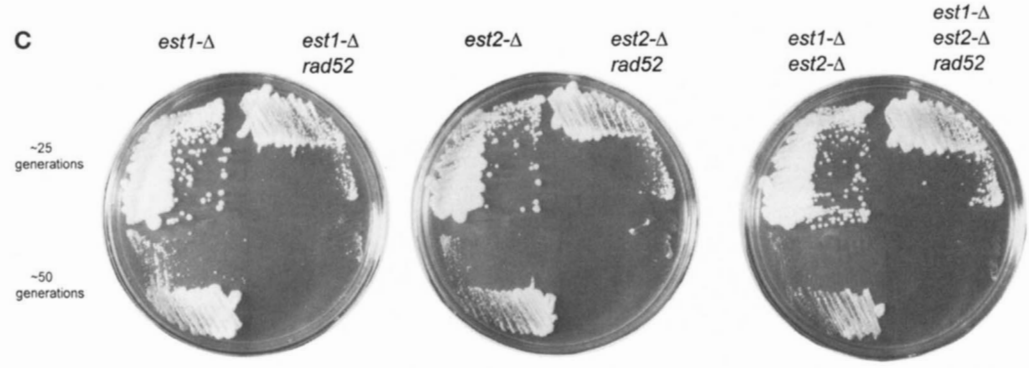

FIGURE 5.—Cloning of the *EST2* gene. (A) Insertional mutagenesis of the 4.4-kb genomic clone, with the position of the *EST2* open reading frame indicated. Each of the 13 $\gamma\delta$ insertions were assayed for complementation of the telomere shortening and senescence phenotypes of an *est2-1* mutant strain; insertions which fully complement the Est phenotype are indicated as ▽, and failure to complement is indicated by ▲. One insertion, 74 bp upstream of the initiating AUG, exhibited an intermediate phenotype, in that the senescence phenotype was complemented but a telomere shortening phenotype was observed (data not shown). The positions of five small ORFs and a poly-A tract in the promoter region of *EST2* are also indicated. (B) The sequence of the 884 amino acid *EST2* open reading frame. (C) Comparison of the viability of *est1*-Δ, *est2*-Δ and *est1*-Δ *est2*-Δ strains (both *RAD52* and *rad52* versions), shown as two successive streak-outs.

poly (dA) tract just upstream of the start of the *EST2* coding region. Poly (dA-dT) tracts have previously been implicated in transcriptional activation (STRUHL 1986; LUE and KORNBERG 1987); the *EST2* poly (dA) tract may also be a promoter element, although it is unusually close to the translational start site.

To determine the null phenotype and confirm that the *EST2* gene had been cloned, an internal deletion

within this ORF was removed and replaced with the *URA3* gene. This construct was introduced into a diploid strain by one-step gene replacement, and the resulting heterozygous strain was sporulated; >80% of the tetrads had four viable spores for each, indicating that this gene was not essential for immediate viability. The haploid strains from 12 tetrads were further analyzed for senescence and telomere length; for all 12 tetrads, an *est* mutant phenotype cosegregated 100% of the time with the *URA3* marker. The senescence phenotype of *est2-Δ1* was identical to that displayed by an *est1-Δ* strain, both in the presence or absence of *RAD52* gene function (Figure 5C); furthermore, consistent with the double mutant analysis conducted above with the *est2-1* point mutation, a strain deleted for both genes showed no enhanced phenotype with regard to senescence (Figure 5C) or telomere length (data not shown). In addition, an *est2-Δ1* strain was capable of giving rise to *RAD52*-dependent survivors with a frequency comparable to that observed for *est1-Δ* and *tlc1-Δ* strains. The *est2-Δ1* mutation failed to complement the *est2-1* mutation for both telomere length and senescence, and the *est2-1* point mutation was shown by plasmid gap-repair to map within the *EST2* coding region (data not shown), indicating that the correct gene had been cloned.

The *EST2* gene encodes a novel 103-kD protein with no similarity to other sequences in the database nor does it possess any motifs that provide clues as to its function. In particular, there was no sequence similarity between Est2p and either of the two protein subunits of the Tetrahymena telomerase enzyme (COLLINS *et al.* 1995). Like Est1, Est2 is an unusually basic protein: both proteins have predicted pIs of 10. Est1 has been shown to bind single-stranded telomeric DNA (V. VIRTA-PEARLMAN, D. K. MORRIS and V. LUNDBLAD, unpublished results); we are currently investigating whether the Est2 protein binds either telomeric DNA or the other *EST* gene products.

DISCUSSION

Using a modification of previous techniques designed to detect chromosome transmission mutants, we have designed a multitiered screen that has identified a large set of mutants defective for telomere replication, long term viability and chromosome stability. These mutants map to four complementation groups: the previously identified *EST1* gene and three new genes, designated *EST2, EST3* and *EST4*. A variety of genetic observations argue that the new *EST* genes function in the same pathway for telomere replication as previously defined by *TLC1* and *EST1*. First, singly mutant strains have highly similar phenotypes when compared with each other (Figure 2). In addition, mutations in all five genes are capable of giving rise to an alternative pathway for telomere maintenance first described for *est1* mutant strains (Figure 3; LUNDBLAD and BLACKBURN 1993). Third, multiple mutant combinations show no enhancement of phenotype, relative to any of the single mutant strains (Figure 4). Epistasis arguments such as this have been used repeatedly to demonstrate that multiple components function in a single pathway or complex (for example, see HOLLINGSWORTH *et al.* 1995; JOHZUKA and OGAWA 1995).

The observation that the *in vivo* defects of *est1, est2* or *est3* mutants are as severe as the phenotype displayed by a mutation in the one known component of telomerase underscores the critical role that each of these genes must play in telomere replication *in vivo*. The recent observation that telomerase activity is present in chromatographic fractions or nuclear extracts prepared from an *est1-Δ* strain indicates that the Est1 protein is not part of the catalytic core of the enzyme (COHN and BLACKBURN 1995; N. F. LUE and V. LUNDBLAD, unpublished results), but does not exclude the possibility that *EST1* is a subunit of telomerase required for some function not revealed by the *in vitro* assay. Alternatively, this protein could regulate telomerase as a constituent of telomeric chromatin, possibly by serving to direct the enzyme onto the end of the chromosome. Consistent with this proposal is the recent demonstration that purified Est1 protein has the properties of a single-strand telomere binding protein (V. VIRTA-PEARLMAN, D. K. MORRIS and V. LUNDBLAD, unpublished results). A similar role as a component of telomeric chromatin has also been proposed for *EST4*. The single *est4-1* allele described in this work has been shown to be a novel allele of the essential gene, *CDC13*. Cdc13p is also a single-strand telomere binding protein, leading us to propose that Cdc13p is required both to protect the chromosomal terminus as well as to regulate access of telomerase to the telomere (NUGENT *et al.* 1996). Whether the other EST genes similarly encode components of telomeric chromatin or telomerase awaits further biochemical characterization.

The *est* mutants as models for mammalian cellular senescence: A recent hypothesis has proposed that the decline of telomeric DNA observed in human somatic cells may be the primary event responsible for determining cellular senescence (COOKE and SMITH 1986; HARLEY *et al.* 1990; HASTIE *et al.* 1990; reviewed by HOLLIDAY 1996). A number of studies have led to the concept of a mitotic clock that is capable of monitoring the number of cellular divisions remaining in a normal cell line. Although the molecular nature of this proposed clock has not been determined, one intriguing hypothesis is that it is the declining length of telomeric repeats found at the end of linear chromosomes that is responsible for "counting" the number of cellular divisions (OLOVNIKOV 1973; HARLEY 1991). Analysis of the *est/tlc1* mutants argues strongly that at least in yeast, a defect in telomere tract maintenance is in fact a causative event in dictating replicative capacity (LUNDBLAD and SZOSTAK 1989; SINGER and GOTTSCHLING 1994; this work). Furthermore, the existence of such mutant yeast

strains provides an easily manipulated system in which to study this proposed mitotic clock at a functional and molecular level.

One application of the yeast *est/tlc1* system to the question of the mitotic clock has been the demonstration that a defect in the ability to replicate telomeres can be bypassed by an alternative, recombination-dependent pathway. A consequence of this pathway is that there is a substantial amplification of both telomeric G-rich repeats and subtelomeric repeats (LUNDBLAD and BLACKBURN 1993; V. LUNDBLAD, unpublished data). This led to the proposal that when the telomerase-based pathway for synthesizing G-rich repeats is absent, recombination can substitute for telomerase in maintaining telomeric sequences. Although this pathway was first described for *est1*-Δ strains, it also occurs in *tlc1*-Δ strains (SINGER and GOTTSCHLING 1994; Figure 3) as well as in every *est2, est3* and *est4* mutant strain tested. Therefore, this bypass is not a specific consequence of the loss of the *EST1* gene, but rather is a general response to the loss of the pathway defined by the five *EST/TLC1* genes for replicating the telomere. A similar *RAD52*-dependent restoration of telomeres is also observed in telomerase-defective strains of *Kluyveromyces lactis* (MCEACHERN and BLACKBURN 1996). Whether this process occurs in mammalian cells remains to be seen, but the identification of cell lines that are telomerase-minus but immortal indicates that there are alternatives to maintaining the telomere in mammalian cells (KIM *et al.* 1994; BRYAN *et al.* 1995). Furthermore, the presence of polymorphisms in the subtelomeric repeat regions of mammalian telomeres (BROWN *et al.* 1990) and evidence for telomere-telomere exchanges (WILKIE *et al.* 1991) argues that recombination events can also occur at mammalian chromosomal termini and therefore have the potential to promote a bypass pathway similar to what has been observed in *S. cerevisiae* and *K. lactis.*

Other studies that have addressed yeast as a model system for cellular aging have proposed that a decrease in telomere length is not a factor in the aging process (D'MELLO and JAZWINSKI 1991; KENNEDY *et al.* 1995; SMEAL *et al* 1996), an apparent contradiction to the work presented here. These groups have shown that telomere length in young mother cells (*e.g.*, newly budded cells) is the same as that in old mother cells (those cells that have generated many daughters) from a wild-type yeast strain. However, the conclusion from these observations, that telomere length is unrelated to aging, depends on whether the individual cells of a unicellular organism such as *S. cerevisiae* can be considered a model for the aging of multicellular organisms. We would argue instead that a comparison of immortal wild-type yeast strains to senescing *est* mutant strains provides a more appropriate model for cellular aging. In other words, the life span of a cell taken from a wild-type culture is the same as that of a cell taken from this same culture hundreds of divisions later. However, during the growth of an *est* mutant culture, the life span of individual cells declines dramatically with continued culturing of the strain (V. LUNDBLAD, unpublished data). This contrast between wild-type and *est/tlc1* yeast cell cultures bears many similarities to the comparison between human immortal cell lines and senescing human diploid fibroblasts. For example, clonal analysis of fibroblasts *in vitro* has demonstrated that the life span of individual fibroblast cells is reduced over time with the mass growth of the culture (SMITH and WHITNEY 1980), in parallel with the observed decline in telomere length (HARLEY *et al.* 1990). In contrast, telomere length of the germ line does not shorten with age (COOKE and SMITH 1986; ALLSOPP *et al.* 1992), similar to the observations described above for telomere length of young and old wild-type yeast cells. This suggests that *est/tlc1* mutants can provide a simple model system for studying the correlation between reduction in telomere length and declining life span in mammalian cells.

Summary: This genetic approach, combined with the discovery of *TLC1* by SINGER and GOTTSCHLING (1994), has led to the identification of a minimum of five genes that are required for the telomerase-mediated pathway of telomere replication. In contrast, purification of the telomerase enzyme from the ciliate Tetrahymena has identified three components associated with this enzyme *in vitro* (GREIDER and BLACKBURN 1989; COLLINS *et al.* 1995). Although biochemical characterization of the individual *EST* gene products will be necessary to elucidate their exact role in telomere replication, our results argue that there may be a large number of genes required for telomerase function *in vivo,* either as actual components of telomerase or as essential regulators of the enzyme.

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