

UNCLASSIFIED

AD NUMBER
ADB257343
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Apr 2000. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.
AUTHORITY
U.S. Army Medical Research and Materiel Command ltr., dtd July 23, 2001.

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-98-1-8020

TITLE: Analysis of the Role of EST1 in Yeast Telomerase

PRINCIPAL INVESTIGATOR: Sara K. Evans
Victoria P. Lundblad, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: April 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Apr 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20000828 184

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

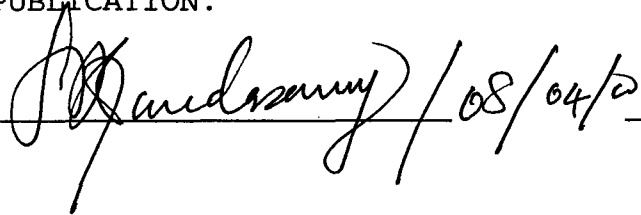
Award Number: DAMD17-98-1-8020

Organization: Baylor College of Medicine

Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

 / 08/04/02

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (23 Mar 99 - 22 Mar 00)	
4. TITLE AND SUBTITLE Analysis of the Role of EST1 in Yeast Telomerase			5. FUNDING NUMBERS DAMD17-98-1-8020	
6. AUTHOR(S) Sara K. Evans Victoria P. Lundblad, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, Texas 77030 E-MAIL: evans@bcm.tmc.edu/lundblad@bcm.tmc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Apr 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				12b. DISTRIBUTION CODE
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 <i>S. cerevisiae</i> . I have previously shown that fusion of Cdc13 to Est1 results in substantial telomere elongation. Fusions consisting of mutant versions of Cdc13 or Est1 confer similar telomere elongation, indicating that close physical proximity can bypass telomerase-defective mutations in either protein. Fusing Cdc13 directly to the catalytic core of telomerase (Est2) allows stable telomere maintenance in the absence of Est1. This study led to the proposal that Est1 and Cdc13 are co-mediators of telomerase access, and has further suggested that Est1 may have a second role in telomere replication, based on the observation that the Cdc13-Est2 fusion is unable to promote telomere elongation in the absence of Est1. Since Est1 is a terminus-specific single-strand DNA binding protein, this second role for Est1 may be to promote accessibility of the telomeric end to the telomerase active site. To explore this second role, I have isolated a set of alanine-scan mutations in Est1 which retain association with telomerase but display telomere replication defects, and have tentatively identified an Est1 mutant that is deficient in this second function.				
14. SUBJECT TERMS telomerase, tumor progression, new drug development			15. NUMBER OF PAGES 40	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

See Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Sara Evans

PI - Signature

April 21/2000

Date

Table of Contents

Cover.....	
SF 298.....	3
Foreword.....	4
Table of Contents.....	5
Introduction.....	6
Research Summary.....	7
Background.....	7
Project I.....	8
Project II.....	9
Project III.....	10
Figures.....	12
Key Research Accomplishments.....	14
Reportable Outcomes.....	14
References.....	15
Appendices.....	

INTRODUCTION

Telomeres, the nucleoprotein complexes present at the ends of chromosomes, perform an essential function in maintaining chromosomal stability. Telomeres are replicated by the reverse transcriptase, telomerase. Telomerase has received a great deal of attention in the field of cancer research, since there is a strong correlation between the presence of telomerase activity and cellular immortalization: telomerase activity is absent in most somatic cells (which have a limited replicative capacity) but is present in the majority of tumors. This observation has led to the model that reactivation of telomerase is a critical event for tumor progression. It has been suggested that inhibitors of the telomerase 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. Identifying additional components of telomerase 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

Telomeres are replicated by the telomerase reverse transcriptase, which catalyzes the synthesis of telomeric repeats using an internal RNA template. In *S. cerevisiae*, *EST1*, *EST2*, *EST3* and *TLC1* are necessary for telomere replication by telomerase. *EST2* and *TLC1* encode the reverse transcriptase and RNA subunits, respectively, of the enzyme catalytic core [1, 2]. *EST1* and *EST3* encode additional components of the yeast telomerase holoenzyme that are required *in vivo* for telomere replication but are dispensable for enzyme catalysis, and therefore function as positive regulators of enzyme function *in vivo* [3] (Hughes et al, submitted). Previous work 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 [4]. These data have led us to propose that Est1 – as a component of the telomerase enzyme complex – functions in delivering or positioning the telomerase enzyme at the end of the chromosome. Presumably, Est1 performs this role via its telomeric DNA binding activity, but this proposed telomere accessing activity may be mediated through protein-protein interactions as well.

A fifth gene, *CDC13*, also required for telomere maintenance, encodes a high affinity single-strand telomeric DNA binding protein that is not tightly associated with the telomerase complex [5, 6] (Hughes et al, submitted). The identification of a telomerase-defective allele of *CDC13* (*cdc13^{est}*) indicated that this end binding protein was also required for telomerase function. We therefore proposed that Cdc13, when bound to the terminus, mediated telomerase access to the telomere.

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. The results of my research during Year I of this proposal, substantiated the role of these two proteins in conferring telomerase access to the telomere, and led to the proposal that Cdc13 mediates access of telomerase to the chromosome terminus through a direct protein-protein interaction with the Est1 protein [6]. This model was based, in part, on the observation that fusion of Est1 to Cdc13 resulted in substantial telomere lengthening. This suggested that the affinity of telomerase for the telomere was greatly enhanced by fusing Cdc13 to a telomerase component. Furthermore, fusion of Cdc13 to Est1 bypassed telomerase defective versions of either protein. A Cdc13-Est2 fusion also resulted in telomere elongation and, strikingly, allowed cells to propagate in the complete absence of Est1 function. However, the Cdc13-Est2 fusion was not able to promote telomere elongation in the absence of Est1, suggesting that Est1 has a positive regulatory role in telomere replication in addition to mediating telomerase access.

This body of work left open two questions regarding aspects of Est1 and Cdc13 function. First, does Est1 have a second role in addition to mediating telomerase access? Second, does Cdc13 mediate telomerase access through direct recruitment of the enzyme? Towards answering these questions, my research in the past year has focused primarily on two projects. Project I reports on a set of experiments to begin investigating the second function of Est1; this is an extension of on-going work using a panel of Est1 mutants generated in Year I of this proposal. Project II outlines an experimental approach designed to test the model that Cdc13 mediates telomerase access through direct recruitment of the telomerase complex. This work is still in progress, but preliminary evidence suggests that the presence of Cdc13 alone may not be sufficient to recruit telomerase to the end of the chromosome. A third project describes a series of fusion experiments extending the principles of the Cdc13-telomerase fusion studies. In Project III, I describe results from a series of experiments employing an Est3-DBD_{Cdc13} fusion, which lends *in vivo* support to previous genetic and biochemical observations for the role of Est3 in telomere replication as a component of the telomerase complex. The results from this latter project have been submitted to Current Biology as part of a manuscript entitled "The Est3 protein is a subunit of yeast telomerase".

Project I: Phenotypic analysis of a panel of *est1* mutants

Rationale

As previously stated, the fusion analysis suggested that Est1 may have a second role in telomere replication, in addition to co-mediating telomerase access. This proposal is based on the observation that fusion of the Est2 subunit of telomerase to Cdc13 partially bypasses the requirement for Est1 in telomere maintenance: telomeres are stably maintained in a strain carrying the Cdc13-Est2 fusion but lacking Est1, but are not elongated to the same degree as observed in the presence of Est1. Since Est1 is a terminus-specific single-strand DNA binding protein, this second proposed role for Est1 may be to promote accessibility of the 3' end of telomeric DNA to the telomerase active site.

One trivial explanation for the failure of the Cdc13-Est2 fusion to promote telomere elongation in an *est1-Δ* strain is that there could be reduced levels of the Cdc13-Est2 telomerase complex in the absence of Est1. To address this possibility, I assayed for the ability of the Cdc13-Est2 fusion to associate with the telomerase RNA in the presence versus absence of Est1. In parallel immunoprecipitation experiments performed in *EST1⁺* and *est1-Δ* strains, there was less than a two-fold difference in the ability of the Cdc13-Est2 protein to co-immunoprecipitate the TLC1 RNA, arguing that the failure to elongate telomeres in an *est1-Δ* strain was not simply due to reduced stability of the Cdc13-Est2 fusion protein levels [6].

In Year I, I generated a panel of Est1 mutants by alanine scanning mutagenesis, and characterized these mutants both genetically for *in vivo* telomere replication phenotypes and biochemically for their association with the telomerase RNA. One particular class of mutants displayed telomere replication defects, although the mutant Est1 proteins still physically associated with telomerase. To explore the proposed second role of Est1, I have tested whether any of these Est1 mutants are also defective in promoting telomere elongation in the presence of the Cdc13-Est2 fusion.

Results and Discussion

One allele, *est1-42*, displays the characteristics expected for an Est1 mutant defective in the second function, in that *est1-42* strains expressing the Cdc13-Est2 fusion protein do not have elongated telomeres (Fig. 1A). Furthermore, this mutant alone has moderately short telomeres and, interestingly, displays a conditional (temperature dependent) senescence phenotype (data not shown and Fig. 1B). Preliminary biochemical characterization of the Est1-42 protein shows that it retains association with telomerase, however the variability in that initial analysis warrants repeating the experiment (currently in progress).

The amino acids that are altered in the *est1-42* mutant do not lie within the mapped DNA binding domain. However we cannot exclude the possibility that determinants required for DNA binding also lie elsewhere in the protein, and that *est1-42* represents a mutant which is defective for DNA binding. Alternatively, since Est1 associates with TLC1 independently of the catalytic subunit, it is possible that Est1 is involved in direct RNA binding, and that the *est1-42* mutant is somehow defective in this activity. Further biochemical analysis of the Est1-42 protein should distinguish between these possibilities, and help define the second function of Est1.

Future Directions

The mutants have thus far only been assayed for association with the telomerase RNA. Since this may or may not reflect an association with an active enzyme, I am currently in the process of testing whether telomerase activity can be detected in each mutant Est1 immunoprecipitate. The genetic and biochemical analyses of these *est1* mutants will form the basis for a manuscript that is

tentatively scheduled to be submitted by the end of the year. In addition, the *est1* mutants remain to be tested for their DNA and RNA binding properties *in vitro*. One ultimate goal of this proposal is to isolate an Est1 mutant that is defective for DNA binding, as it remains unknown what the *in vivo* consequences of a DNA binding defect are. Thus, if none of the mutants are defective, then I plan to randomly mutagenize the DNA binding domain using PCR-based techniques, and assay these mutants for their *in vitro* DNA binding properties and *in vivo* telomere replication phenotypes. High copy suppression genetic screens employing a subset of these *est1* mutants have so far been uninformative.

Project II: Does Cdc13 mediate telomerase access by directly recruiting the telomerase enzyme?

Rationale and Experimental strategy

The results of the fusion protein studies lead us to favor a model in which Cdc13 mediates telomerase access through a direct interaction with a component of the enzyme, likely Est1. However, the natural telomere consists of many other protein factors that could potentially aid Cdc13 in its recruitment function. I therefore wanted to pursue the model that Cdc13 is sufficient for telomerase recruitment, by directing Cdc13 to a non-telomeric location and asking if it brings telomerase along with it. The experimental approach involves artificially directing Cdc13 to a double strand break site (created by the site specific HO endonuclease) by virtue of the LexA DNA binding system, and determining if telomerase is recruited to the break by monitoring if telomeric sequences are added to the *de novo* chromosome end.

The strains I have constructed and employed in this experiment have an HO cut site inserted approximately 20kb from the end of chromosome VIII. The terminal 20kb of this chromosome does not contain any essential genes, and therefore the cell is able to maintain viability in the absence of this DNA, if the end is healed in such a manner as to create a stable chromosome (by telomere addition, for example). In this strain, the HO endonuclease gene has been placed under the inducible galactose promoter. Cleavage by the HO endonuclease creates a four base pair G-rich overhang, which can be elongated by the telomerase enzyme [7]. Prior experiments in yeast have shown that the presence of a telomere "seed" sequence near the chromosome break site will efficiently promote telomere addition [7, 8]. Therefore, as a control to ensure my system was working, I engineered a strain with G₁₋₃T sequences adjacent to the HO site. This strain displayed equivalent growth on media containing either glucose or galactose, indicating that telomere healing had indeed efficiently occurred, such that there was no decrease in viability when the terminal portion of chromosome VII was lost. I then constructed strains where the G₁₋₃T sequences were replaced with three tandem Lex sites, and tested for the ability of the Cdc13-Lex_{DBD} fusion protein to recruit telomerase by assaying for viability on galactose containing media.

Results and Discussion

The G₁₋₃T containing strain, as expected, retained 100% viability when plated on media containing galactose (data not shown). However, strains containing the Lex sites (zero or three) - but lacking the G₁₋₃T sequences - displayed a marked decrease in viability on media containing galactose (0.1% viability); the residual growth presumably arises from colonies where HO cleavage has not happened, or where other events (such as non-homologous end joining) have occurred to heal the chromosome at a low frequency. To assay whether Cdc13 could recruit telomerase, I transformed the strain harboring LexA binding sites with a high copy plasmid expressing an epitope tagged, functional Cdc13-LexA_{DBD} fusion protein; this fusion protein complements the *cdc13-Δ* mutation and interacts with LexA binding sites *in vivo* (data not shown). Following HO endonuclease induction, no increase in viability was observed on galactose containing media.

Therefore, at least in this preliminary system, it appears that the presence of Cdc13 at a chromosome end is not sufficient to promote telomere elongation. One critical control, currently being tested, is whether a Cdc13-Est1-LexA_{DBD} fusion is capable of promoting increased viability.

Future directions

One drawback of the current experimental strategy is that the assay for viability may be too stringent; perhaps telomere addition in this experimental system does occur, but is too inefficient to form a functional telomere and permit colony growth. Therefore molecular methods will be used to determine if telomeric sequences are indeed added onto the chromosome end. If it is determined that Cdc13 is not sufficient for telomerase recruitment, this system can easily be manipulated to include other heterologous DNA binding sites at the chromosome break site, which may then be used - in combination with the LexA binding sites - to screen for additional factors required for telomerase recruitment.

A second aspect of the Cdc13-Est1 recruiting model that I am pursuing is whether a physical interaction between Cdc13 and Est1 can be detected; two approaches will be used to examine this. Based on the observation that a Cdc13-telomerase interaction cannot be detected by co-immunoprecipitation, we have proposed the model that a Cdc13-telomerase interaction is weak or transient. However, thus far we have only performed the immunoprecipitation experiments in unsynchronized cultures. Since telomerase is thought to replicate the telomere late in S phase, perhaps a Cdc13-telomerase association can be detected in a population of cells enriched in this stage of the cell cycle. In a second approach examining this interaction, *in vitro* pull-down assays will be used to detect a direct interaction between the Cdc13 and Est1 proteins.

Note: Several fusion protein-related projects were outlined in the Future Directions part of the Year I annual report, and the majority of these projects are currently being conducted by other members of the laboratory.

Project III: The Est3-DBD_{Cdc13} fusion protein

Rationale

Work from our lab has shown that Est1 and Est3 associate with the telomerase core enzyme (Hughes et al, submitted). I have previously shown that telomerase access to the telomere can be greatly augmented by fusing the high affinity DNA binding domain of Cdc13 (DBD_{Cdc13}) to the telomerase-associated Est1 protein [6]. In those experiments, the Est1-DBD_{Cdc13} fusion, expressed under the *EST1* promoter and on a single copy plasmid, conferred substantial telomere lengthening in both wild type and *est1-Δ* strains. Since Est3, like Est1, is telomerase-associated, one would predict that fusion of the Cdc13 DNA binding domain to Est3 should similarly enhance telomerase access and consequently increase telomere length.

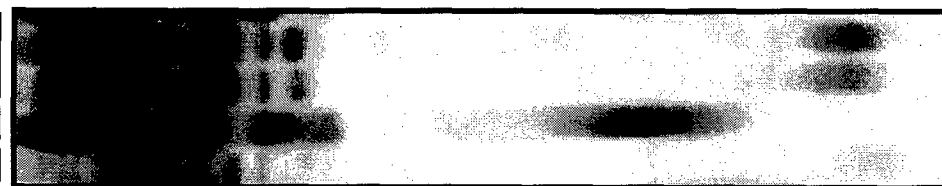
Results and Discussion

To test this, I constructed an Est3-DBD_{Cdc13} fusion expressed under the *EST3* promoter and on a single copy plasmid; this Est3 fusion protein complemented the senescence phenotype of an *est3-Δ* strain (Fig. 3B), demonstrating that Est3 function had not been abolished in the context of the fusion. Introduction of the Est3-DBD_{Cdc13} fusion protein into a wild type strain resulted in substantial telomere elongation, comparable to the lengthening conferred by the Est1-DBD_{Cdc13} fusion (Fig. 3A). Telomere elongation was further enhanced in an *est3-Δ* strain (Fig. 3A, lanes 6 and 7), presumably due to the

absence of the competing wild-type Est3 protein. This effect was dependent on the presence of an intact functional catalytic core, as the Est3-DBD_{Cdc13} fusion failed to bypass the telomere replication defect of an *est2-Δ* strain (Fig. 3B). I therefore have shown that telomerase access to the chromosome end can be greatly increased by fusing either of two telomerase-associated proteins, Est1 or Est3, to the Cdc13 DNA binding domain.

I further tested the ability of the Est3-DBD_{Cdc13} fusion to confer enzyme access in the absence of the telomerase-mediating functions of Cdc13 and Est1. Two previously described mutations, *cdc13-2^{est}* and *est1-47*, appear to specifically compromise the ability of telomerase to access the end of the chromosome. This conclusion is based in part on previous observations showing that (i) the *cdc13-2^{est}* mutant phenotype can be bypassed in a strain carrying the Est1-DBD_{Cdc13} fusion, and (ii) the telomere replication defect of the Est1-47 protein can be bypassed by fusion of Est1-47 to the DBD_{Cdc13}. Fusion of Est3 to DBD_{Cdc13} was similarly effective in bypassing the senescence phenotype of both the *cdc13-2^{est}* and *est1-47* mutations (Fig. 3C). This indicates that fusing the DBD_{Cdc13} to a telomerase-associated protein can alleviate the requirement for the telomerase accessing functions of either Cdc13 or Est1. However, although the Est3-DBD_{Cdc13} fusion bypassed the telomerase-accessing function of Est1, it was not capable of rescuing the telomere replication defect of an *est1-Δ* null strain (data not shown). An Est1-DBD_{Cdc13} fusion is similarly unable to rescue an *est3-Δ* strain (data not shown). Thus, although enzyme access can be increased when either Est1 or Est3 is fused to DBD_{Cdc13}, neither fusion is sufficient to bypass the complete function of the other protein. Therefore, the roles of these two telomerase-associated proteins do not overlap. More specifically, these results demonstrate that Est3 does not function in the telomerase recruitment role previously proposed for Est1. These results have been submitted for publication in Current Biology.

est1-Δcdc13-est2
EST1+/cdc13-est2
est1-Δ2/cdc13-est2



1 2 3 4
 — *est1-Δ* —
 — *cdc13-Δ* —

Figure 1. The *est1-42* mutant does not promote telomere elongation by the Cdc13-Est2 fusion protein. Genomic Southern blot hybridized with a telomere specific probe. Plasmids expressing the *EST1* or *est1-42* allele (or empty vector) were cotransformed with a plasmid expressing the Cdc13-Est2 fusion into an *est1-Δ cdc13-Δ/pCDC13* strain. Genomic DNA was prepared ~25 generations of growth following eviction of the *CDC13* plasmid.

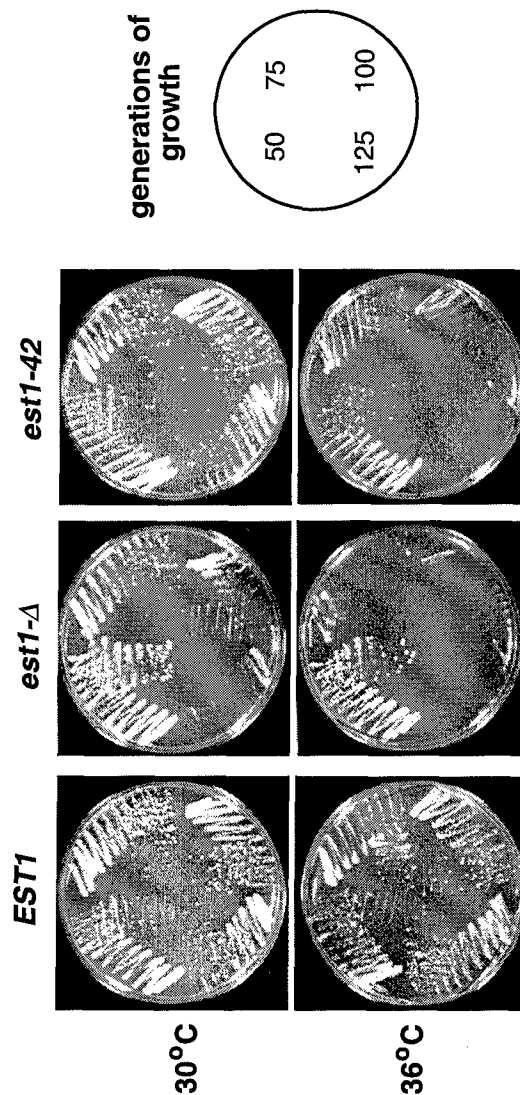


Figure 2. Conditional senescence phenotype of the *est1-42* mutant. Each plate represents an assembled set of streakouts, grown for a number of generations as depicted in the schematic to the right. The conditional senescent mutant displays near wild type growth at 30°C, but is senescent at the higher temperature.

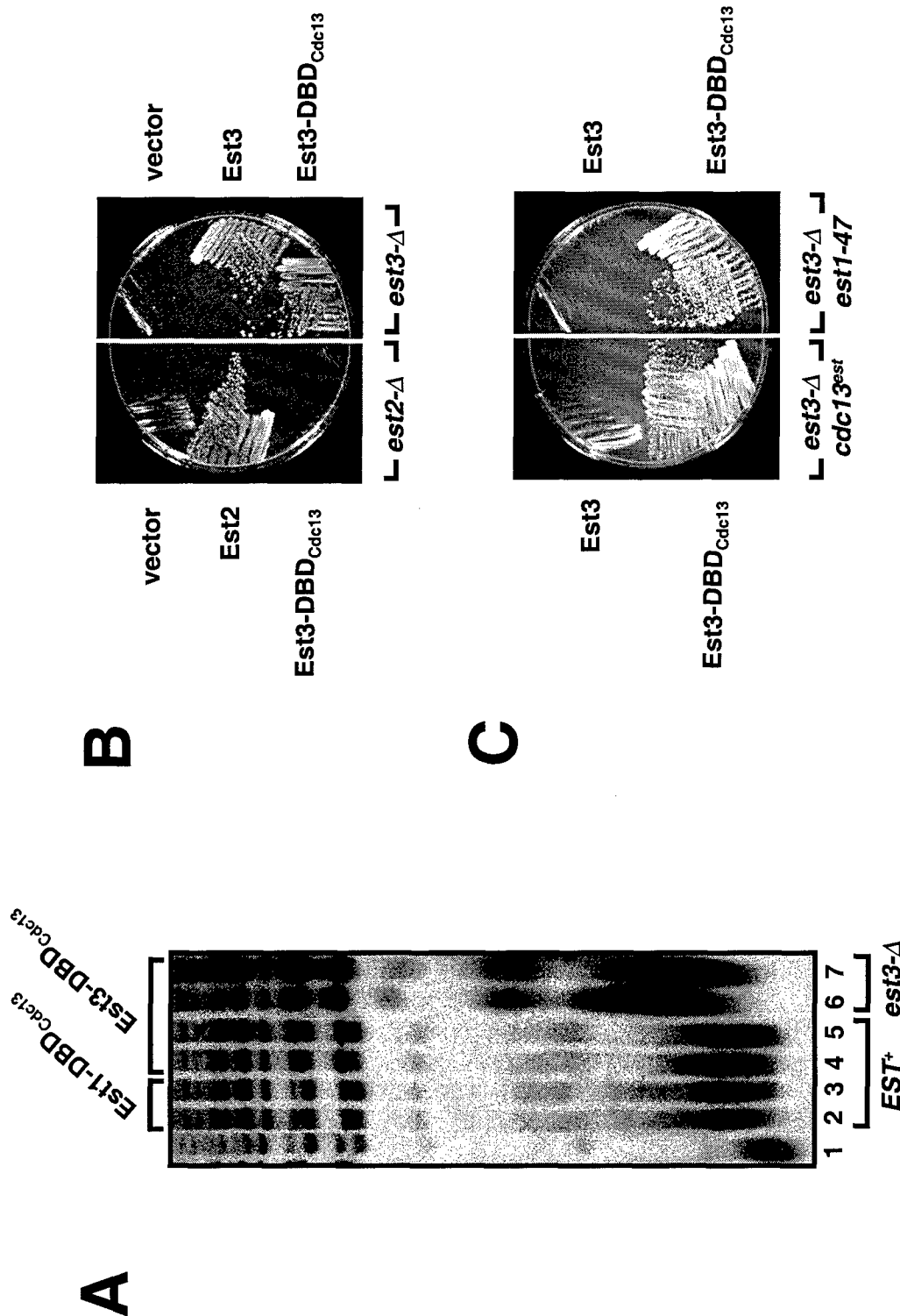


Figure 3. The Est3-DBD_{Cdc13} fusion increases access of telomerase to the chromosome terminus. (A) Genomic Southern blot performed as in Fig. 1; DNA was prepared ~ 50 generations of growth following plasmid transformation. *EST*⁺ control (lane 1); *EST*⁺/ Est1-DBD_{Cdc13} (lanes 2, 3); *EST*⁺/ Est3-DBD_{Cdc13} (lanes 4, 5); *est3*^{-Δ}/ Est3-DBD_{Cdc13} (lanes 6, 7); all plasmids were single copy CEN vectors and expressed under the native *EST* gene promoter. (B) Growth after approximately 50 generations of *est2*^{-Δ} or *est3*^{-Δ} strains harboring either vector or single copy plasmids expressing the appropriate wild type Est protein or the Est3-DBD_{Cdc13} fusion. (C) Growth after ~ 50 generations of an *est3*^{-Δ} *cdc13*^{est} strain or an *est3*^{-Δ} *est1*-47 strain with plasmids expressing the wild type Est3 protein or the Est3-DBD_{Cdc13} fusion. These strains expressing the Est3-DBD_{Cdc13} fusion have been propagated a further 75 generations with no signs of senescence.

SUMMARY APPENDICES

Key research accomplishments:

- Screened a panel of Est1 mutants for those that do not promote telomere elongation by the Cdc13-Est2 fusion, and identified one candidate mutant that may be used to further define the auxiliary role for Est1.
- Devised an experimental system to test the hypothesis that Cdc13 mediates telomerase access by recruiting the enzyme to the end of the chromosome, and tentatively assessed that Cdc13 is not sufficient for this recruiting role. This system may be expanded to screen for factors that are required in conjunction with Cdc13 for telomerase recruitment.
- Provided *in vivo* support for the role of Est3 as a subunit of telomerase through the use of Est3-DBD_{Cdc13} fusions.

Reportable outcomes:

A. Manuscripts:

1. Evans SK, Lundblad V. (1999) Est1 and Cdc13 as Co-mediators of Telomerase Access. Science 286, 117-120.
2. Hughes TR, Evans SK, Weilbaecher RG, Lundblad V. The Est3 protein is a subunit of yeast telomerase. (submitted to Current Biology).

B. Abstracts and Presentations:

Analysis of Est1 and Cdc13: Co-mediators of telomerase access
Hal Weintraub Graduate Student Award Presentation, May 5-6, 2000
Platform Presentation

Analysis of the Roles of *EST1* and *CDC13* in Telomere Replication
Era of Hope Department of Defense Breast Cancer Research Program Meeting, June 8-12, 2000
Poster Presentation

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

REFERENCES

1. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR: Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* 1997, 276:561-567.
2. Singer MS, Gottschling DE: TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* 1994, 266:404-409.
3. Lingner J, Cech TR, Hughes TR, Lundblad V: Three Ever Shorter Telomere (*EST*) genes are dispensable for in vitro yeast telomerase activity. *Proc Natl Acad Sci U S A* 1997, 94:11190-11195.
4. Virta-Pearlman V, Morris DK, Lundblad V: Est1 has the properties of a single-stranded telomere end-binding protein. *Genes Dev* 1996, 10:3094-3104.
5. Nugent CI, Hughes TR, Lue NF, Lundblad V: Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science* 1996, 274:249-252.
6. Evans SK, Lundblad V: Est1 and Cdc13 as comediators of telomerase access. *Science* 1999, 286:117-120.
7. Diede SJ, Gottschling DE: Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta. *Cell* 1999, 99:723-733.
8. Kramer KM, Haber JE: New telomeres in yeast are initiated with a highly selected subset of TG1-3 repeats. *Genes Dev* 1993, 7:2345-2356.

ANALYSIS OF THE ROLES OF *EST1* AND *CDC13* IN YEAST TELOMERE REPLICATION

Sara K. Evans and Victoria Lundblad

Department of Molecular and Human Genetics,
Baylor College of Medicine, Houston, TX 77030

se691525@bcm.tmc.edu

Telomerase is a reverse transcriptase that specifically replicates the end of the chromosome, thereby counterbalancing the terminal sequence loss that occurs due to semi-conservative replication. In *Saccharomyces cerevisiae*, *TLC1* and *EST2* encode the templating RNA and reverse transcriptase subunits of telomerase, respectively, and are required both *in vivo* for telomere replication and *in vitro* for telomerase enzyme activity. In contrast, *EST1*, *EST3* and *CDC13* are required *in vivo* for telomerase function but are dispensable for catalytic activity *in vitro*. Both Est1p and Est3p are stably associated with the telomerase enzyme. Est1 is a single-stranded telomeric DNA-binding protein, suggesting that Est1 promotes accessibility of the chromosome terminus to the active site of telomerase. Cdc13 is a high-affinity single-stranded telomeric DNA-binding protein that has an essential function at the telomere, presumably in protecting the end of the chromosome, as well as a role in telomere replication. The prior identification of a telomerase-defective allele (*cdc13^{est}*) of a telomere end-binding protein suggested a regulatory role for Cdc13 in telomere replication, perhaps in mediating the process by which telomerase interacts with chromosome termini. Thus, Est1 and Cdc13p display 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 proteins at the telomere, I have pursued two experimental approaches. The first has been a detailed site-directed mutational analysis of the Est1 protein, which has yielded three particularly informative classes of mutants. One particular allele, *est1-47*, displays a telomere replication defect, although the mutant Est1-47 protein still retains association with telomerase. One possibility for this mutant phenotype is that the Est1-47 protein is defective in its telomerase accessing function. In a second approach, I have examined the consequences of physically tethering telomerase components to the telomere as protein fusions with Cdc13p (or, alternatively, with the high affinity DNA-binding domain of Cdc13p). Strikingly, tethering Est1p, Est2p or Est3p in this manner confers a substantial telomere lengthening phenotype, suggesting that telomerase access to the terminus has been greatly increased. Furthermore, fusion proteins consisting of mutant versions of Cdc13p (*Cdc13^{est}*) or Est1p (*Est1-47*) confer similar telomere elongation, indicating that close physical proximity can bypass telomerase-defective mutations in either protein. Fusing Cdc13p directly to the catalytic subunit of telomerase (*Est2p*) allows stable telomere maintenance in the complete absence of Est1p, consistent with a role for Est1p in mediating telomerase access. These experiments support a model in which Cdc13p mediates telomerase access by a direct interaction with the enzyme. Furthermore, these data indicate that Est1p is a comediator of this accessing function.

The U.S. Army Medical Research and Material Command under DAMD 17-98-1-8020 supported this work.

Description of Thesis Work — Sara Kaye Evans

Telomerase is a reverse transcriptase that specifically replicates the end of the chromosome, thereby counterbalancing the terminal sequence loss that occurs due to semi-conservative replication. In *Saccharomyces cerevisiae*, *TLC1* and *EST2* encode the templating RNA and reverse transcriptase subunits of telomerase, respectively, and are required both *in vivo* for telomere replication and *in vitro* for telomerase enzyme activity. In contrast, Est1 is required *in vivo* for telomerase function but is dispensable for catalytic activity *in vitro*. Previous work in the Lundblad laboratory has shown that Est1 is a single-stranded telomeric DNA-binding protein that is stably associated with the telomerase enzyme. These findings suggest that Est1 promotes accessibility of the chromosome terminus to the active site of telomerase.

To define more clearly the mechanistic function of Est1, I have pursued two experimental approaches. The first has been a detailed site-directed mutational analysis of the protein, which has yielded three distinct classes of mutants. Detailed biochemical and genetic analysis of these mutants is one of the remaining goals of my thesis. One particular allele, *est1-47*, displays a telomere replication defect, although the mutant Est1-47 protein still retains association with telomerase. One possibility for this mutant phenotype is that the Est1-47 protein is defective in its telomerase accessing function.

In a second approach, I have examined the consequences of physically tethering telomerase to the telomere as a protein fusion with Cdc13. Cdc13 is a high-affinity single-stranded telomeric DNA-binding protein that has an essential function at the telomere, presumably in protecting the end of the chromosome, as well as a role in telomere replication. The prior identification of a telomerase-defective allele (*cdc13^{est}*) of a telomere end-binding protein suggested a regulatory role for Cdc13 in telomere replication, perhaps in mediating the process by which telomerase interacts with chromosome termini. Strikingly, a Cdc13-Est1 fusion confers a substantial telomere lengthening phenotype, suggesting that tethering Est1 to Cdc13 greatly increases access of telomerase to the terminus. Fusion proteins consisting of mutant versions of Cdc13 (Cdc13^{est}) or Est1 (Est1-47) confer similar telomere elongation, indicating that close physical proximity can bypass telomerase-defective mutations in either protein. Fusing Cdc13 directly to the catalytic subunit of telomerase (Est2) allows stable telomere maintenance in the complete absence of Est1, which is consistent with a role for Est1 in mediating telomerase access. These experiments support a model in which Cdc13 mediates telomerase access by a direct interaction with the enzyme (Evans and Lundblad, 1999). Furthermore, these data indicate that Est1 is a comediator of this accessing function; experiments are currently in progress to determine if Est1 is the direct binding partner of Cdc13.

In a separate set of experiments, I have shown that increased expression of *EST1*, *EST2*, and *TLC1* also causes significant telomere elongation (Hughes et al., submitted). In conjunction with the fusion experiments mentioned above, these studies suggest that telomere length homeostasis is maintained in part by restricting the access of telomerase to chromosome termini, but this limiting situation can be overcome either by directly tethering telomerase to the telomere, or by increasing the levels of functional telomerase in the cell.

Reprint Series
1 October 1999, Volume 286, pp. 117-120

Science

Est1 and Cdc13 as Comediators of Telomerase Access

Sara K. Evans and Victoria Lundblad*

Est1 and Cdc13 as Comediators of Telomerase Access

Sara K. Evans and Victoria Lundblad*

Cdc13 and Est1 are single-strand telomeric DNA binding proteins that contribute to telomere replication in the yeast *Saccharomyces cerevisiae*. Here it is shown that fusion of Cdc13 to the telomerase-associated Est1 protein results in greatly elongated telomeres. Fusion proteins consisting of mutant versions of Cdc13 or Est1 confer similar telomere elongation, indicating that close physical proximity can bypass telomerase-defective mutations in either protein. Fusing Cdc13 directly to the catalytic core of telomerase allows stable telomere maintenance in the absence of Est1, consistent with a role for Est1 in mediating telomerase access. Telomere length homeostasis therefore is maintained in part by restricting access of telomerase to chromosome termini, but this limiting situation can be overcome by directly tethering telomerase to the telomere.

In most species, telomeres are composed of G-rich repetitive sequences that are elongated by telomerase (1). Several factors govern the balance between sequence addition and loss to maintain telomeres at a stable length, including positive and negative regulation of telomerase access to the chromosome terminus (2–4). In *S. cerevisiae*, five genes are required for the telomerase pathway (4–7). *TLC1* and *EST2* encode the RNA and reverse transcriptase subunits of telomerase, respectively, and as expected for subunits that are essential for catalysis, telomerase activity is absent in extracts from strains defective in *EST2* or *TLC1* (7–9). In contrast, mutations in *EST1*, *EST3*, and *CDC13* do not eliminate enzyme activity in vitro (9, 10), despite the fact that strains carrying mutations in any of these three genes have the same severe telomere replication defect as *est2-Δ* or *tlc1-Δ* strains (6, 10).

Both Cdc13 and Est1 bind single-strand telomeric DNA (4, 11, 12), although they make separate contributions to telomere replication and stability. Est1 is required solely for the telomerase pathway (11), whereas

Cdc13 has an essential function at the telomere, presumably in protecting the end of the chromosome (13), as well as a role in telomere replication (4). This latter activity was revealed by a telomerase-defective allele of Cdc13, called *cdc13^{est}* [originally named *est4* (6)], leading to the proposal that Cdc13, like Est1, mediates telomerase access (4). The two proteins also display different biochemical properties. Est1, but not Cdc13, requires a free single-strand 3' terminus for DNA binding and binds telomeric DNA with a 500-fold reduced affinity compared with Cdc13 (4, 11). In addition, Est1 is associated with telomerase, whereas Cdc13 does not exhibit a detectable interaction with the enzyme (14).

These results suggest that telomerase is recruited to the telomere due to a direct (but weak) protein-protein interaction between Cdc13 and the enzyme, and the telomere shortening in the *cdc13^{est}* mutant strain is due to a further reduction in this interaction. This model predicts that increasing the association between Cdc13 and telomerase would increase telomere length. To test this, we examined the consequences of fusing Cdc13 to the telomerase-associated protein Est1 (15). Introduction of the gene encoding this Cdc13-Est1 fusion, present on a single-copy plasmid and expressed by the *CDC13* promoter, into a *CDC13⁺* strain resulted in substantial telomere elongation (Fig. 1A, lanes 2

Department of Molecular and Human Genetics, and Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030, USA.

*To whom correspondence should be addressed: E-mail: lundblad@bcm.tmc.edu

and 3). This effect was dependent on functional telomerase, because telomeres were not elongated when the fusion was introduced into an *est2*- Δ strain (Fig. 1A, lanes 14 to 16). Telomere elongation was even more pronounced in a *cdc13*- Δ strain, in which telomere length increased by ~ 800 base pairs after ~ 100 generations of growth (Fig. 1A, lanes 4 and 5); continued propagation resulted in even further telomere lengthening (16).

Several experiments indicated that both Cdc13 and Est1 retained function in the context of the fusion. First, the essential function of *CDC13* was fully complemented by the fusion protein (Fig. 1B). The Cdc13-Est1 fusion also complemented the senescence phenotype of an *est1*- Δ strain (Fig. 1C). This was not due to a general bypass of the telomerase pathway, because this same fusion did not complement *est2*- Δ or *est3*- Δ strains (Fig. 1C). The complementation behavior of the fusion protein indicates that Est1 and Cdc13 normally function in temporal and physical proximity in their respective roles in telomere replication. Telomerase also has been shown to coimmunoprecipitate with Est1 but not with Cdc13 (Fig. 1D) (14). The Cdc13-Est1 fusion protein coimmunoprecipitated the RNA subunit of the telomerase complex (Fig. 1D) and enzyme activity (17), indicating that telomerase is associated with the fusion protein.

These results suggest that the proposed

recruitment function of Cdc13 can be enhanced by fusing it to a telomerase component, and predict that the telomerase-defective *cdc13^{est}* mutation would be bypassed in a fusion. Consistent with this prediction, a fusion containing the mutant Cdc13^{est} protein behaved indistinguishably from the wild-type (WT) fusion: Telomere elongation occurred

to the same degree in both *CDC13⁺* and *cdc13*- Δ strains (Fig. 1A, compare lanes 6 to 9 and 2 to 5), and no senescence was observed when the Cdc13^{est}-Est1 fusion was introduced into a *cdc13*- Δ strain (17). Similar results were observed in a reciprocal experiment with a mutant allele of *EST1* (*est1-47*); this mutation disrupts telomere replication

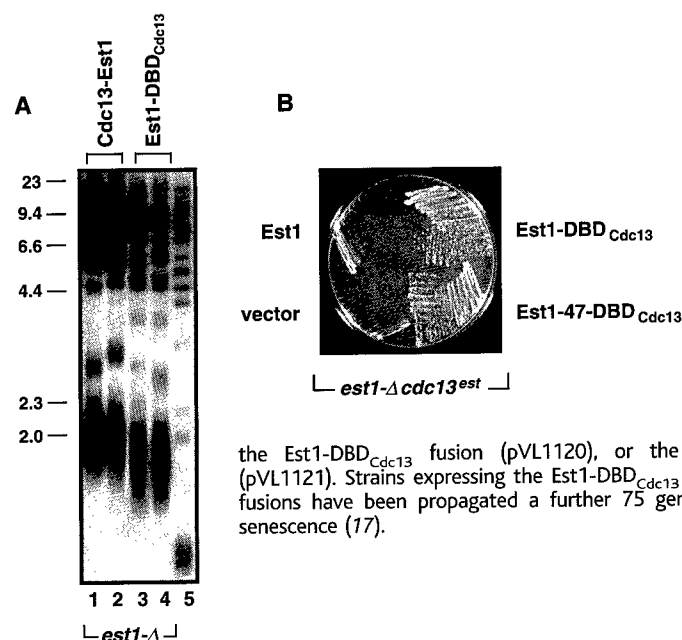
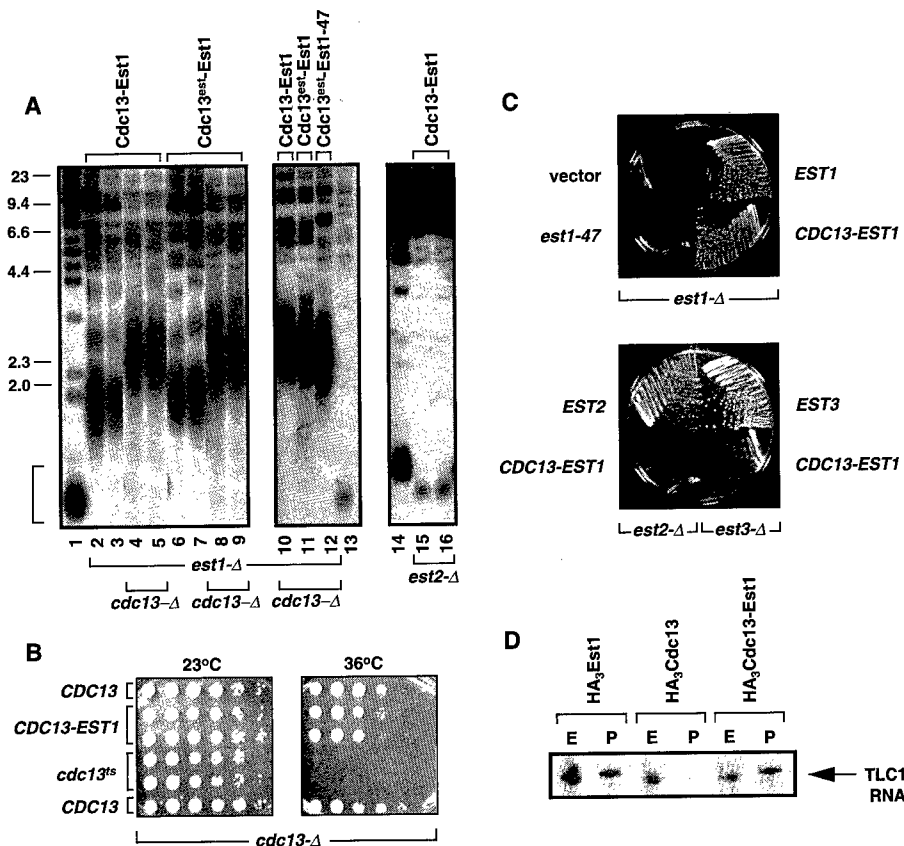


Fig. 2. The DNA binding domain of Cdc13 is sufficient to deliver telomerase to the telomere. (A) Telomere length after ~ 100 generations of growth. Lanes 1 and 2, *est1*- Δ /pVL1091; lanes 3 and 4, *est1*- Δ /pVL1120 (expressing the Est1-DBD_{Cdc13} fusion); lane 5, *EST1⁺* control. (B) Growth after ~ 50 generations of an *est1*- Δ *cdc13^{est}* strain with plasmids expressing the Est1 protein (pVL499), the Est1-DBD_{Cdc13} fusion (pVL1120), or the Est1-47-DBD_{Cdc13} fusion (pVL1121). Strains expressing the Est1-DBD_{Cdc13} and the Est1-47-DBD_{Cdc13} fusions have been propagated a further 75 generations with no signs of senescence (17).

Fig. 1. The Cdc13-Est1 fusion confers telomere elongation. (A) Telomere Southern (DNA) blots were performed as in (6). The bracket indicates a heterogeneous telomeric band that represents about two-thirds of the telomeres in this strain background. Cultures for lanes 1 to 13 were grown for ~ 100 generations before DNA preparation. Lanes 1, 13, and 14, *CDC13⁺ EST⁺* control strain; lanes 2 to 5, 10, 15, and 16, pVL1091 (expressing the Cdc13-Est1 fusion); lanes 6 to 9 and 11, pVL1092 (Cdc13^{est}-Est1); lane 12, pVL1098 (Cdc13^{est}-Est1-47); the relevant genotypes of the strains are indicated. Plasmids were introduced into strains deleted for *CDC13* for this and subsequent figures by first transforming into a *cdc13*- Δ /pVL438 (*CDC13⁺ URA3⁺*) strain followed by subsequent eviction of pVL438 by plating on media containing 5-fluoro-orotic acid. Molecular sizes are indicated on the left (in kilobase pairs). (B) *cdc13*- Δ strains containing pVL648 (*CDC13⁺*), pVL1091, or pVL762 (*cdc13^{ts}*) (13) were grown at 23°C, and equivalent numbers of cells, as serial 10-fold dilutions, were plated at 23° and 36°C. (C) Growth after ~ 50 generations of *est1*- Δ , *est2*- Δ , or *est3*- Δ strains, with single-copy plasmids bearing the *CDC13-EST1* fusion gene or the appropriate WT *EST* gene (each under their native promoter). (D) Immunoprecipitation from extracts prepared from strains expressing proteins with a triple hemagglutinin epitope (HA₃) introduced at the NH₂-terminus: HA₃Est1 (pVL1106), HA₃Cdc13 (pVL841), or HA₃Cdc13-Est1 (pVL1102), followed by detection of the telomerase RNA (TLC1) levels in the extract (E) and immunoprecipitates (P) by Northern (RNA) blotting (27).



(Fig. 1C), although the mutant Est1 protein still physically associates with telomerase (18), suggesting a defect in the same telomerase-accessing function that is altered by the *cdc13^{est}* allele. This defect was bypassed when the Est1-47 protein was fused to Cdc13. In fact, the double-mutant fusion protein (Cdc13^{est} fused to Est1-47) conferred the same degree of telomere elongation as the WT fusion protein, even in a strain deleted for both *est1-Δ* and *cdc13-Δ* (Fig. 1A, lane 12); moreover, this strain did not exhibit senescence (17). The ability of the double-

mutant fusion to complement a *cdc13-Δ est1-Δ* strain indicates that the fusion is acting as a dimeric molecule that bridges telomerase and the telomere.

One alternative interpretation of our data is that telomere elongation is due to perturbation of chromatin structure, rather than to increased access of telomerase to the telomere. In particular, telomere lengthening could be a secondary consequence of altered Cdc13 function, because mutations in *CDC13* have been identified that increase telomere length (19, 20). However, these recessive alleles of *CDC13* have a set of genetic and biochemical features that distinguish them from the gain-of-function properties of the Cdc13-Est1 fusion (17, 20). In addition, fusion of several unrelated protein sequences, or an inactive telomerase subunit (see below), to the COOH-terminus of either the WT Cdc13 protein or the mutant Cdc13^{est} protein does not increase telomere length (17, 21). The most direct argument against this alternative interpretation is the result of an experiment in which we examined the behavior of a fusion in which only the high-affinity DNA binding domain of Cdc13 (DBD_{Cdc13}) was fused to Est1 (15). This experiment was based on our previous demonstration that DBD_{Cdc13} can be expressed as a stable, functional subdomain (22) and therefore could be used as an alternative means of directing Est1 to the telomere with high efficiency, while leaving the full-length Cdc13 protein intact. As predicted, the Est1-DBD_{Cdc13} fusion conferred extensive telomere lengthening in a *CDC13⁺* strain (Fig. 2A) and bypassed senescence of a *cdc13^{est}* strain (Fig. 2B). Further-

more, fusion of the defective Est1-47 protein to DBD_{Cdc13} bypassed both *est1-Δ* and *cdc13^{est}* mutations (Fig. 2B), even though the *est1-47* allele fails to complement either mutation (Fig. 1C) (17). Expression of either Est1 or DBD_{Cdc13} had no effect on telomere length or viability in *CDC13⁺* or *cdc13^{est}* strains, and the Est1-DBD_{Cdc13} fusion failed to rescue the inviability of a *cdc13-Δ* strain (Fig. 2A) (17). Thus, the telomere lengthening properties of these fusions are likely to be a consequence of delivery of telomerase to the telomere, rather than a perturbation of Cdc13 function.

We next fused Cdc13 directly to Est2, the catalytic subunit of telomerase (8). The Cdc13-Est2 fusion (15) resulted in telomere lengthening to levels comparable to that of the Cdc13-Est1 fusion (Fig. 3A, lanes 3 and 4). The fusion complemented *cdc13-Δ* and *est2-Δ* null mutations, and telomere elongation occurred to the same degree in *est2-Δ* and *EST2⁺* strains (17). A Cdc13-Est2_{D670A} fusion, containing an Asp to Ala mutation at position 670 in the active site of Est2 (8), did not confer extensive telomere elongation but instead maintained telomere length at WT levels in a *cdc13-Δ EST2⁺* strain (Fig. 3A, lanes 5 and 6) (23), showing that telomere elongation is only observed when a catalytically active version of telomerase is tethered to the telomere. Strikingly, the Cdc13-Est2 fusion allowed cell growth in the complete absence of Est1 function, because an *est1-Δ* strain carrying this fusion was viable for more than 250 generations (Fig. 3B) (24). Long-term propagation in the absence of Est1 was not due to a previously described alternative pathway that can maintain telomeres in the absence of telomerase function (6, 25): Telomeres in an *est1-Δ* strain carrying the Cdc13-Est2 fusion were stably maintained at a length slightly below that of WT telomere length (Fig. 3C), with none of the striking changes in telomere structure that characterize the alternative pathway (6, 25). The ability of the Cdc13-Est2 fusion to maintain an *est1-Δ* strain required tethering of a functional telomerase, because an *est1-Δ* strain carrying the Cdc13-Est2_{D670A} fusion exhibited senescence (Fig. 3B). This supports the hypothesis that a critical function of the Est1 protein is to mediate access of telomerase to the telomere. Notably, neither the Cdc13-Est1 fusion nor the Cdc13-Est2 fusion bypassed the requirement for Est3 (Fig. 1C) (17), showing that Est1 and Est3 perform functionally distinct roles in telomere replication.

Our results are consistent with a model in which Cdc13 mediates telomerase access by a direct interaction with the enzyme (Fig. 4). Furthermore, these data indicate that Est1 is a comediator of this "accessing" function, potentially as a direct binding partner of Cdc13, although we cannot rule out the possibility of

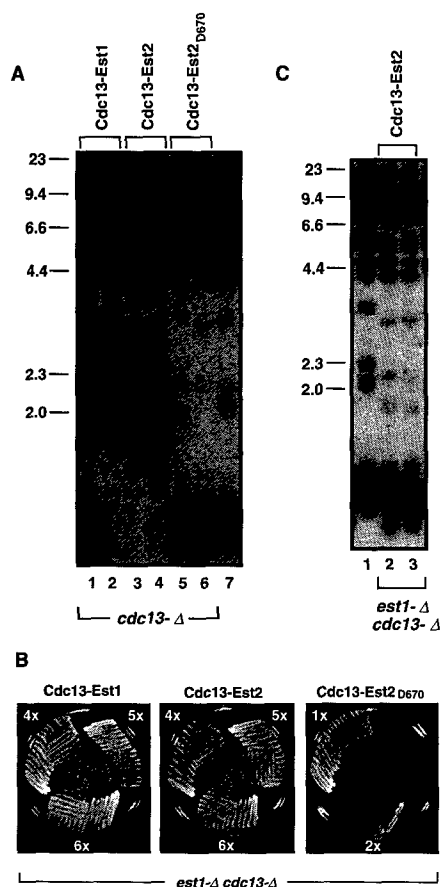


Fig. 3. Fusing Cdc13 to the catalytic subunit of telomerase bypasses the requirement for Est1. (A) Telomere length, after ~75 generations of growth. Lanes 1 and 2, *cdc13-Δ*/pVL1091; lanes 3 and 4, *cdc13-Δ*/pVL1107 (expressing the Cdc13-Est2 fusion); lanes 5 and 6, *cdc13-Δ*/pVL1111 (Cdc13-Est2_{D670A}); lane 7, *CDC13⁺* control strain. (B) Growth of an *est1-Δ cdc13-Δ* strain with either pVL1091 or pVL1107 for ~100 (4x) or ~150 (6x) generations after eviction of the *CDC13* plasmid. The *est1-Δ cdc13-Δ*/pVL1111 (Cdc13-Est2_{D670A}) strain (constructed by dissection of a *est1-Δ/EST1⁺ cdc13-Δ/CDC13⁺* diploid strain with pVL1111) is shown after ~25 and ~50 generations of growth. (C) Telomere length, after ~150 generations of growth, of a *CDC13⁺* control strain (lane 1) or an *est1-Δ cdc13-Δ*/pVL1107 strain (lanes 2 and 3). This strain has been propagated for an additional ~125 generations with no signs of senescence or changes in telomere length (17).

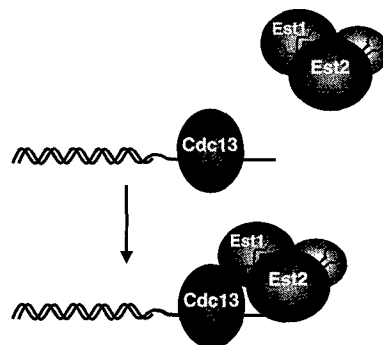


Fig. 4. Model for Cdc13 and Est1 as positive regulators of telomerase function. Cdc13 is proposed to bind the single-stranded overhang present at the ends of chromosomes and to mediate telomerase access by a direct but weak protein interaction with a component of the telomerase holoenzyme, possibly Est1. Telomerase is shown as a multisubunit RNA-containing complex that may include additional proteins such as Est3 (14). In addition to the positive regulation described here, telomeres are also subject to negative length regulation in both yeast and human cells, which has been proposed to be mediated by cis-inhibition of telomerase through the action of duplex telomere DNA binding proteins (3). Whether Est1 and Cdc13 are the direct recipients of such negative regulators is an intriguing question.

additional intervening protein or proteins. These experiments may have also uncovered an additional role for Est1 in telomerase function, as the Cdc13-Est2 fusion was not capable of promoting extensive telomere elongation in the absence of Est1 (Fig. 3C) (26). Because Est1 is a terminus-specific DNA binding protein (11), we speculate that this second role may be to promote accessibility of the 3' terminus to the active site of telomerase.

References and Notes

- C. I. Nugent and V. Lundblad, *Genes Dev.* **12**, 1073 (1998).
- J. Shampay and E. H. Blackburn, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 534 (1988).
- S. Marcand, E. Gilson, D. Shore, *Science* **275**, 986 (1997); B. van Steensel and T. de Lange, *Nature* **385**, 740 (1997); S. Marcand, V. Brevet, E. Gilson, *EMBO J.* **18**, 3509 (1999).
- C. I. Nugent, T. R. Hughes, N. F. Lue, V. Lundblad, *Science* **274**, 249 (1996).
- V. Lundblad and J. W. Szostak, *Cell* **57**, 633 (1989).
- T. S. Lendvay, D. K. Morris, J. Sah, B. Balasubramanian, V. Lundblad, *Genetics* **144**, 1399 (1996).
- M. S. Singer and D. E. Gottschling, *Science* **266**, 404 (1994).
- J. Lingner *et al.*, *ibid.* **276**, 561 (1997).
- M. Cohn and E. H. Blackburn, *ibid.* **269**, 396 (1995).
- J. Lingner, T. R. Cech, T. R. Hughes, V. Lundblad, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11190 (1997).
- V. Virta-Pearlman, D. K. Morris, V. Lundblad, *Genes Dev.* **10**, 3094 (1996).
- J. J. Lin and V. A. Zakian, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13760 (1996).
- B. Garvik, M. Carson, L. Hartwell, *Mol. Cell. Biol.* **15**, 6128 (1995).
- T. R. Hughes, S. K. Evans, R. G. Weilbaecher, V. Lundblad, in preparation.
- In-frame fusion proteins were constructed as follows: The Cdc13-Est1 fusion (pVL1091) fused Cdc13₁₋₉₂₄-Est1₆₋₆₉₉; the Cdc13-Est2 (pVL1107) and Cdc13-Est2_{D670A} (pVL1111) proteins fused Cdc13₁₋₉₂₄-Est2₁₋₈₈₄. All three constructs were expressed from the genomic *CDC13* promoter and derived from the single-copy *CEN* plasmid, pRS415. The Est1-DBD_{Cdc13} and Est1-47-DBD_{Cdc13} fusions (pVL1120 and pVL1121, respectively), expressed in single copy from the *EST1* promoter, fused the Cdc13 DNA binding domain (22) to the COOH terminus of Est1, to generate Est1₁₋₆₉₉-Cdc13_{2-21,452-693}. Telomere elongation by any of these fusion proteins is not due to increased protein expression in the context of the fusion, because overexpression of Est1, Est2, or Cdc13, or expression of Est1 by the *CDC13* promoter, has little or no effect on telomere length (8, 11, 17, 19).
- We have also observed up to a ~4-kb increase in the length of a single telomere (chromosome III_L). Strains with greatly elongated telomeres do not exhibit any discernable growth defect but were not examined for more subtle defects (such as alterations in cell cycle progression). We have not investigated yet whether the increase in telomere length in strains carrying Cdc13-telomerase fusions occurs at a constant rate, or if there is some influence of cis-inhibition on elongation, as previously observed (3).
- S. K. Evans and V. Lundblad, unpublished data.
- The *est1-47* mutation is one of a panel of alanine-scan mutations in *EST1* (17); the Est1-47 mutant protein still retains association with telomerase (at 20% of WT levels), as assessed by coimmunoprecipitation with the TLC1 RNA (27).
- N. Grandin, S. I. Reed, M. Charbonneau, *Genes Dev.* **11**, 512 (1997).
- A. Chandra, T. R. Hughes, V. Lundblad, unpublished data.
- C. I. Nugent, E. Pennock, V. Lundblad, unpublished data.
- T. R. Hughes, R. G. Weilbaecher, V. Lundblad, in preparation.
- Previous work showed that high-level expression of the Est2_{D670A} mutant protein (under the control of the *ADH* promoter, on a 2 μ high-copy plasmid) resulted in substantially shorter telomeres in an *EST2*⁺ strain (8). The lack of an effect of the Cdc13-Est2_{D670A} fusion on WT telomere length (Fig. 3A) is presumably a consequence of the lower levels of this fusion protein (confirmed by protein immunoblotting analysis), due to single-copy plasmid expression by the *CDC13* promoter. As expected, the Cdc13-Est2_{D670A} fusion failed to complement an *est2*- Δ strain.
- Bypass of *est1*- Δ senescence was not simply a consequence of increased Est2 levels (due to possible minimal increase in expression of *EST2* by the *CDC13* promoter), because even higher level expression of *EST2* by the constitutive *ADH* promoter (8) was not sufficient to allow an *est1*- Δ strain to grow (17).
- V. Lundblad and E. H. Blackburn, *Cell* **73**, 347 (1993).
- Association of Cdc13-Est2 fusion protein with the TLC1 RNA was reduced by less than twofold in the absence of Est1, as assessed by immunoprecipitation (27), arguing that the failure to elongate telomeres in an *est1*- Δ strain is not simply due to reduced stability of the Cdc13-Est2 telomerase complex.
- For each sample, cells were grown in selective media to an optical density (600 nm) of 1.0. Cells were harvested by centrifugation and the cell pellets were washed in water and then in TMG 300+ [10 mM tris-HCl (pH 8.0), 1 mM MgCl₂, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 300 mM NaCl]. Cell extracts were prepared by five repeated cycles of freezing and grinding in liquid nitrogen. Extracts were cleared twice by centrifugation for 10 min at 14,000 rpm at 4°C and immunoprecipitated with an antibody to hemagglutinin (HA) (16 β 12, Babco) and protein A/G agarose beads (Calbiochem). RNA was prepared by SDS-phenol-chloroform extraction, and TLC1 was detected on 7 M urea-4% polyacrylamide gel as described (8). The efficiency of TLC1 recovery in immunoprecipitates is typically less than 2%; the recovery with untagged proteins was less than 0.05%.
- We thank T. Hughes, R. Weilbaecher, and L. Zumstein for critical review of the manuscript. Supported by NIH grant R01GM55867 (V.L.) and by a U.S. Army Breast Cancer Research Predoctoral Traineeship (S.K.E.).

9 June 1999; accepted 30 August 1999

The Est3 protein is a subunit of yeast telomerase

Timothy R. Hughes^{1,4,5}, Sara K. Evans^{2,5}, Rodney G. Weilbaecher³ and Victoria Lundblad^{1,2,3,6}

¹Program in Cellular and Molecular Biology, and Departments of ²Biochemistry and Molecular Biology and ³Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030 USA.

⁴Present address: Rosetta Inpharmatics, Kirkland, WA 98034 USA

⁵These authors contributed equally to this work

⁶Corresponding author.

E-MAIL lundblad@bcm.tmc.edu; (713) 798-3454; FAX (713) 798-5386.

***EST1*, *EST2*, *EST3* and *TLC1* function in a single pathway for telomere replication in the yeast *Saccharomyces cerevisiae*, as would be expected if these genes all encode component of the same complex. Previous work has shown that Est2p, the reverse transcriptase protein subunit, and TLC1, the templating RNA, are subunits of the catalytic core of yeast telomerase. We show here that Est3p is a stable component of the telomerase holoenzyme and furthermore, association of Est3p with the enzyme requires an intact catalytic core. As predicted for a telomerase subunit, fusion of Est3p to the high affinity Cdc13p telomeric DNA binding domain greatly increases access of telomerase to the telomere. Like Est3p, Est1p is also tightly associated with telomerase, although in contrast to Est3p, Est1p is capable of forming a stable TLC1-containing complex even in the absence of Est2p or Est3p. Yeast telomerase therefore contains a minimum of three Est proteins for which there is both in vivo and in vitro evidence for their role in telomere replication as subunits of the telomerase complex.**

Results and Discussion

Maintenance of chromosome termini, or telomeres, requires the action of the telomerase reverse transcriptase, which counterbalances the terminal sequence loss that occurs due to incomplete semi-conservative replication. Telomere length is normally maintained at a stable set point for a given organism, indicating that there is a regulated balance between sequence loss and addition [1]. One way of modulating the extent of telomere addition is by regulating access of telomerase to the end of the chromosome through both positive and negative mechanisms [2-6]. Telomerase-associated proteins may function in such regulation. Thus, identifying and characterizing such factors is a logical starting point for developing a more complete understanding of the mechanisms of telomere length regulation.

In the budding yeast *S. cerevisiae*, the reverse transcriptase catalytic subunit and the templating RNA are encoded by the *EST2* and *TLC1* genes, respectively [7,8]. Strains deleted for either of these two genes exhibit in vivo telomere replication defects [8,9], and extracts prepared from these strains are deficient for telomerase activity [7,10]. Mutations in *EST1*, *EST3* or *CDC13* also eliminate telomere replication in vivo [5,9,11-13] but are dispensable for in vitro telomerase catalytic activity [10,14]. However, this does not rule out the possibility that one or more of these three proteins performs its role as a component of the telomerase ribonucleoprotein particle (RNP). Consistent with this premise, Est1p is a single strand telomeric DNA binding protein [15] that has been shown to associate with telomerase, both when over-expressed [16-18] and in single copy (Evans and Lundblad 1999; this work). Cdc13 also binds single strand telomeric DNA [5,19] but is not tightly associated with telomerase (Evans and Lundblad 1999; this work), suggesting that it instead is bound to chromosome termini [20]. We have recently proposed that Est1p and Cdc13p, as components of telomerase and telomeric chromatin, respectively, cooperate to directly recruit telomerase to the end of the chromosome [6].

In contrast to Est1p and Cdc13p, Est3p has not yet been biochemically characterized and thus its specific role in telomere replication is unclear. To address whether Est3p is a component of the telomerase enzyme, we examined whether it is associated with the TLC1 RNA and with enzyme activity, in parallel with Est1p, Est2p and Cdc13p. In order to avoid artifactual associations that might arise from over-expression, we constructed a set of strains containing epitope-tagged versions of each gene integrated into the respective endogenous locus under the control of the native promoter (Table 1). The identical HA₃ epitope was introduced into a region of each protein such that the activity of the tagged protein was not overtly affected, as assessed by examining the telomere length of these strains. Figure 1 shows that little or no alterations in telomere length were observed in strains bearing integrated versions of HA₃-*EST1*, HA₃-*EST2*, *EST3*-HA₃ or HA₃-*CDC13*.

Immunoprecipitates from extracts prepared from these four individually tagged strains were assayed for the presence of the relevant recombinant protein, the telomerase RNA subunit (TLC1) and telomerase activity (Fig. 2). HA₃-Est1, HA₃-Est2, and HA₃-Cdc13 proteins could be detected in the immunoprecipitates (Fig. 2A, top panel), but were not visible in the starting extracts (data not shown), due to the low levels of each of these proteins when expressed under their native promoter. The Est3-HA₃ protein co-migrates with the antibody heavy chain and therefore was masked in this particular western blot (however, see Fig. 3A for an alternative means of Est3p detection). The TLC1 RNA subunit was readily detectable in Est1p, Est2p and Est3p immunoprecipitates by northern analysis, but was not present in immunoprecipitates prepared from an untagged strain (Fig. 2A, middle panel). The interactions with TLC1 were specific for the telomerase RNP, since the U1 small nuclear RNP (snRNP) RNA was not present in each immunoprecipitate (Fig. 2A, bottom panel). In multiple repeats of this experiment in which the two RNA species were monitored quantitatively, typically 1-5% of the

telomerase RNA present in the starting extract was immunoprecipitated, whereas the more abundant U1 snRNP RNA was recovered at an efficiency of $\leq 0.02\%$.

This interaction between the Est proteins and the TLC1 RNA also reflected an association with an active telomerase complex, since enzyme activity was identified in each of the Est protein immunoprecipitates (Fig. 2B). Two related activity assays were performed using the primer substrate (5' -TGTGGTGTGTGTGGG-3'); both assays, as well as the primer, have previously been used to monitor telomerase activity from *S. cerevisiae* [7,10,14]. In the first assay, enzyme extension products were assayed in the presence of the chain-terminating ddGTP nucleotide, which results in extension of this primer by two nucleotides. This concentrates the signal in the product labeled "+2" and thus provides a sensitive measure for detection of enzyme activity [7,14]. Telomerase activity was readily observed in each Est protein immunoprecipitate using this assay (Fig. 2B, lanes 1-5); activity was RNase A-sensitive and primer-dependent in each case (data not shown). In several repetitions of this experiment, the levels of enzyme activity, which correlated directly with the levels of TLC1 that were recovered, varied by no more than 2-fold when compared among the three Est protein immunoprecipitates (data not shown), as would be predicted if these three proteins, each containing the identical HA₃ epitope, were subunits of the same complex. Enzyme activity was also monitored using a non-terminating reaction (Fig. 2B, lanes 6-10) that generates a ladder of reaction products representing the incomplete extension of a primer across the TLC1 template [10,21]. Telomerase activity (which was RNase A-sensitive; data not shown) was again detected in HA₃-Est1p and Est3p-HA₃ immunoprecipitates at roughly the same levels as observed in HA₃-Est2p immunoprecipitates (compare lanes 6 and 7 with lane 10). In both sets of assays, immunoprecipitates were subjected to relatively stringent wash conditions (0.4 M NaCl) prior to enzyme assays. Therefore, Est3p is tightly associated with an active telomerase complex, demonstrating that Est3p plays its role in telomere replication as a subunit of telomerase. These experiments also show that Est1p is a

stable component of the enzyme, confirming and extending previous observations that showed that Est1, when over-expressed, was associated with the TLC1 RNA [16-18].

In contrast, neither the TLC1 RNA subunit (Fig. 2A) nor enzyme activity (Fig. 2B) were detectable in Cdc13p immunoprecipitates performed under the same high stringency conditions. Even under less stringent conditions, telomerase activity was not detected in Cdc13p immunoprecipitates (data not shown). Thus, Cdc13p is not tightly associated with telomerase, although these observations do not exclude the possibility of a transient interaction.

To determine the requirements for interaction of Est3p with telomerase, we examined whether Est3p association with a TLC1-containing complex could be retained in the absence of either Est1p or Est2p. A GST-pulldown assay was used to determine levels of the Est3 protein in the deletion strains used in this experiment, since Est3p comigrated with the immunoglobulin heavy chain when immunoprecipitated. Deletion of *EST1* or *EST2* did not affect the level of Est3-HA₃-GST protein recovered from whole cell extracts on glutathione beads (Fig. 3A, lower panel). In addition, the steady state levels of TLC1 RNA, which were normalized to the levels of U1 RNA, were also unchanged in extracts prepared from strains that lacked either Est1p, Est2p or Est3p (Fig. 3B). When immunoprecipitated, Est3p retained association with a TLC1-containing complex in a strain deleted for *EST1* (Fig. 3A, lanes 3 and 4). However, the ability of Est3p to co-immunoprecipitate with TLC1 was abolished in the absence of the Est2p subunit (Fig. 3A, lanes 5 and 6), indicating that an intact catalytic core was a requirement for the interaction of Est3p with the telomerase complex. Whether this reflects a direct interaction between the Est2 and Est3 proteins has not yet been determined; two hybrid tests intended to detect such an interaction have been so far negative (D.K. Morris and V.L., unpublished data).

We similarly investigated the requirement for the association of Est1p with telomerase. For these experiments, we constructed a strain containing differentially tagged versions of Est1p

and Est2p (HA₃-Est1p and myc₃-Est2p) integrated into the genome and expressed under the respective native *EST1* or *EST2* promoters. Est3p is not required for the interaction of Est1p with the enzyme (Fig. 3C, lane 7), indicating that Est1p and Est3p associate with telomerase independently of each other. Unlike Est3p, however, the Est1 protein was capable of forming a TLC1-containing complex even in the absence of the catalytic Est2p subunit (Fig. 3C, lane 5), indicating that Est1p and Est2p interact with the TLC1 RNA independently of one another. Similar results for Est2p- and Est3p-independent association of Est1p with a TLC1 complex have been reported, using an over-expressed LexA-Est1 protein fusion [18].

We have previously shown that telomerase access to the telomere can be greatly augmented by fusing the high affinity DNA binding domain of Cdc13p (DBD_{Cdc13}) to the telomerase-associated protein Est1p [6]. In those experiments, the Est1-DBD_{Cdc13} fusion, expressed under the *EST1* promoter and on a single copy plasmid, conferred substantial telomere lengthening in both wild type and *est1-Δ* strains [6]. The above experiments demonstrating that Est3p, like Est1p, is telomerase-associated predict that fusion of the Cdc13 DNA binding domain to Est3p should similarly enhance telomerase access and consequently increase telomere length. To test this, we constructed an Est3-DBD_{Cdc13} fusion expressed under the *EST3* promoter and on a single copy plasmid; this Est3 fusion protein complemented the senescence phenotype of an *est3-Δ* strain (Fig. 4B), demonstrating that *EST3* function had not been abolished in the context of the fusion. Introduction of the Est3-DBD_{Cdc13} fusion protein into a wild type strain resulted in substantial telomere elongation, comparable to the lengthening conferred by the Est1-DBD_{Cdc13} fusion (Fig. 4A). Telomere elongation was further enhanced in an *est3-Δ* strain (Fig. 4A, lanes 6 and 7), presumably due to the absence of the competing wild-type Est3 protein. This effect was dependent on the presence of an intact functional catalytic core, as the Est3-DBD_{Cdc13} fusion failed to bypass the telomere replication defect of an *est2-Δ* strain (Fig. 4B). We therefore have shown that telomerase access to the chromosome end can be greatly increased by fusing either of two telomerase-associated proteins,

Est1p or Est3p, to the Cdc13 DNA binding domain. This is not a non-specific effect of the DBD_{Cdc13} but instead is a specific consequence of fusing the DBD_{Cdc13} to a telomerase subunit, since expression of either the DBD_{Cdc13} alone, or as a fusion to two other proteins implicated in telomere length maintenance (Stn1 and Pol1; [22,23]), does not result in telomere elongation (S.K.E., A. Chandra, E. Pennock and V.L., data not shown).

We further tested the ability of the Est3-DBD_{Cdc13} fusion to confer enzyme access in the absence of the telomerase-mediating functions of Cdc13p and Est1p. Two previously described mutations, *cdc13-2^{est}* and *est1-47*, appear to specifically compromise the ability of telomerase to access the end of the chromosome [6]. This conclusion is based in part on previous observations showing that (i) the *cdc13-2^{est}* mutant phenotype can be bypassed in a strain carrying the Est1-DBD_{Cdc13} fusion, and (ii) the telomere replication defect of the Est1-47 protein can be bypassed by fusion of Est1-47p to the DBD_{Cdc13} [6]. Fusion of Est3p to DBD_{Cdc13} was similarly effective in bypassing the senescence phenotype of both the *cdc13-2^{est}* and *est1-47* mutations (Fig. 4C). This indicates that fusing the DBD_{Cdc13} to a telomerase-associated protein can alleviate the requirement for the telomerase accessing functions of either Cdc13 or Est1. However, although the Est3-DBD_{Cdc13} fusion bypassed the telomerase-accessing function of Est1p, it was not capable of rescuing the telomere replication defect of an *est1-Δ* null strain (data not shown). An Est1-DBD_{Cdc13} fusion is similarly unable to rescue an *est3-Δ* strain (data not shown). Thus, although enzyme access can be increased when either Est1p or Est3p is fused to DBD_{Cdc13}, neither fusion is sufficient to bypass the complete function of the other protein. Therefore, the roles of these two telomerase-associated proteins do not overlap. More specifically, these results demonstrate that Est3p does not function in the telomerase recruitment role previously proposed for Est1p.

The demonstration that the Est3 protein is a component of telomerase completes the prediction for the functions of *EST1*, *EST2*, *EST3* and *TLC1* based on genetic observations.

Previous epistasis analysis had shown that these four genes function in a single pathway for telomere replication, as would be expected if these genes each encode subunits of the same enzyme complex [9,14]. The work presented here, combined with previous observations, shows that all four genes encode components of the telomerase enzyme. The polymerase core is generally considered to be the minimum subset of components required to perform the basic catalytic action which reflects that of the enzyme *in vivo*, whereas the holoenzyme has either additional properties or augmented activity, such as the ability to respond appropriately to different substrates. The association of the Est1 and Est3 proteins with the telomerase catalytic core, and the requirement for Est1p and Est3p *in vivo* to mediate telomerase function, mirrors the behavior of holoenzyme components of other polymerases. A role for the Est1 protein subunit has been previously proposed, as a bridging protein between telomerase and the telomere. Additional genetic and biochemical investigation should help reveal the precise biochemical activity of the Est3 telomerase protein subunit.

Materials and methods

Yeast strains and plasmids

AVL78 (*MATa leu2 trp1 ura3-52 prb prc pep4-3*) and isogenic derivatives, as described below, were used for all experiments in Figures 1, 2 and 3. Replacement of native chromosomal sequence with the epitope-tagged version was achieved by a pop-in/pop-out strategy, as described previously. Integrating constructs (derived from YIplac211) are as follows: pVL816 (*HA₃-EST1*), pVL846 (*HA₃-EST2*), pVL845 (*myc₃-EST2*), pVL901 (*EST3_{fsc}-HA₃-GST*; *EST3_{fsc}* is a frameshift corrected version of *EST3* [12]), pVL983 (*EST3_{fsc}-HA₃-HMK-His₆*), pVL842 (*HA₃-CDC13*). The *HA₃* tag was the same for all these constructs [(YPYDVDPDYA)₂RSMYPYDVDPDYA] and was inserted in frame at the amino terminus of Est1p (between aa 1 and 2), Est2p (between aa 11 and 12) or Cdc13p (between aa 1 and 2) or at the carboxy terminus of Est3p (after the terminal aa). The *myc₃* tag [(EQKLISEEDL)₃] was inserted at the amino terminus of Est2p (between aa 11 and 12). In each case, insertion of the DNA sequence encoding the epitope tag introduced a few additional amino acids, which are not indicated. Deletion derivatives for the experiments in Fig. 3 were made by one-step gene disruption directly into tagged haploid strains, and cultures were grown from freshly disrupted strains; these deletion strains were not permanently stored due to their senescent characteristics. *EST1* was disrupted with a kanamycin cassette derived by PCR of plasmid pFA6a.kanMX2 [24] which removed 89% of the *EST1* coding region. *EST2* was disrupted with pVL363 (*est2-Δ1::URA3*, which removed 76% of the coding sequence) or by PCR-mediated gene deletion (*est2-Δ2::kan*, derived from pFA6a.kanMX2, which removed the entire *EST2* coding region, 51 bases of upstream sequence, and 49 bases of downstream sequence). *EST3* was disrupted with pVL418 (*est3-Δ2::URA3*, which removed 63% of the coding sequence).

Haploid strains for Figure 4 were generated from dissection of diploid strains DVL172 (to generate *est2-Δ*), DVL322 (to generate *est3-Δ* and *est1-Δ est3-Δ*) and DVL323 (to generate

est3-Δ cdc13-2^{est}). Plasmids used in these experiments expressed each gene on a single copy plasmid under the appropriate *EST* gene native promoter: pVL499 (Est1), pVL1293 (Est1-47), pVL1048 (Est2), pVL1005 (Est3_{fsc}-HA₃-HMK-His₆), pVL1120 (Est1-DBD_{Cdc13}), pVL1292 (Est3_{fsc}-HA₃-DBD_{Cdc13}). pVL1292 was constructed by inserting the DBD of Cdc13p (aa 2-19, 452-693) in frame at the end of Est3_{fsc}-HA₃.

Immunoprecipitations

For western and northern analysis, extracts were prepared by lysis with glass beads essentially as previously described [14], with the exception that extracts were prepared in TMG (10 mM Tris-HCl pH 8.0, 1 mM MgCl₂, 5% glycerol) plus 300 mM NaCl, 0.5% Tween-20, 1 mM PMSF, 1 u/ml RNase inhibitor (RNasin, Promega), and 0.1 mM DTT, and immunoprecipitates were washed three times in TMG plus 300 mM NaCl and once in TMG. For telomerase activity assays, cultures were grown at 30°C in YPD and harvested at an OD₆₀₀ of 1.0. All subsequent steps were performed at 4°C. The cell pellet was washed once in water, once in TMG plus 300 mM NaCl, and resuspended in 2 pellet volumes TMG plus 300 mM NaCl, 0.5 mM DTT, and 0.25 mM PMSF. Cells were frozen in liquid nitrogen and homogenized with a mortar and pestle. RNasin (40 units / ml) and Tween-20 (0.5% final concentration) were added to the crude extract before clearing twice by centrifugation for 15 minutes at 14,000 rpm. ~2.5 mg extract in 0.5 ml was incubated for 45 min with 1 µl anti-HA high affinity antibody (Boehringer Mannheim). 20 µl settled volume Protein G Plus / Protein A Agarose was added and incubated for an additional 90 min. After gentle pulse centrifugation, the beads were washed 3 times with TMG plus 400 mM NaCl, 0.5% Tween-20, 0.5 mM DTT, 40 units/ml RNasin, one time with TMG plus 50 mM NaCl, 0.5 mM DTT, 40 units/ml RNasin, and finally resuspended with an equal volume of the latter buffer. Telomerase activity recovered by this method retains at least 50% activity following a freeze-thaw cycle with storage at -70°C. Telomerase activity was assayed by incubating 5 µl of the agarose bead slurry containing HA-

immune complexes with 5 μ l of a 2x reaction mixture containing 5 μ M substrate oligonucleotide [5' -TGTGGTGTGTGTGGG-3'], 80 mM Tris-Cl (pH 8.0), 100 mM NaCl, 10% glycerol, 5 mM MgCl₂, 1 mM spermidine, 1 mM DTT, and either 200 μ M TTP and 1.5 μ l [α^{32} -P] dGTP (3000 Ci/mmol), or 800 μ M ddGTP and 1.5 μ l [α^{32} -P] TTP (3000 Ci/mmol). The reaction proceeded for 20 minutes at 30°C and was stopped by the addition of EDTA to 25 mM. Reaction products were extracted with phenol/chloroform, ethanol precipitated and resolved in a 15% acrylamide / 7 M urea sequencing gel.

Acknowledgements

We thank Ted Wensel, Chris Cowan and members of the Lundblad lab for helpful discussions and advice. This work was supported by a U.S. Army Medical Research and Materiel Command Breast Cancer Research Predoctoral Fellowship to S.K.E., a National Institutes of Health NRSA fellowship to R.G.W. and a National Institutes of Health grant AG11728 and an SRA from Geron Corporation to V.L.

References

1. Shampay J, Blackburn EH: **Generation of telomere-length heterogeneity in *Saccharomyces cerevisiae*.** *Proc Natl Acad Sci U S A* 1988, **85**:534-538.
2. Marcand S, Gilson E, Shore D: **A protein-counting mechanism for telomere length regulation in yeast.** *Science* 1997, **275**:986-990.
3. Marcand S, Brevet V, Gilson E: **Progressive *cis*-inhibition of telomerase upon telomere elongation.** *EMBO J* 1999, **18**:3509-3519.
4. van Steensel B, de Lange T: **Control of telomere length by the human telomeric protein TRF1.** *Nature* 1997, **385**:740-743.
5. Nugent CI, Hughes TR, Lue NF, Lundblad V: **Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance.** *Science* 1996, **274**:249-252.
6. Evans SK, Lundblad V: **Est1 and Cdc13 as comediators of telomerase access.** *Science* 1999, **286**:117-120.
7. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR: **Reverse transcriptase motifs in the catalytic subunit of telomerase.** *Science* 1997, **276**:561-567.
8. Singer MS, Gottschling DE: **TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase.** *Science* 1994, **266**:404-409.
9. Lendvay TS, Morris DK, Sah J, Balasubramanian B, Lundblad V: **Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional *EST* genes.** *Genetics* 1996, **144**:1399-1412.
10. Cohn M, Blackburn EH: **Telomerase in yeast.** *Science* 1995, **269**:396-400.
11. Lundblad V, Szostak JW: **A mutant with a defect in telomere elongation leads to senescence in yeast.** *Cell* 1989, **57**:633-643.

12. Morris DK, Lundblad V: **Programmed translational frameshifting in a gene required for yeast telomere replication.** *Curr Biol* 1997, **7**:969-976.
13. Diede SJ, Gottschling DE: **Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta.** *Cell* 1999, **99**:723-733.
14. Lingner J, Cech TR, Hughes TR, Lundblad V: **Three Ever Shorter Telomere (*EST*) genes are dispensable for in vitro yeast telomerase activity.** *Proc Natl Acad Sci U S A* 1997, **94**:11190-11195.
15. Virta-Pearlman V, Morris DK, Lundblad V: **Est1 has the properties of a single-stranded telomere end-binding protein.** *Genes Dev* 1996, **10**:3094-3104.
16. Lin JJ, Zakian VA: **An in vitro assay for *Saccharomyces* telomerase requires *EST1*.** *Cell* 1995, **81**:1127-1135.
17. Steiner BR, Hidaka K, Futcher B: **Association of the Est1 protein with telomerase activity in yeast.** *Proc Natl Acad Sci U S A* 1996, **93**:2817-2821.
18. Zhou J, Hidaka K, Futcher B: **The Est1 subunit of yeast telomerase binds the Tlc1 telomerase RNA.** *Mol Cell Biol* 2000, **20**:1947-1955.
19. Lin JJ, Zakian VA: **The *Saccharomyces* CDC13 protein is a single-strand TG₁₋₃ telomeric DNA-binding protein in vitro that affects telomere behavior in vivo.** *Proc Natl Acad Sci U S A* 1996, **93**:13760-13765.
20. Bourns BD, Alexander MK, Smith AM, Zakian VA: **Sir proteins, Rif proteins, and Cdc13p bind *Saccharomyces* telomeres in vivo.** *Mol Cell Biol* 1998, **18**:5600-8.
21. Prescott J, Blackburn EH: **Telomerase RNA mutations in *Saccharomyces cerevisiae* alter telomerase action and reveal nonprocessivity in vivo and in vitro.** *Genes Dev* 1997, **11**:528-540.
22. Grandin N, Reed SI, Charbonneau M: **Stn1, a new *Saccharomyces cerevisiae* protein, is implicated in telomere size regulation in association with Cdc13.** *Genes Dev* 1997, **11**:512-527.

23. Adams AK, Holm C: **Specific DNA replication mutations affect telomere length in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1996, **16**:4614-4620.
24. Wach A, Brachat A, Pohlmann R, Philippsen P: **New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*.** *Yeast* 1994, **10**:1793-808.

Figure 1. Addition of epitope tags to *EST1*, *EST2*, *EST3*, and *CDC13* has a minimal effect on telomere length. Genomic DNA was prepared from strains carrying integrated, epitope tagged versions of each gene as indicated, and subjected to Southern blotting and hybridization with a telomere-specific probe as described previously [9]. Telomeric restriction fragments are indicated by arrows and a bracket. Each pair of lanes is derived from cells grown approximately 70 and 100 generations following replacement of the wild-type allele. Strains are as follows: untagged (AVL78; lanes 1, 2, 11, 12); HA₃-Est1 (TVL288; lanes 3, 4); HA₃-Est2 (TVL292; lanes 5, 6); Est3-HA₃-GST (TVL293; lanes 7, 8); HA₃-Cdc13 (TVL290; lanes 9, 10).

Figure 2. Est1, Est2 and Est3 proteins associate with an active telomerase enzyme complex. (A) Extracts from strains, each bearing an HA₃ epitope tag on a single gene as indicated, were immunoprecipitated and analyzed by western blotting to detect HA tagged protein (*top*) and northern blotting to detect TLC1 RNA (*middle*) or U1 RNA (*bottom*). HA₃-Est1 (TVL288; lane 2); HA₃-Est2 (TVL292; lane 3); Est3-HA₃-GST (TVL293; lane 4); HA₃-Cdc13 (TVL290; lane 5); untagged (AVL78; lane 6). TLC1 and U1 RNA levels in crude extracts prepared from an untagged strain, prior to immunoprecipitation, are shown in lane 1; the amount of extract represents 4% of the amount loaded in the IP lanes. Lanes 2 - 6 are equivalently loaded, as assessed by total protein. The Est3-HA₃-GST polypeptide co-migrates with the antibody heavy chain (which is not shown) and is not detectable on this particular blot. (B) Immunoprecipitates from each tagged strain were assayed for telomerase activity using either a chain terminating reaction in the presence of ddGTP (lanes 1 - 5; the band representing the +2 product is indicated) or non-chain terminating reaction (lanes 6 - 10). The strains used in this experiment are identical to those used in (A), with the exception of the Est3-HA₃ strain (TVL307).

Figure 3. The association of Est3p with the telomerase RNP requires an intact catalytic core.

(A, *top*) Extracts prepared from an Est3-HA₃-GST strain (TVL293) or deletion derivatives of TVL293 were immunoprecipitated, and TLC1 RNA was detected by northern blotting. TVL293

(lanes 1, 2); *est1*- Δ derivative of TVL293 (lanes 3, 4); *est2*- Δ derivative of TVL293 (lanes 5, 6); untagged (AVL78; lanes 7, 8). (Bottom) Extracts were purified on glutathione sepharose and analyzed by western blotting with an antibody to detect the HA₃-tagged Est3 protein.

(B) Extracts prepared from *est1*- Δ (lane 2), *est2*- Δ (lane 3), *est3*- Δ (lane 4) and *tlc1*- Δ strains (lane 5), in parallel with an *EST*⁺ strain (lane 1), were analyzed by northern blotting to detect TLC1 RNA and U1 RNA, followed by quantitation of the relative RNA levels by PhosphorImager analysis. The abundance of TLC1 RNA was reduced by no more than three-fold in the three *EST* deletion strains, relative to the ratio of the two RNAs in a wild type strain.

(C) Extracts from TVL300 (containing HA₃-Est1p and myc₃-Est2p) or deletion derivatives of strain TVL300 were immunoprecipitated with either anti-myc or anti-HA antibodies and analyzed by northern blotting to detect *TLC1* RNA. TVL300 (lanes 1, 2); *est1*- Δ derivative of TVL300 (lanes 3, 4); *est2*- Δ derivative of TVL300 (lanes 5, 6); *est3*- Δ derivative of TVL300 (lanes 7, 8); *tlc1*- Δ derivative of TVL300 (lanes 9, 10); untagged (AVL78; lanes 11, 12). The variations in TLC1 signal in HA versus myc immunoprecipitations are due, at least in part, to the fact that the myc₃ tag is a more efficiently immunoprecipitated epitope, as myc₃-Est2p co-immunoprecipitates more telomerase RNA than HA₃-Est2p (data not shown).

Figure 4. The Est3-DBD_{Cdc13} fusion increases access of telomerase to the chromosome terminus. (A) Genomic Southern blot performed as in Fig. 1; DNA was prepared ~ 50 generations of growth following plasmid transformation. *EST*⁺ control (lane 1); *EST*⁺/pVL1120 (Est1-DBD_{Cdc13}; lanes 2, 3); *EST*⁺/pVL1292 (Est3-DBD_{Cdc13}; lanes 4, 5); *est3*- Δ /pVL1292 (Est3-DBD_{Cdc13}; lanes 6, 7); all plasmids were single copy CEN vectors and expressed under the native *EST* gene promoter. (B) Growth after approximately 50 generations of *est2*- Δ or *est3*- Δ strains harboring either vector or single copy plasmids expressing the appropriate wild type Est protein or the Est3-DBD_{Cdc13} fusion. (C) Growth after ~ 50 generations of an *est3*- Δ *cdc13*^{est} strain or an *est3*- Δ *est1*-47 strain with plasmids expressing the wild type Est3 protein or

the Est3-DBD_{Cdc13} fusion. These strains expressing the Est3-DBD_{Cdc13} fusion have been propagated a further 75 generations with no signs of senescence.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

Rec'd
7/23/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

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

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management