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13. ABSTRACT *(Maximum 200 words)*

My research proposal focuses on characterizing the roles of proteins that are responsible for mediating access of telomerase to the telomere in the yeast *S. cerevisiae*. We have previously demonstrated that both Est1 and Cdc13 have biochemical and genetic properties that are consistent with those expected for proteins that mediate telomerase access. To define more clearly the mechanistic function of these two proteins at the telomere, I have pursued two experimental approaches over the past year. The first has been a detailed site directed mutational analysis of *EST1*, with has yielded three particularly informative classes of mutants and has provided critical groundwork for the future Specific Aims of my research. In a second approach, I have examined the consequences of fusing Est1 to Cdc13, and have found that this fusion protein results in substantial telomere lengthening. The results of this second approach greatly support our model for the roles of Est1 and Cdc13 as co-mediators of telomerase access to the chromosome terminus. In addition, it has also provided us with a powerful tool to further genetically define regions of both of these two proteins involved in telomere maintenance.

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Sara Evans 19 APR 99

PI - Signature Date

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INTRODUCTION

The postulation that reactivation of telomerase is a critical event for tumor progression has suggested that inhibitors of the enzyme may be employed for therapeutic use against epithelial cancers, including breast cancer. Experimental results indicate that certain reverse transcriptase inhibitors, which target the catalytic subunit of telomerase, may indeed be a successful approach for cancer treatment (1). Identifying additional components of telomerase, or telomerase associated proteins, may further characterize other potential anti-telomerase targets. Towards this goal, my research proposal focuses on examining the role of a telomerase associated protein, Est1, in telomere maintenance in the yeast *Saccharomyces cerevisiae*. The combined genetic and biochemical approaches that can be used with a yeast model system allows dissection of the molecular details of telomerase function – an important first step in characterizing potential targets in a mammalian system.

RESEARCH SUMMARY

BACKGROUND

Five genes have been identified that are required for telomere replication by telomerase in *S. cerevisiae*: *EST1, EST2, EST3, TLC1* and *CDC13* (2-5). *EST2* and *TLC1* comprise components of the catalytic core of the yeast enzyme: *EST2* encodes the reverse transcriptase catalytic subunit and *TLC1* encodes the templating RNA (5, 6). Mutations in either of these two genes results in progressively shorter telomeres and cellular senescence (collectively termed the *est* phenotype, for ever shorter telomeres), as well as an absence of detectable *in vitro* telomerase activity. However, *EST1*, *EST3* and *CDC13*^{est}, which are also required for *in vivo* replication, are dispensable for catalytic activity *in vitro* (7). We have proposed that these three proteins perform essential regulatory roles *in vivo*.

Immunoprecipitation experiments performed in our lab have demonstrated that Est1 and Est3, like Est2, are associated with an active telomerase enzyme complex (8). The interaction between Est1 and the telomerase RNA is independent of Est2, suggesting that the association of Est1 with telomerase is mediated through protein-RNA interactions rather than Est1-Est2 interactions. Previous work also has shown that Est1 has the properties of a single-strand telomere DNA binding protein, with a strong preference for a free single stranded 3' end (9). These data have led us to propose that Est1 is a telomerase-associated protein that functions in mediating enzyme access to the end of the chromosome. Presumably, Est1 performs this role via its telomere DNA binding activity, but this proposed telomere accessing activity may be mediated through protein-protein interactions as well. One candidate for an Est1-interacting protein is another single-strand telomere DNA binding protein, Cdc13.

Insight into the role of Cdc13 in telomere maintenance has come from both genetic and biochemical experiments. Work from both our lab and the Zakian lab has shown that Cdc13 is a single-strand telomere DNA binding protein *in vitro*. (4, 10). While bound to the telomere, Cdc13 appears to play to roles, based on genetic analysis of two distinct mutations: (i) the *cdc13-1*^{ts} allele defines an essential role for Cdc13 in protecting the end of the chromosome (11), whereas (ii) the *cdc13-2*^{est} mutation defines a role in telomerase function. This *est* mutation confers progressive telomere shortening and senescence, although *in vitro* telomerase activity is still present in extracts made from *cdc13-2*^{est} strains. Cdc13 does not appear to be tightly associated with telomerase, as Cdc13 does not co-immunoprecipitate telomerase at detectable levels (8). Therefore, if Cdc13 mediates access of telomerase to the chromosome terminus through a direct protein-protein interaction, this interaction must be relatively weak.

My research proposal focuses on characterizing the role(s) of proteins that are responsible for mediating access of telomerase to the telomere, with a focus on *EST1* and *CDC13*. To define more clearly the mechanistic function of Est1 at the telomere, I have pursued two experimental approaches over the past year. Project I has been a detailed site directed mutational analysis of *EST1*, with has yielded three particularly informative classes of mutants and has provided critical groundwork for the future Specific Aims of my research.

In a second approach, Project II, I have examined the consequences of fusing Est1 to Cdc13, and have found that this fusion protein results in substantial telomere lengthening. The results of this second approach, described in more detail in the Research Summary, Project II section, greatly support our model for the role of Est1 and Cdc13 as co-mediators of telomerase access to the chromosome terminus. In addition, it has also provided us with a powerful tool to further genetically define regions of both of these two proteins in telomere maintenance.

Project I: Mutational analysis of *EST1*

Rationale

There are two main undefined areas with regard to Est1 function: first, proteins that directly interact with Est1 remain largely unknown, and second, we do not know the *in vivo* role of the *in vitro* defined DNA and RNA binding activities of Est1 (i.e. what the *in vivo* consequences of mutations conferring a DNA and/or RNA binding defect would be). As a first step towards addressing these two aspects, and to define more clearly the role of Est1 in telomere maintenance, I have carried out a mutational analysis of the protein. In Specific Aim 2 of my original research proposal, I had proposed to mutate specific residues in the putative DNA/RNA binding motifs of Est1 in an attempt to establish their functional significance. At the onset of this project, I decided to substantially broaden that approach and conduct a site-directed mutational analysis on the entire protein. There are two primary goals of this project. First, I hope to identify and analyze functional domains of Est1 by identifying not only the nucleic acid binding residues, but also identifying other domains of the protein that may be involved in interacting with telomerase or components of telomeric chromatin. The second goal of this project is to generate reagents that will be useful for identifying Est1-interacting factors; appropriate alleles from this mutagenesis will be used in suppression screens to isolate possible Est1-interacting proteins, as outlined in Specific Aim 3 of my original research proposal. In addition, such alleles should be useful in other genetic analyses of the role of Est1 in telomere replication, as described in more detail in Project II of this Research Summary.

Strategy and Methods for mutant isolation

The approach used for the mutagenesis was a directed charged-cluster to alanine scan, using charged residues that are conserved between *EST1* homologs from *S. cerevisiae* and the related yeast, *S. carlesbergensis* to dictate many of the residues that were mutated. A total of 23 clustered mutations (changing two to four residues at a time) and five single mutations were made: 14 in the amino terminal portion of the protein, 12 in the nucleic acid binding domain, and two in the carboxyl terminus. Mutations were created in an amino-terminal epitope tagged (HA_3) *EST1* CEN plasmid, and subsequently cloned into overexpression and *in vitro* expression vectors. Each mutant was analyzed for *in vivo* complementation of *EST1* function (senescence and telomere length), an overexpression dominant negative phenotype, and association with the telomerase RNA.

Results and Discussion

This approach has yielded three interesting classes of mutants, as summarized in Table 1 and described in more detail below. Of the 28 *est1* mutants made, 11 mutants have phenotypes that are particularly instructive, 16 have no overt *est* phenotypes, and one makes no detectable protein.

Class I (6 mutants): These mutants have an *in vivo* telomerase defective phenotype: they display senescence (with one exception) and short telomeres. However, they appear to associate with the telomerase RNA at levels comparable to wild type. Since these mutations all reside within the mapped DNA binding domain, it is possible that these mutations confer a defect in DNA binding, and therefore these mutants will be assayed for DNA binding *in vitro*. These mutants also have a dominant negative phenotype when overexpressed, suggesting that overexpression of mutant forms of Est1 which are unable to bind DNA may titrate out components which are required for telomerase function. This is one class of mutants that may be used in high copy suppression screens to isolate putative Est1-interacting factors.

Class II (2 mutants): Two of the conditional senescence mutants fall into this class, which behave like null mutations at 36°C, but are semi-permissive at 30°C. These mutants still associate with TLC1 at 30°C, and preliminary evidence suggests this association is retained at 36°C. There are two possible models for how these mutants affect Est1 function. One is that the mutant protein remains associated with telomerase, but may be defective for an interaction with another factor required for telomere maintenance. If so, mutants of this class can also be used in screens to identify potentially a different class of Est1 interactors than those defined by the Class I mutations. Alternatively, I cannot rule out the possibility that although these mutations fall outside of the mapped nucleic acid binding region, that the mutations define a domain which is still required for DNA binding or binding specificity. *In vitro* DNA binding experiments should help distinguish between these two possibilities.

Class III (3 mutants): Three mutants fall into this class, which is defined by a substantial reduction in the association with the telomerase complex (as assessed by co-immunoprecipitation with the telomerase RNA). These mutants have slightly different *in vivo* phenotypes:

est1-38: not senescent, short telomeres, synthetic with *yku70Δ*

est1-39: conditional senescence at 36°C, short telomeres even at 30°C, synthetic with *yku70Δ*

est1-41: not senescent, short telomeres, but not synthetic with *yku70Δ*

Like the mutants in Class II, there are two possible models for how these mutants affect Est1 function. One is that these mutations define a protein-protein interaction domain required for telomerase association. A second possibility is that the determinants for direct RNA binding, or RNA binding specificity, lie within this region.

Conclusions and Future Directions

The mutagenesis project was successful in generating many useful reagents for further analysis of Est1. Future directions include characterizing these *est1* mutants by: (i) examining the DNA and RNA binding properties of the mutants using *in vitro* nucleic acid binding assays, and (ii) performing allele-specific suppression screens of certain *est1* mutants to search for Est1-interacting factors. This project will be supplemented by two additional approaches designed to isolate particularly informative subsets of *est1* mutants: (i) a mutagenized *EST1* library will be used to screen for dominant negative alleles, and (ii) mutants defective in mediating telomerase access will be obtained using the reagents and techniques described in Project II in next section.

Project II: Tethering telomerase to the telomere

Rationale

We previously proposed that the phenotypes of the telomerase-defective allele of *CDC13* (*cdc13-2^{est}*) were due to the inability of telomerase to access the chromosome terminus, although the precise molecular mechanism by which Cdc13 mediated telomerase access was not defined. One possible mechanism is through a direct protein-protein interaction between Cdc13 and telomerase, and therefore, the phenotypes of the *cdc13-2^{est}* allele could be due to reduced interaction between these two factors. The reciprocal experiment would be to examine the consequences of increasing the affinity between Cdc13 and telomerase. I decided to test this in a somewhat unusual way, by fusing Cdc13 directly to Est1. As described below, this protein fusion resulted in greatly elongated telomeres, suggesting that we had physically tethered telomerase to the telomere via the high affinity telomere DNA binding protein, Cdc13. As outlined in more detail in the Future Directions section of this Project, this result has provided us with both a powerful tool for dissecting the individual components of telomerase.

Results and Discussion

Figure 1 shows the striking result of fusing Cdc13 and Est1, which is that the Cdc13-Est1 fusion protein conferred greatly elongated telomeres (Fig. 1, lanes 2 and 3), compared to wild type (Fig. 1, lane 1). Telomere lengthening was even more pronounced in a *cdc13-Δ* strain where the only copy of Cdc13 present was part of the fusion protein (Fig. 1, lanes 4 and 5). This telomere elongation was dependent on functional telomerase, as lengthening was not observed when the fusion was introduced into a strain deleted for the catalytic subunit of enzyme (data not shown). Control experiments demonstrated that each half of the fusion protein still retained function: the fusion protein complemented either null mutation, and importantly, the Est1 portion retained TLC1 association when present in the fusion (Fig. 2, lane 6). We interpreted these initial results to suggest that we had greatly increased the affinity of telomerase for the telomere by fusing Cdc13 to a telomerase component.

This initial observation allowed us to genetically test our model that the $Cdc13^{est}$ mutant protein had a reduced affinity for telomerase. One testable prediction of this model is that fusing Est1 to the telomerase defective version of Cdc13 should bypass the consequences of this mutation. Figure 1 shows that the $Cdc13^{est}$ defect was indeed bypassed, as this mutant version of the protein behaved exactly as the wild type version: telomeres lengthened in both a *CDC13+* strain (Fig. 1, lanes 7 and 8) and in a *cdc13-Δ* strain (Fig. 1, lanes 9 and 10), and no senescence was observed (data not shown). This shows that physically putting Est1 in the proximity of Cdc13 does in fact bypass the telomerase defect of the mutant Cdc13 protein.

Reagents that had been generated from the site directed mutational analysis of Est1 (Project I) also allowed us to ask the reciprocal experiment: can Est1 mutants be suppressed by fusion to Cdc13? A subset of *est1* mutants isolated from the site directed mutagenesis displayed both short telomeres and senescence phenotypes, but retained association with the telomerase RNA at levels comparable to the wild type protein. One of these mutants, *est1-47*, was chosen for the fusion experiments because it had a strong senescence phenotype *in vivo*. However, when fused to Cdc13, this *est1* defect was now bypassed and the mutant fusion protein also behaved exactly as the wild type fusion, as expected if the ability of Est1 to get to the telomere had been restored (data not shown).

Taking the fusion experiments one step further, we asked what the consequences were of fusing Cdc13 directly to the catalytic subunit of telomerase. This Cdc13-Est2 fusion also gave dramatic telomere lengthening (Fig. 3, lanes 3 and 4), comparable to the Cdc13-Est1 fusion (Fig. 3, lanes 1 and 2). The use of Est2 in these experiments allowed an important control fusion to be tested: fusion of a catalytically inactive version of Est2 (conferred by a mutation in an active site residue, D670A) to Cdc13 did not result in telomere lengthening, but instead resulted in wild type telomere length (Fig. 3, lanes 5 and 6).

Finally, the Cdc13-Est2 fusion has allowed us to ask if Est1 function can be bypassed when telomerase is tethered to the telomere. Strikingly, the Cdc13-Est2 fusion now allowed cells to propagate in the complete absence of *EST1* function: in a strain where both *CDC13* and *EST1* were deleted, the senescence phenotype of an *est1-Δ* mutation was bypassed, and the Cdc13-Est2 fusion could maintain healthy growth for at least up to 150 generations (Fig. 4). Est1 function was not completely bypassed, however, since telomere length was short, but stably maintained (data not shown). At present, we are unable to determine if this incomplete bypass is due to imperfection in the Cdc13-Est2 fusion protein or if Est1 has other roles at the telomere in addition to mediating telomerase access. Interestingly, the Cdc13-Est2 fusion did not bypass the requirement for Est3 (data not shown).

One alternative interpretation of the above data was that we had perturbed some function of Cdc13, as mutations in *CDC13* have been identified which cause telomere lengthening. I have constructed several control fusion proteins to address this concern, and one particularly informative fusion (the Cdc13-$Est2^{D670A}$ fusion) has already been mentioned. In a second informative experiment, instead of fusing intact Cdc13 to Est1, an alternative Est1 fusion was constructed.

Previous work in our lab has shown that the DNA binding domain of Cdc13 can be expressed as a stable, discrete sub-domain (8). The premise behind this fusion experiment is that the affinity of Cdc13 for yeast telomeric DNA is approximately 500 fold higher than that of Est1. Therefore, fusing the Cdc13 DNA binding domain to Est1 should deliver Est1, and hence telomerase, to the telomere by virtue of the much higher affinity DNA binding domain. This modified version of Est1 is capable of suppressing the senescence phenotype of the telomerase defective *cdc13-2*^{est} allele (Fig. 5), as predicted if telomerase is now getting to the telomere independent of the telomerase function of Cdc13.

Conclusions and Future Directions

Collectively, these data show that Cdc13 and Est1 are co-mediators of telomerase access to the telomere and also provide a molecular basis for the telomerase-defective Cdc13 mutant. These results argue that Cdc13 mediates telomerase access via a protein-protein interaction, presumably via contact with Est1. Furthermore, we propose that a primary role for Est1 is to directly mediate telomerase access, based in part by the observation that it can be bypassed by fusing Cdc13 directly to telomerase.

These fusion proteins now provide us with an extremely powerful tool to mechanistically dissect interesting aspects of telomerase function. The following is a short list of projects that use the principles and reagents generated from the fusion experiments:

1. Identifying additional mutations in Est1 or Cdc13 that are suppressed in the fusion protein. As an extension of Project I, this will be used to further define domains in both proteins that are required to mediate telomerase access.
2. Defining the minimum regions of Est2 and TLC1 required for catalysis. Since the domains required for association with Est1 and/or other telomerase-recruiting proteins can now be dispensed with by fusing portions of Est2 to Cdc13, we can define the minimum component requirements for telomerase activity *in vivo*.
3. Testing whether directing Cdc13 to an HO-endonuclease-induced double strand break (by virture of the LexA system) is sufficient to recruit telomerase to that break and see healing/telomere addition at a location other than at the telomere. This experiment directly tests the model that the presence of the Cdc13 protein is a mediator of telomerase access.
4. Extending this line of experimentation to mammalian cells. In collaboration with Ronald DePinho's laboratory, we are constructing Cdc13-hTERT fusions to ask whether such a fusion will similarly confer telomere lengthening in human cells (based on our prior observations that Cdc13 can bind human telomeric DNA).

Project III: Role of telomerase in telomeric silencing

Results and Discussion

We have previously shown that Cdc13p and the heterodimer Ku are required, along with telomerase, for full telomere function, and we have proposed that Ku and Cdc13p contribute distinct roles in end protection. Ku has recently been shown to exhibit defects in transcriptional repression of telomere proximal genes, known as telomere position effect (TPE), or telomeric silencing. In collaboration with other members of our lab, I investigated whether alterations in genes involved in the telomerase pathway also exhibit TPE defects. We found that deletion or overexpression of *EST1*, *EST2* or *TLC1* does not significantly affect telomeric silencing. However, telomeric silencing is derepressed upon overexpression of dominant negative alleles of each. In addition, we determined that overproduction of telomerase pathway components partially alleviates the TPE defect in *hdf1Δ* cells. This indicates that there is genetic crosstalk between these two telomere maintenance pathways, and suggests that overproduction of telomerase pathway components may at least partially compensate for the loss of Ku in maintaining telomeric silencing. These findings were published last year (12).

Table 1: Summary of the est1 mutants obtained in the targeted charged cluster to alanine scan

	allele	In vivo senescence phenotype in wild type[1]	in *yku70 Δ*[2]	telomere length	dominant negative phenotype[4]	co-IP with TLC1[6]	full length protein upon overexpression
CLASS I	*est1-46*	near wild type	null	short	very strong	yes	not determinable
	est1-47	near null	null	short	very strong	yes	yes
	est1-49	near null	null	medium short	weak	yes	yes
	est1-52	null	null	very short	weak	yes	yes
	est1-54	partial	null	short	strong	yes	not tested
	est1-55	near null	null	short	strong	yes	not tested
CLASS II	*est1-34*	temp sensitive[3]	null	short	no at 30°C, unclear at 36°C[5]	yes at 30°C, 36°C	not tested
	est1-42	temp sensitive[3]	null	medium short	no at 30°C, unclear at 36°C[5]	yes, may be reduced	not tested
CLASS III	*est1-38*	near wild type	null	medium short	no	no	yes
	est1-39	temp sensitive[3]	null	medium short	no at 30°C, unclear at 36°C[5]	no	yes
	est1-41	wild type	wild type	wild type	no	no	yes

1. Assayed after a plasmid shuffle following approximately 75 generations of growth
2. Assayed after a plasmid shuffle. The *yku70 Δ* strains is more sensitive to defects in telomerase, and thus was used to detect weaker mutant alleles.
3. Normal or near wild type growth (but short telomeres) at 30°C; senescence comparable to the null at 36°C
4. Each allele was overexpressed (behind the *ADH* promoter on a 2μ plasmid) in a *yku80 Δ EST1+* strain and assayed for a rapid senescence phenotype. Plating cells at 10-fold serial dilutions allowed a somewhat quantitative analysis of the strength of the dominant negative phenotype.
5. For technical reasons, I am unable to conclusively determine if they display a dominant negative phenotype at 36°C.
6. Assayed by IP Northern experiments. Failure to associate with TLC1 is defined by less than 1% of wild type levels.

Figure 1: Fusing Cdc13 or Cdc13^{est} to Est1 results in substantial telomere lengthening. Genomic Southern blot, hybridized with a telomere-specific probe. Wild type telomere length is represented in lanes 1 and 6. Single copy plasmids carrying the Cdc13-Est1 fusion (lanes 2-5) or the Cdc13^{est}-Est1 fusion (lanes 7-10) were transformed into either an *est1-Δ CDC13+* strain (lanes 2, 3, 7 and 8) or shuffled in an *est1-Δ cdc13-Δ* strain (lanes 4, 5, 9 and 10). All lanes represent equivalent generations of growth.

Figure 3: Fusing Cdc13 to Est2, but not a catalytically inactive version of Est2, results in telomere elongation. Wild type telomere length is represented in lane 7. Plasmids carrying the Cdc13-Est1 fusion (lanes 2 and 3), Cdc13-Est2 fusion (lanes 3 and 4), or Cdc13-Est2^{D670A} (lanes 5 and 6) were transformed into a *cdc13-Δ* strain.

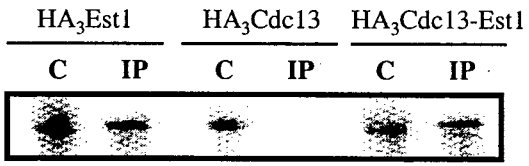

Figure 2. The Cdc13-Est1 fusion protein interacts with telomerase. Single copy plasmids carrying HA_3EST1 (lanes 1 and 2), HA_3CDC13 (lanes 3 and 4), and HA_3CDC13-EST1 (lanes 5 and 6) were transformed into a protease deficient strain. Following preparation of crude extracts (C) and immunoprecipitation (IP), RNA was extracted by SDS/phenol chloroform treatment and detected by Northern blotting as described in reference 6.

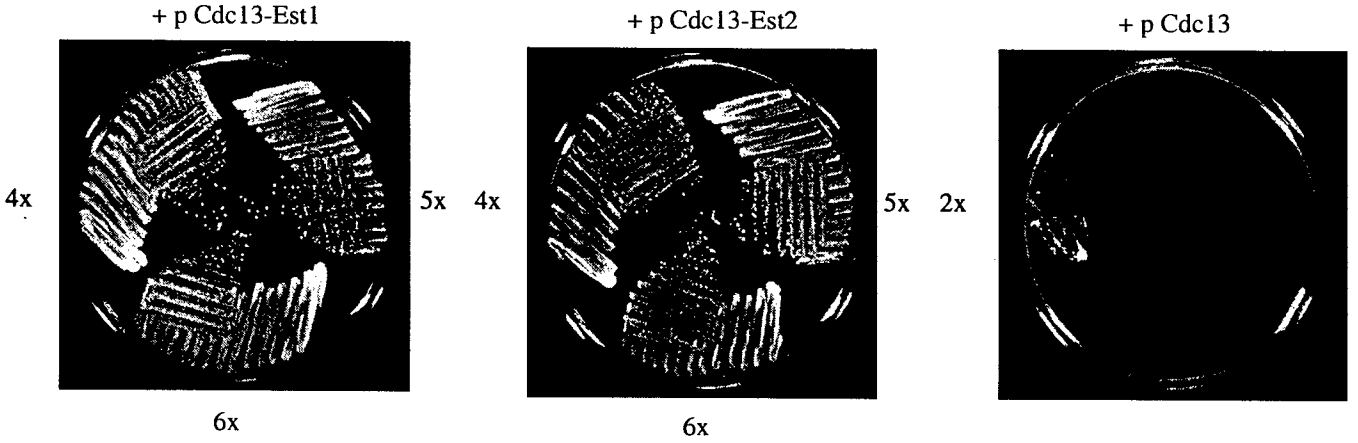

Figure 4: The Cdc13-Est2 fusion bypasses senescence of an *est1-Δ* strain. Plasmids carrying the Cdc13-Est1 fusion (left panel), the Cdc13-Est2 fusion (middle panel) or Cdc13 alone (right panel) were shuffled into an *est1-Δ cdc13-Δ* strain. 1"x" is equivalent to approximately 25 generations of growth.

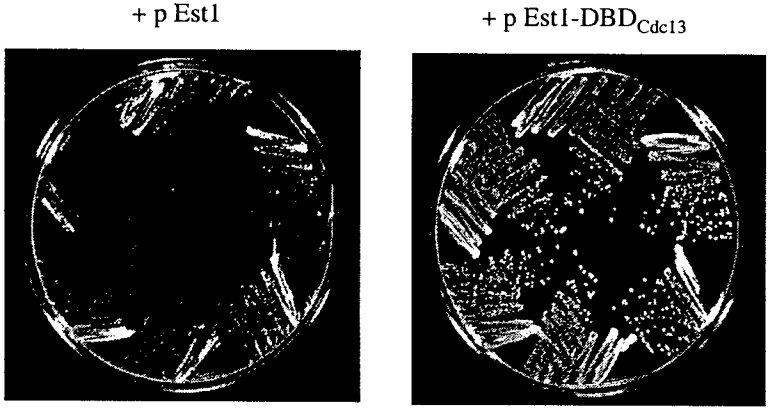

Figure 5: Fusing the high affinity DNA binding domain of Cdc13 to Est1 results in suppression of the telomerase defective *cdc13-2^{est}* mutant. Plasmids carrying either wild type Est1 (left panel) or the modified Est1 fusion (right panel) were transformed into an *est1-Δ cdc13-2^{est}* strain. Six independant isolates are shown.

SUMMARY APPENDICES

Key research accomplishments:

- completed a detailed site directed mutational analysis of *EST1*, and isolated three informative mutant classes that may be used to further define Est1 function and to isolate Est1-interacting factors.
- provided strong evidence further supporting our model that Cdc13 and Est1 are co-mediators of telomerase access to the chromosome terminus based on the observations:
  - fusing Cdc13 to Est1 or Est2 results in substantial telomere lengthening, arguing that tethering telomerase to Cdc13 results in increased access of telomerase to the telomere.
  - fusing Est1 to the telomerase defective Cdc13 mutant bypasses the consequences of that mutation, indicating that the molecular basis for telomerase defective Cdc13 mutant likely results from the inability of Cdc13 to directly mediate telomerase access by loss of protein-protein interaction with telomerase.
  - fusing Cdc13 directly to the catalytic subunit of telomerase partially bypasses the requirement for Est1, suggesting that the primary role of Est1 is to mediate telomerase access to the telomere.

Reportable outcomes:

A. Manuscripts:

1. Evans SK, Sistrunk ML, Nugent CI, Lundblad V. Telomerase, Ku and telomeric silencing in *Saccharomyces cerevisiae*. Chromosoma (1998) 107:352-358
2. Evans SK, Lundblad V. Tethering telomerase to the telomere: Est1 and Cdc13 are comediators of telomerase access. (in preparation, to be submitted to Science)

B. Abstracts and Presentations:

EST1: Mediator of telomerase access to the chromosomal terminus?
Cold Spring Harbor Meeting on Telomeres and Telomerase, March 25-28, 1999
Platform presentation

C. Copies of the above cited manuscripts and abstracts are attached.

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CHROMOSOMA

Telomerase, Ku, and telomeric silencing in *Saccharomyces cerevisiae*

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Abstract. Telomeres comprise a specialized chromosome end structure distinct from the standard nucleosomal architecture of the remainder of the genome. Telomere maintenance and chromosome stability require both replication of telomeric sequences by telomerase and telomeric end protection through binding of proteins. We have shown that Cdc13p and the heterodimer Ku are required, along with telomerase, for full telomere function, and we have proposed that Ku and Cdc13p contribute distinct roles in end protection. Ku has recently been shown to exhibit defects in transcriptional repression of telomere-proximal genes, known as telomere position effect (TPE), or telomeric silencing. We investigate here whether alterations in genes involved in the telomerase pathway also exhibit TPE defects and find that deletion or overexpression of *EST1* or *EST2* does not significantly affect telomeric silencing. However, telomeric silencing is derepressed upon overexpression of certain nonfunctional alleles of each. In addition, we determined that overproduction of telomerase pathway components partially alleviates the TPE defect in *hdf1*Δ cells. This indicates that there is genetic crosstalk between these two telomere maintenance pathways, and suggests that overproduction of telomerase pathway components may at least partially compensate for the loss of Ku in maintaining telomeric silencing.

Introduction

Telomeres, as the ends of linear chromosomes, are critical structures that prevent such catastrophic events as end-to-end fusions and chromosome loss via nucleolytic digestion. Telomeres also circumvent loss of sequence in successive replication cycles, known as the "end-replication problem", by employing a specialized telomere-specific DNA polymerase called telomerase. Telomerase is a ribonucleoprotein complex that utilizes its intrinsic RNA subunit as a template for telomere repeat synthesis (for review see Nugent and Lundblad 1998). The telomerase catalytic protein subunit was originally identified in *Euplotes aediculatus* and *Saccharomyces cerevisiae* using biochemical and genetic approaches (Lendvay et al. 1996; Lingner and Cech 1996). The *Euplotes* p123 and the Est2p of *S. cerevisiae* both contain a set of motifs common to known reverse transcriptases; these motifs comprise a protein fold, which forms the reverse transcriptase active site. When potential active site residues are altered in the Est2 protein, telomerase activity is disrupted in vitro and subsequent in vivo telomere replication defects are also exhibited (Lingner et al. 1997a). Since identification of the catalytic component in *Euplotes* and *S. cerevisiae*, this catalytic subunit (called TERT, for telomerase reverse transcriptase) has subsequently been identified in humans, *Schizosaccharomyces pombe* (Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Harrington et al. 1998; Nakayama et al. 1998), *Tetrahymena thermophila* and *Oxytricha trifallix* (Bryan et al. 1998; Collins and Gandhi 1998).

Although the RNA subunit and then TERT protein appear to be sufficient to provide core enzymatic activity in reticulocyte lysates (Weinrich et al. 1997), it is likely that additional components comprise a holoenzyme complex that is required in vivo. These proteins may function to regulate telomerase activity or mediate contact between the core enzyme and the end of the telomere. These additional factors could be components of telomeric chromatin or alternatively could be associated with the core telomerase components to form a telomerase holoenzyme complex. In *Tetrahymena*, the p80 and p95 proteins have been shown to be telomerase associated and are proposed to be involved in DNA-substrate recognition and telomerase-RNA interaction (Collins et al. 1995; Gandhi and Collins 1998). Telomerase-associated mammalian homologs of the *Tetrahymena* p80 protein have also been iden-

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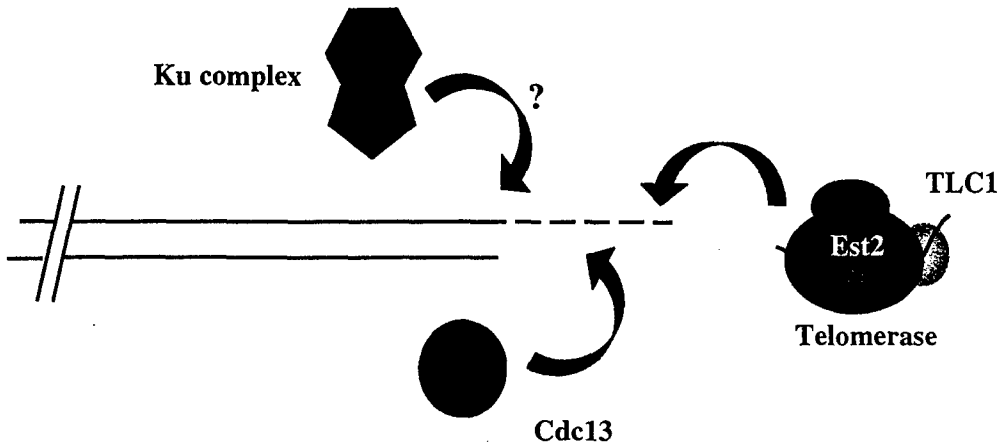

Fig. 1. Three distinct activities are required for telomere function. Ku and Cdc13p have been proposed to have distinct functions at the telomere, perhaps binding the terminal duplex DNA or single-strand G-tail extensions, respectively, at temporally discrete periods in the cell cycle (for review see Bertuch and Lundblad, in press). The telomerase holoenzyme is drawn for purposes of illustration with multiple subunits, although only the templating RNA (TLC1) and catalytic subunits (Est2p) of telomerase have been shown to be components of the enzyme (Singer and Gottschling 1994; Lingner et al. 1997a; Counter et al. 1997)

tified (Harrington et al. 1997; Nakayama et al. 1977). In *S. cerevisiae*, genetic screening for telomere replication mutants has led to the identification of three genes, *EST1, EST3* and *CDC13*, along with *EST2*. Mutations in any of these three genes result in the same phenotype as observed for a defect in the core enzyme: progressive telomere shortening and a gradual decline in cell viability (Lendvay et al. 1996). However, unlike strains defective for either component of the core enzyme (Est2p and TLC1), strains carrying mutations in *EST1, EST3,* and *CDC13* still retain enzyme activity in vitro (Cohn and Blackburn 1995; Lingner et al. 1997b). Est1p binds single-strand G-rich telomeric DNA in vitro, and associates with the telomerase RNA in vivo, suggesting that Est1p may function in bringing the chromosome terminus substrate into the enzyme active site (Lin and Zakian 1995; Steiner et al. 1996; Virta-Pearlman et al. 1996). Cdc13p, as defined by the *cdc13-2*^{est} mutation, also has a role in positive regulation of telomerase (Nugent et al. 1996).

One function vital to maintaining the integrity of chromosome ends, which the cell theoretically could perceive to be DNA breaks, is the protection of these specialized ends from degradation or recombination. In addition to its suggested role in telomerase function, Cdc13p has been proposed to have a second role in maintaining telomere integrity by protecting telomeric termini via end-binding. The *cdc13-1*^{ts} mutation suggests a role for Cdc13p in end protection, because it confers the loss of the telomeric C-rich strand and results in subsequent cell death (Garvik et al. 1995). In addition, Cdc13p binds with high affinity to single-strand telomeric DNA substrates in vitro (Nugent et al. 1996). Since in *S. cerevisiae* the G-rich strand has been shown to exist as a single-strand extension late in S-phase (Wellinger et al. 1993), Cdc13p may function as a cap to telomeric ends at certain stages of the cell cycle.

Another potential player in telomeric end protection is the Ku heterodimer, comprising the *HDF1* and *YKU80* gene products. Ku has been shown previously to bind DNA ends and to mediate nonhomologous end-joining (NHEJ) in both yeast and mammalian cells (reviewed in Jin et al. 1997). Recent evidence suggests that Ku also appears to play an important role in telomeric end protection in yeast. First, absence of either *YKU80* or *HDF1* incurs telomere shortening (Porter et al. 1996; Boulton and Jackson 1998; Nugent et al. 1998). Second, crosslinking experiments suggest Ku80p is physically associated with telomeric chromatin in vivo (Gravel et al. 1998). One hypothesis is that a more discretely defined physical telomeric location for Ku may in fact be the terminal duplex ends, as opposed to a part of internal duplex chromatin (see Bertuch and Lundblad 1998). Finally, further evidence that Ku function is important for establishing or maintaining telomeric chromatin structure is the change in expression of telomere-localized genes in cells lacking Ku function (Boulton and Jackson 1998; Gravel et al. 1998; Laroche et al. 1998; Nugent et al. 1998).

Ku and Cdc13p have been shown to interact genetically: mutations in *YKU80* have been isolated from a screen designed to identify genes that function in parallel with *CDC13* (Nugent et al. 1998). Their roles in end protection, however, are distinct since growth in *cdc13-1*^{ts} *ku*$^{-}$ cells is greatly reduced compared with cells harboring the individual mutations (Nugent et al. 1998; Polotnianka et al. 1998). This argues that Cdc13p and Ku exhibit two genetically separable activities required for complete telomere function. Growth defects of either *cdc13-1*^{ts} or *ku*$^{-}$ *(either hdf1*Δ or *yku80*Δ) cells are more severe in cells also lacking functional telomerase (Nugent et al. 1996; Gravel et al. 1998; Nugent et al. 1998), suggesting that Ku and Cdc13p are involved in a telomere maintenance pathway separate from telomerase-mediated telomere replication (Fig. 1).

Although telomere replication by telomerase is a genetically distinct activity from the end-protection functions of Ku and Cdc13p, telomerase function may also be important for maintaining the structure of telomeric ends. In cells overexpressing truncated *TLC1* cDNAs,

telomeres become shorter and telomeric silencing is disrupted (Singer and Gottschling 1994). One explanation for the loss of silencing in cells overexpressing these alleles of *TLC1* is that mutant forms of the RNA titrate away telomerase components, thereby effectively reducing the amount of active telomerase in the cell. This, in turn, would cause shortening of telomeres by preventing telomeric replication. Subsequent attrition of binding sites for silencing proteins may result in loss of telomeric silencing. One prediction of this model is that any strain with short telomeres would have derepressed silencing. An alternative model for the disruption of telomere position effect (TPE) upon overexpression of these truncated alleles of *TLC1* is that they are titrating out chromatin components that are required for telomeric silencing, and that telomere shortening is not a prerequisite to disruption of silencing.

To distinguish between these two models, we determined whether strains deleted in telomerase pathway genes, known to exhibit short telomeres (Lundblad and Szostak 1989; Singer and Gottschling 1994; Lendvay et al. 1996), demonstrate defects in telomeric silencing. We determined that *est1*Δ, *est2*Δ, or *tlc1*Δ exhibit no significant defects in TPE. To ascertain whether the TPE defect upon overexpression of mutant alleles is unique to the telomerase RNA, we examined whether overproduction of other nonfunctional telomerase pathway components would similarly disrupt telomeric silencing. We found that overexpression of certain mutant alleles of *EST1* or *EST2*, but not the wild-type genes, also disrupts telomeric silencing. Finally, we have previously shown that overproduction of telomerase pathway components can suppress a temperature-sensitive growth defect, which displays a phenotypic lag, in *yku80*Δ cells (Nugent et al. 1998). Given this precedent, we therefore wanted to determine whether overproduction of telomerase pathway components could also suppress the silencing defect in *hdf1*Δ cells, and determined that overexpression of *EST1, EST2,* and *TLC1* partially alleviates the defect.

Materials and methods

Yeast strains. The *EST1* and *EST2* genes were disrupted in the haploid silencing strain UCC3505 (*MATa ura3–52 lys2–801 ade2-101 trp1–Δ63 his3–Δ200 leu2–Δ1 ppr::HIS3 adh4::URA3-TEL DIA5–1*) using gene disruptions generated from polymerase chain reaction amplification of the kanMX2 cassette (Singer and Gottschling 1994; Wach et al. 1994). Primer pairs for each gene were designed with 46 bp homology to regions at the start and stop codons of the *EST1* and *EST2* open reading frame. For the telomeric silencing assays shown in Figs. 2 and 4, the strain UCC3505 (Singer and Gottschling 1994) was used. For the sectoring assays shown in Fig. 3, the strain UCC41 (*MATα lys2 his4 trp1Δ ade2 leu2–3,112 ura3–52* with *URA3* and *ADE2* at telomere VII–L) (Gottschling et al. 1990) was used.

Genetic methods. For the growth assays shown in Figs. 2 and 4, serial tenfold dilutions of haploid strains of the desired genotype were spotted onto appropriate medium and incubated at differing temperatures. To ensure that equivalent numbers of cells were compared for each strain, initial cell density was determined using a hemocytometer. For analysis of high-copy suppression of *hdf1*Δ temperature sensitivity of telomeric silencing, at least two transformants from a minimum of two independent transformations were examined for each plasmid/strain combination. This assay was performed prior to manifestation of the temperature-sensitive growth defect of the *hdf1*Δ strain. Yeast transformations were performed using standard genetic techniques. The *hdf1*Δ strains were transformed with: vector alone (pVL399), pVL784 (2μ p*ADH-EST1*), pVL999 (2μ p*ADH-EST2*), or pVL799 (2μ p*ADH-TLC1*). Wild-type cells were transformed with vector control or pJBN155 (2μ p$ADH\text{-}SIR4_{CT}$). Cells were grown in selective medium and examined at 23° C (after 5 days) and 36° C (after 2.5 days).

For the adenine sectoring assays shown in Fig. 3, the silencing strain was transformed with: vector alone (pVL248 or pVL399), pVL249 (2μ p*ADH-EST1*), pVL306 (2μ p*ADH-est1–7*), pVL999 (2μ p*ADH-EST2*), or pVL1030 (2μ p*ADH-est2–5*) and plated on the appropriate selective medium containing 40 μg/ml adenine. Following another passage of growth on 40 μg/ml adenine plates, cells were plated on media containing 10 μg/ml adenine. Colonies were allowed to grow for 5–7 days at 30° C prior to a 48 h exposure at 4° C to allow full color development.

Results

Absence of telomerase pathway components does not affect TPE

We tested the effect of the disruption of telomerase function on telomeric silencing using two different approaches. The first method examined telomeric silencing in strains deleted in *EST1, EST2* or *TLC1*. Telomeric silencing was measured by assaying the transcriptional activity of a telomere-proximal reporter gene, *URA3* (Gottschling et al. 1990). Cells expressing *URA3* are unable to grow on medium containing the drug 5-FOA (5-fluoro-orotic acid). In wild-type cells, the telomere-proximal *URA3* gene is transcriptionally repressed and cells are able to survive in the presence of 5-FOA (Gottschling et al. 1990). However, when telomeric chromatin is disrupted, the *URA3* gene is expressed, causing lethality on medium containing 5-FOA. In this experiment, we used a silencing strain in which the *URA3* transactivator, *PPR1*, was absent. In a *ppr1*$^-$ strain, the telomeric *URA3* gene is more sensitive to parameters affecting telomeric silencing (Renauld et al. 1993). In this strain background, deletion of either *EST1, EST2* or *TLC1* had no significant effect on TPE, as evidenced by equivalent growth on non-selective (YPD) and 5-FOA-containing medium (Fig. 2). These platings represent cultures that have grown for approximately 40 generations following loss of the gene, yet before the senescence phenotype of these strains is manifested (Singer and Gottschling 1994; Lendvay et al. 1996; Virta-Pearlman et al. 1996). In contrast, overproduction of the C-terminal portion of Sir4p in this experiment completely abolishes telomeric silencing, as previously reported (Cockell et al. 1995).

TPE is disrupted in cells overexpressing certain mutant alleles of telomerase pathway components

A second strategy that we employed to determine whether the disruption of telomerase function could affect TPE was to overproduce particular mutant forms of the Est1

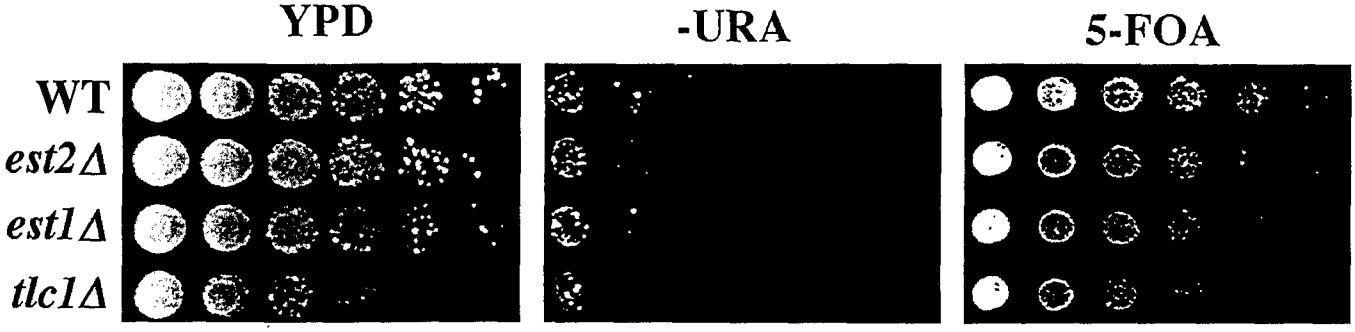

Fig. 2. *EST1*, *EST2*, and *TLC1* are not required for silencing of telomere-proximal genes. Serial tenfold dilutions of cells from freshly grown wild-type, *est1*Δ, *est2*Δ, or *tlc1*Δ strains were plated on complete medium (YPD) in order to monitor total cells viability, on medium lacking uracil (–URA) to assess the extent of derepression of *URA3* transcription, and one medium containing 5-fluoro-orotic acid (5-FOA) to determine the proportion of cells able to repress *URA3* transcription. Plates were incubated at 30° C for 3 days

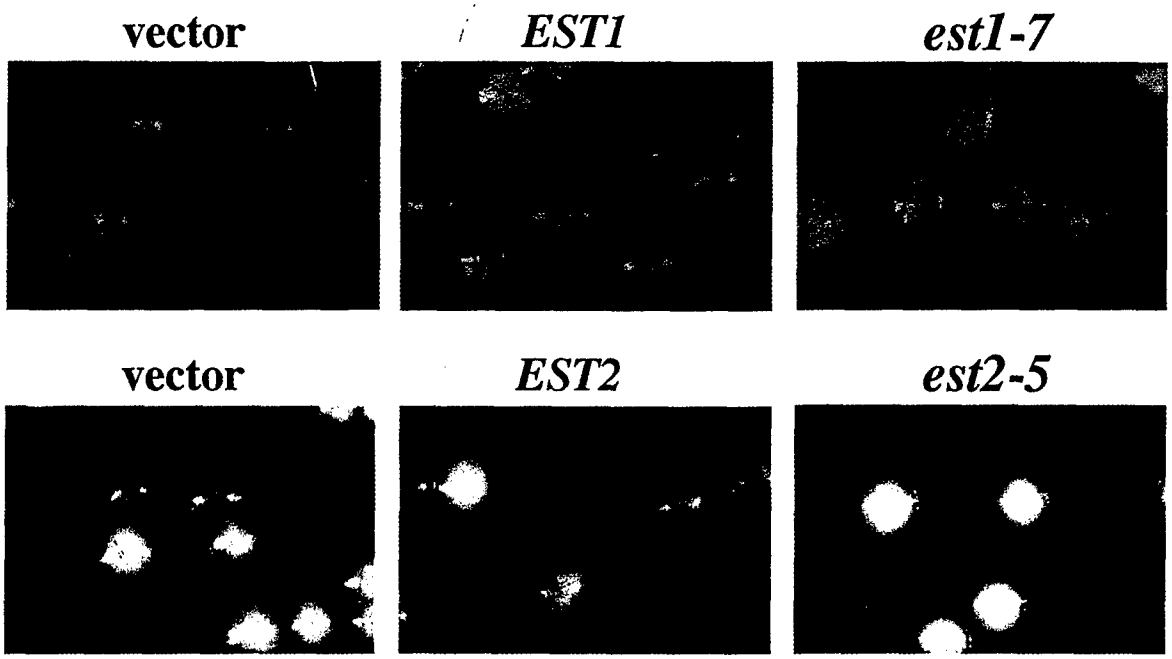

Fig. 3. Overexpression of certain mutant alleles of *EST1* or *EST2*, but not wild-type *EST1* or *EST2*, disrupts telomeric silencing. Wild-type cells harboring plasmids overexpressing wild-type *EST1* or *EST2* or the nonfunctional alleles *est1–7* (Virta-Pearlman et al. 1996) and *est2–5* (Lingner et al. 1997a) or vector control were assayed for the expression state of a telomere-proximal *ADE2* marker. Repression of *ADE2* transcription, reflecting the silencing state, produces red colonies, whereas derepression of *ADE2* transcription, reflecting loss of silencing, produces white colonies

and Est2 proteins. This approach differs from the first experiment in that, although wild-type Est1 or Est2 proteins are still present, the overproduction of nonfunctional forms may be titrating out factors required for telomeric silencing. Thus while the complete absence of the proteins may have insignificant effects on telomeric silencing, it is possible that overproduction of these mutant forms could disrupt telomeric chromatin. As previously stated, overexpression of detective alleles of *TLC1* derepresses telomeric silencing and moderately shortens telomeres, an effect that could be unique to the telomerase RNA component. When particular mutant alleles of either *EST1* or *EST2* are overexpressed in wild-type cells, telomeres also shorten (Virta-Pearlman et al. 1996; Lingner et al. 1997a). We have previously reported that in a *PPR1*$^+$ strain overexpression of certain mutant alleles of *EST1* disrupts telomeric silencing (approximately 500-fold), while overexpression of wild-type *EST1* results in a modest increase in telomeric silencing (Virta-Pearlman et al. 1996). However, Est1 exhibits single-strand telomere binding activity (Virta-Pearlman et al. 1996) and we cannot exclude a role for Est1 as a component of telomeric chromatin. Therefore, we wished to determine whether overproduction of a nonfunctional form of a known protein component of telomerase would have similar effects. We assayed this by taking advantage of a strain with a telomere-proximal *ADE2* marker. In this strain, telomeric silencing is evident by the present of red-sectored colonies (the *ade2*$^-$ phenotype), while disruption of telomeric silencing is manifested as white colonies (the *ADE2*$^+$ phenotype). Figure 3 shows that, like *TLC1* and *EST1*, overexpression of a dominant negative allele of

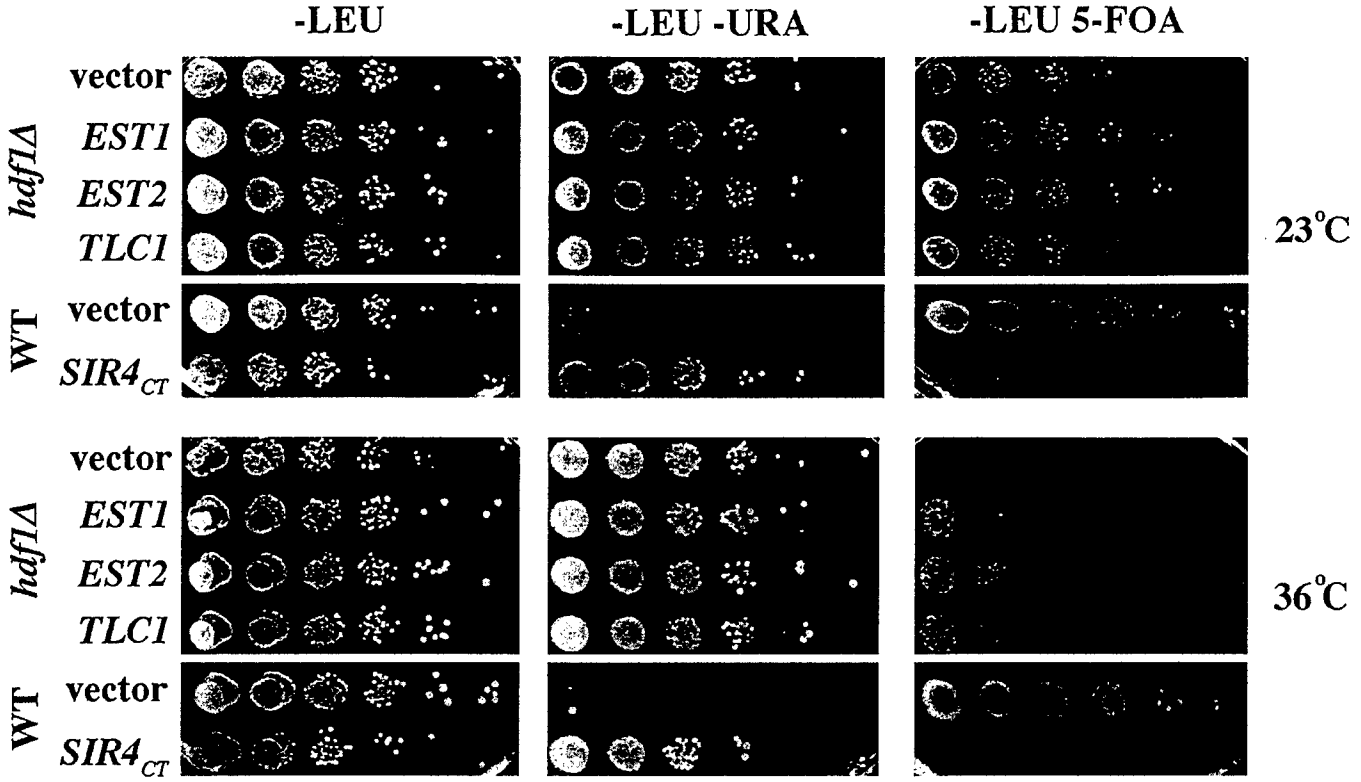

Fig. 4. Overexpression of *EST1, EST2,* and *TLC1* partially alleviates a temperature-sensitive Ku telomeric silencing defect. *hdf1*Δ strains were transformed at 23° C with plasmids overexpressing *EST1, EST2,* or *TLC1,* or a vector control. Wild-type strains were transformed with a plasmid overproducing the C-terminus of Sir4p or its vector control. Serial tenfold dilutions of freshly grown cells were plated on medium lacking leucine to maintain growth in the presence of the respective tester plasmids. Dilutions were grown on plates also lacking uracil (–LEU –URA) to assess the extent of derepression of *URA3* transcription or on medium also containing 5-FOA (–Leu 5-FOA) to determine the proportion of cells able to repress *URA3* transcription. Plates were incubated at either 23° C (5 days) or 36° C (2.5 days)

EST2 also disrupts telomeric silencing, while overexpression of wild-type *EST2* does not. This demonstrates that TPE can be disrupted by overproduction of mutant forms of either of the core catalytic components of telomerase.

Overproduction of telomerase pathway components partially alleviates a Ku TPE defect

Strains deleted for *HDF1* or *YKU80* have been previously shown to have a temperature-sensitive growth phenotype: cells can grow at 23° C but exhibit delayed lethality at 36° C (Feldmann and Winnacker 1993; Barnes and Rio 1997). *ku*⁻ cells also exhibit a temperature-sensitive defect in expression of a telomere-proximal *URA3* reporter gene (Gravel et al. 1998; Nugent et al. 1998). Since the absence of Ku confers a defect that is seen at high temperatures, this suggests the existence of a thermolabile factor that is partially redundant with Ku at low temperatures. To determine whether altering expression levels of telomerase pathway components affects Ku-dependent TPE activities, we overexpressed *EST1, EST2,* and *TLC1* in *hdf1*Δ cells. Figure 4 shows that *hdf1*Δ cells exhibit a modest TPE defect at 23° C, but at 36° C telomeric silencing is completely abolished, such that cells are unable to grow on 5-FOA, as previously reported (Nugent et al. 1998). However, in a *hdf1*Δ strain overexpressing *EST1, EST2,* or *TLC1,* the temperature-sensitive TPE defect is partially alleviated.

Discussion

The question of whether the disruption of telomerase function would lead to alterations in telomeric chromatin was assayed utilizing two separate approaches. First, we tested the ability of cells to repress *URA3* transcription in the complete absence of the Est1 and Est2 proteins. In our strain background, we did not observe a noticeable defect in telomeric silencing upon deletion of *EST1, EST2* or *TLC1.* This is in contrast to deletion of known chromatin components such as the Sir proteins, where silencing is completely abolished (Aparicio et al. 1991). This suggests that telomerase is not a substantial component of telomeric chromatin, and that the physical presence of the telomerase enzyme is not required to maintain telomeric silencing. We cannot exclude the possibility that there would be long-term effects of the loss of telomerase on silencing, but we have not to addressed this issue because of the decrease in viability in late generation *est/tlc1* cultures.

Second, we tested the effects on telomeric chromatin of overexpression of both wild-type and nonfunctional al-

leles of *EST1* and *EST2*. Overproduction of mutant forms of these proteins in wild-type strains disrupted silencing, whereas overproduction of wild-type proteins did not have a significant effect. There are two possible explanations for this. One is that overproduction of mutant forms of the proteins titrates out other telomerase components that are required for telomerase activity, causing telomere shortening and disruption of telomeric chromatin. However, we think this explanation is unlikely since the complete absence of telomerase components, which also causes telomere shortening (Lendvay et al. 1996; Virta-Pearlman et al. 1996), does not lead to the same phenotype. Instead, a more likely explanation is that overproduction of mutant forms of telomerase components titrates out some factor(s) that is required for telomeric silencing. Candidate factors include Ku itself, or possibly one of the Sir proteins. One way to address which factor is titrated is to determine whether the effects of overexpression of these nonfunctional alleles are telomere specific, or have global consequences in silencing at other loci. Another informative experiment would be to determine whether there is a synergistic TPE defect upon overexpressing the mutant *est* alleles in *ku*$^-$ cells: if the factor being titrated is not Ku, then one would expect a more severe defect upon overexpression of *est1* or *est2* because now both Ku and the candidate silencing factor would be absent from the telomere. One alternative explanation for the TPE defect in cells overexpressing dominant negative alleles of *EST1* and *EST2* may be that they are indirectly affecting TPE by titrating away Ku or some other factor important for telomeric clustering (Laroche et al. 1998).

Although it appears that the telomerase pathway components *EST1, EST2* and *TLC1* are not required directly for telomeric silencing, we questioned whether overproduction of these factors could compensate for the TPE defect observed in *hdf1*Δ strains. To circumvent the possibility that overexpression of *EST/TLC1* genes partially alleviates the temperature-sensitive TPE defect merely by relieving the growth defect seen at 36° C, we assayed repression of TPE at a time point prior to the manifestation of the growth defect. The demonstration that overexpression of these telomerase pathway genes alleviates the Ku silencing defect suggests that there is genetic crosstalk between these two telomere maintenance pathways. Perhaps overexpression of these genes subtly alters telomere structure, or alters the ratio of one type of end structure to another, thereby allowing differential recruitment of particular factors.

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Telomeres and Telomerase Meeting Evans
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EST1: MEDIATOR OF TELOMERASE ACCESS TO THE CHROMOSOMAL TERMINUS?

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There are two known components of *S. cerevisiae* telomerase, *TLC1*, the RNA subunit, and *EST2*, the protein catalytic subunit, which are required both in vivo for telomere replication and in vitro for enzyme activity. In contrast, *EST1* and *EST3* are required in vivo for telomerase function but are dispensable for catalytic activity in vitro. Previous work in our lab has shown that Est1p is a single-strand telomere DNA binding protein; Est1 also co-immunoprecipitates the yeast telomerase RNA in an Est2-independent manner. These data suggest that Est1 assists telomerase in bringing the chromosome terminus to the enzyme active site.

To define more clearly the mechanistic function of Est1 at the telomere, I have pursued two experimental approaches. The first has been a site-directed mutational analysis of the protein, which has yielded three classes of mutants. Class I mutants, which map within the DNA binding domain of Est1, have an in vivo *est1* phenotype but retain association with the telomerase RNA. Class II mutants are conditional for senescence, and association with TLC1 is retained in these mutants at all temperatures. Class III mutants are defined by their inability to associate with the telomerase RNA at detectable levels. Experiments are in progress employing these alleles in suppression screens to identify Est1-interacting factors.

In a second approach, I am examining whether Est1 can be physically tethered to the telomere as a protein fusion with Cdc13p. Strikingly, a Cdc13-Est1 fusion complements either null mutation. Furthermore, a $Cdc13^{est}$-Est1 fusion behaves comparable to the wild type Cdc13-Est1 fusion, arguing that tethering Est1 to Cdc13 is sufficient to bypass the *est* defect of Cdc13. Both fusions also confer a substantial telomere lengthening phenotype, suggesting that tethering Est1 to Cdc13 increases access of telomerase to the terminus. I am currently testing whether telomerase activity co-immunoprecipitates with the Cdc13-Est1 fusion, and whether any of the Class I, II or III *est1* mutations, discussed above, can similarly be bypassed when present as a Cdc13-Est1 fusion

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ATTENTION OF:

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MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

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