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13. ABSTRACT ***(Maximum 200 Words)***

Telomeres, specialized structures at the ends of chromosomes, help maintain the stability of the genome and are essential for continued cell proliferation. The purpose of this research has been to understand the processes involved in maintaining the length and integrity of chromosome ends using the yeast *S. cerevisiae* as a model system. This work has helped to define three genetic pathways that are critical for telomere function and replication; one defined by the Ku complex, a second comprises telomerase and *MRE11*, *RAD50*, and *XRS2*, and the third identified by *CDC13* and *STN1*. Although chromosome ends must be functionally distinct from DNA double strand breaks, genes involved in DNA double-strand break repair were found to play critical roles in two of these genetic pathways for maintaining telomeres. Characterization of Cdc13p, a telomeric single-stranded binding protein, revealed that it may be required at telomeres in a cell-cycle specific manner, dependent upon active DNA replication. Intriguingly, this analysis also suggested that Cdc13p may also function elsewhere in the genome. To further unravel how telomerase activity is regulated, a number of telomerase interacting proteins were also identified. These proteins may be components or regulators of the telomerase holoenzyme.

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Constance Nugent 7/30/2000

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RESEARCH SUMMARY

INTRODUCTION

The ability to preserve the stability of the genome is crucial for the successful propagation of an organism. Maintaining linear chromosomes poses an intrinsic challenge for the cell; the chromosome ends must be functionally distinct from DNA double-strand breaks, and in highly proliferative cells such germ cells, the terminal sequences need to be duplicated without a loss of genetic material. The enzymes that replicate the bulk of the genome are not capable of fully duplicating the ends of the chromosomes. Therefore, without a mechanism to replenish these sequences, the chromosomes will inexorably shorten through replicative cycles. The telomeres of most eukaryotes examined are composed of short, repetitive GT/CA sequence tracts. The enyzme telomerase, an RNA-dependent DNA polymerase, extends the GT repeat unit to allow complete terminal replication. For multicellular organisms, controlling telomere length is thus one potential means to limit the number of times a cell can divide. It has been proposed that for either normal or transformed cells to proliferate indefinitely, the cells must stabilize their chromosome ends. My research has been aimed at investigating the processes involved in maintaining the length and integrity of chromosome ends using the yeast *S. cerevisiae* as a model system. The mechanisms that yeast cells employ to regulate telomere length and suppress cellular senescence may be similar to those utilized by cancer cells to override their normally finite lifespan and continue unregulated proliferation. The long-term objective of this proposal is to further elucidate the role of telomere length regulation in replicative senescence and in cellular immortalization.

BODY

Background and Overview

The specific goals of this grant were to use genetic means to find genes that are important for mediating telomere function, and, through analysis of these genes, to begin to determine how telomere replication is regulated in yeast. The approach for identification of these genes was crafted through screens aimed at determination of the genes and processes implicated with *CDC13* in maintaining telomere function. Genetic and biochemical analysis has suggested that the Cdc13 protein is critical for telomere replication and length regulation. *CDC13* is a gene essential for cell viability. Analysis of the *cdc13-1*^{ts} allele has revealed that when Cdc13p is absent, telomere proximal regions become extensively single-stranded (Garvik et al. 1995). This suggests either that the replication of the chromosome ends is crippled, unable to properly complete lagging strand synthesis, or that the ends are subject to degradation by nucleases. The *cdc13-2*^{est} allele displays a phenotype similar to that of a telomerase-defective strain, although the level of telomerase activity *in vitro* is normal (Nugent et al. 1996). *In vitro*, Cdc13p binds preferentially to single-stranded G-rich oligomers (Nugent et al. 1996, Lin and Zakian 1996). Thus, Cdc13p appears to be a single-stranded telomere binding protein with a function critical for maintaining proper telomere length and integrity.

Identification and analysis of genetic pathways required for telomere maintenance

One approach that I undertook was to analyze mutations synthetically lethal with *cdc13-1^{ts}*. Epistasis analysis revealed that genes encoding telomerase components, the Ku complex and Cdc13p each appear to contribute separate roles required for telomere replication and cell viability. Interestingly, this work also suggested that *MRE11*, *RAD50* and *XRS2*, a set of genes required with the Ku complex for DNA repair via non-homologous end-joining, are in the genetic pathway for telomere replication via telomerase. This data was described in my 1997-1998 annual report and have been published in Current Biology (Nugent et al. 1998). These experiments accomplished the goals stated for my second objective. Human homologs of the *YKU70*, *YKU80*, *MRE11*, and *RAD50* have already been identified. The functional homolog of *XRS2* is likely to be the human Nbs1 gene (Carney et al. 1998). In the future it will be interesting to further explore the role of these genes in maintaining telomeric function in humans. Understanding how these complexes function at telomeres as well as at internal sites of DNA double-strand breaks could help elucidate how cells distinguish telomeres from damaged DNA that is in need of repair.

Identification and analysis of Cdc13p-interacting proteins

A second method used to identify genes involved with *CDC13* in maintaining telomere function was a two-hybrid assay for Cdc13p-interacting proteins. This screen identified Stn1p, a protein that participates with Cdc13p in regulating telomere replication and in maintaining genome integrity (Grandin et al. 1997). The experiments undertaken for this screen were described in my 1997-1998 annual report, and addressed tasks one to five as described for the grant's first objective. Genetic and biochemical analysis of both *CDC13* and *STN1* were continued to meet the objectives of task five, and the beginning of that analysis was described in the 1998-1999 annual report. Experiments completed in the past year have been directed at further characterization of both the *in vivo* regulation of Cdc13p and the interaction of Cdc13p and Stn1p; this data is detailed in the next section. Currently, homologs of neither Cdc13p nor Stn1p have been identified in any organism. Since these genes are both essential for growth in *S. cerevisiae* and have profound effects on telomere function, it will be important to continue pursuit of the identification of functional homologs of these genes in higher eukaryotes.

Identification and analysis of telomerase-associated proteins

An objective of this grant was to identify additional telomerase components; obviously, analysis of these genes would be particularly important for evaluating regulation of telomerase activity. Thus, I undertook a two-hybrid analysis using the catalytic component of yeast telomerase, *EST2*, as my "bait" to find interacting proteins. This screen was expected to identify potential telomerase components, regulators, or other proteins critical for telomere replication. The initial portion of this screen was described in the 1997-1998 annual report. The screen has been completed, and has led to the identification of a number of genes that potentially have important roles in regulating telomerase activity (see Table 1 for summary). Interestingly, the second protein identified from the Cdc13 two-hybrid screen, *SBA1* or *YKL117w*, was observed to also have a two-hybrid interaction with Est2. The human homolog of this protein, p23, has now been shown to associate with telomerase in human cells as well as in reticulolysates (Holt et al. 1999). Since p23 associates with Hsp70 and Hsp90, it has been proposed to function as a chaperone for telomerase (Holt et al. 1999). If *SBA1* is essential for activities of either telomerase or Cdc13p *in vivo*, it must be redundant with other genes as *sba1* null mutations have no detectable phenotype that is suggestive of a role in telomere maintenance (see 1997-1998

annual report). Further analysis of the genes identified in this screen will be a subject of my future research.

Experimental Summary for 7 / 1999 - 6 / 2000

Cdc13p association with telomeres is cell cycle dependent

In the 1998-1999 annual report, data from chromatin-immunoprecipitation experiments was presented showing that Cdc13p crosslinks with telomeric DNA during S phase of the cell cycle, suggesting that Cdc13p can bind telomeric DNA *in vivo* and this binding is cell-cycle dependent. Further data demonstrated that Cdc13p may not associate exclusively with telomeres, but may also associate with at least some centromeric regions in a cell cycle dependent manner. Experiments in the past year have been directed toward further understanding the regulation of the association of Cdc13p with DNA.

Cdc13p function is required during S phase of the cell cycle.

Different models for *CDC13* function make predictions for when its activity is required in the cell cycle. A role in protecting chromosome ends from degradation might be expected to be required throughout the cell cycle whereas a role in telomere replication would be expected to be required during S phase. To determine when *CDC13* is required during the cell cycle an execution point experiment was done. Loss of *CDC13* function has been shown to activate the DNA damage checkpoint response, resulting in cell cycle arrest at G2/M. While bulk DNA replication appears to be complete at the *cdc13-1^{ts}* arrest, single-stranded DNA can be detected at telomere proximal regions (Garvik et al. 1995, Lydall and Weinert 1995); it has been postulated that this single-stranded DNA activates the checkpoint response (Garvik et al. 1995, Lydall and Weinert 1995).

To assess when *CDC13* function is required, *cdc13-1^{ts}* cells were shifted to restrictive temperature while blocked at various stages in the cell cycle, and then released from the arrest at permissive temperature. If *CDC13* function is required to prevent DNA damage during or just following the arrest, one would expect to observe a delay in the ability of the cells to proceed through the cell cycle once they are released at permissive temperature. After shifting cells back to permissive temperature, a short period of time may be required to refold or resynthesize functional protein. As shown in Figure 1, the *cdc13-1^{ts}* cells that were shifted to restrictive temperature while arrested in S phase with hydroxyurea experienced a substantial delay (at least 2.5 hours) in cell cycle progression relative to cells maintained at permissive temperature. A proportion of *cdc13-1^{ts}* cells that were arrested in G1 also showed a delay in their progression through the cell cycle after the temperature shift, but this delay was of much different magnitude, approximately 20-40 minutes. *cdc13-1^{ts}* cells that were shifted to restrictive temperature while arrested in G2/M by nocodazole treatment did not show an alteration in cell cycle kinetics, suggesting that threshold levels of DNA damage were not created in the absence of *CDC13* during or after the arrest. This data is most consistent with a requirement for *CDC13* function during S phase progression. Taken together, these results are most consistent with a requirement for *CDC13* just prior to and during replicative processes, rather than with a requirement throughout the cell cycle as a block to degradation of the DNA from ends.

Cdc13p chromatin association is sensitive to HU in a checkpoint dependent manner

As previously reported (annual report 1998-1999), chromatin immunoprecipitation experiments revealed that Cdc13p is maximally associated with telomeric or centromeric regions in S phase of the cell cycle. To further examine the cell cycle dependency of this crosslinking, I observed that treatment of cells with hydroxyurea (HU), a drug that inhibits progression through S-phase, abrogates association of both telomeric and *CENIII* regions with Cdc13p (Figure 2 and data not shown). The inhibition of crosslinking by hydroxyurea contrasts with the association detected in progression through an unperturbed S phase. It has been shown that treatment of *S. cerevisiae* with HU results in the prevention of late origin firing through S-phase checkpoint genes such as *MEC1* and *RAD53* (Santocanale and Diffley 1998, Shirahige et al. 1998). Removal of the checkpoint pathway through mutation of either of these genes relieves the block to late origin firing in the presence of HU (Santocanale and Diffley 1998, Shirahige et al. 1998), and also permits Cdc13p association with telomeric (and *CENIII*) regions (Figure 3). Thus, the HU sensitivity of the Cdc13p association with telomeres, regions known to replicate late, is consistent with a role for Cdc13p at telomeres during, or just subsequent to, the process of DNA replication. Two models for Cdc13p function could be consistent with this finding. The first is that Cdc13p is enriched at telomeres during the process of DNA replication, and associates with single-stranded telomeric DNA, possibly to aid the lagging strand replication machinery. Alternatively, if Cdc13p associates with telomeric regions solely for fill-in synthesis after bulk DNA replication and telomerase extension are complete, then the HU sensitivity may reflect inhibition of these processes at a point before Cdc13p acts. The first model would suggest a possible general role for Cdc13p in replicative processes, at least in certain chromatin contexts. The latter model would suggest a telomere specific function for Cdc13p, and would need to be expanded to account for its observed centromeric association and the fact that *CDC13* is an essential gene.

Genetic regulation of Cdc13p telomeric association

The different mutant alleles of *CDC13* provide an opportunity to further test models for Cdc13p function at telomeres. The alleles with short telomere mutant phenotypes (*cdc13-2*^{est} and *cdc13-8*, an allele created through site-directed mutagenesis) would be predicted to be capable of telomere association but might show defective regulation of their chromatin association through the cell cycle. Within the limits of the chromatin IP assay, the data suggests that these mutant proteins associate with telomeric DNA to a similar extent and timing compared with wild-type protein (Figure 4). Thus, if these alleles are defective for an interaction responsible for regulating their chromatin association, this assay did not detect it. The *cdc13-1*^{ts} allele would be predicted to be defective for crosslinking with any genomic region at high temperature since it is thought that the mutation destabilizes the protein at high temperature. Since the protein is likely to be absent, and the epitope tag further disrupts the function of the mutant protein, this experiment has not been done.

To further understand what factors regulate Cdc13p DNA binding *in vivo*, the crosslinking of Cdc13p was examined in *YKU80* and *stn1-13* mutant backgrounds. Null mutants of yku80 have been shown to have short telomeres that terminate with a single-stranded tract of telomeric ($G_{1\text{-}3}T$) DNA (Gravel et al. 1998). This single-stranded region has been observed in cells from all stages of the cell cycle, and does persist in cells treated with hydroxyurea (Gravel et al. 1998). *Stn1-13* mutant cells contain telomeres that are elongated, with significant single-stranded nature as well (Grandin et al. 1997, Nugent, unpublished data). In both mutant

backgrounds the extent of Cdc13p crosslinking with telomeric DNA was increased (Figure 5). Interestingly, the extent of crosslinking also increased at centromere III and IV in the *stn1-13* mutant background; this effect was not general since other loci tested did not show a similar increase in crosslinking (data not shown). Thus, *YKU80* and *STN1* are formally negative regulators of Cdc13p telomere association. This effect could be indirect, mediated through the increase in telomeric single-strandedness present in the mutant cells. It remains to be determined what effect the *stn1* mutation has upon centromeric regions. The data relating to the analysis of the cell-cycle dependent telomeric association of Cdc13p is currently being assembled into a manuscript for submission.

Analysis of STN1

STN1 was identified in the two-hybrid screen for Cdc13 interacting proteins (see 1997-1998 report). It is an essential gene, and was originally identified in a screen for low copy suppressors of the temperature sensitivity of *cdc13-1*^{ts} cells (Grandin et al. 1997). The low copy suppression and Cdc13p two-hybrid interaction data suggest that Stn1p and Cdc13p function as a complex. High copy expression of *STN1* can lead to telomere shortening whereas mutant *STN1* alleles can display extremely elongated telomeres (Grandin et al. 1997, Table 2), suggesting that *STN1*, like *CDC13*, is required for proper telomere length regulation. The single-stranded nature of the elongated telomeres in the *stn1-13* mutant suggests that *STN1* may be required for either complete replication of telomeres or protection from nucleolytic processing.

In order to further understand the function of Stn1p and its role in telomere maintenance, both genetic and biochemical approaches are being undertaken. One goal is to identify gene products that interact with *STN1;* identifying alleles that will be useful in genetic screens is thus one objective. I have generated *STN1* alleles by random PCR mutagenesis as well as by site directed mutagenesis (alanine scanning). These mutant alleles have been characterized with respect to their growth and telomere related phenotypes, as summarized in Table 2. From analysis of these alleles, it is clear that *STN1* is a negative regulator of telomere length. A second goal is to better understand the functional significance of the interaction between Cdc13p and Stn1p. To that end, the panel of *stn1* mutant alleles have been characterized in the two hybrid assay with Cdc13 and have additionally been tested for their ability to suppress the temperature sensitive phenotype of the *cdc13-1*^{ts} allele (Table 2). There is not a clear correlation between the extent of high-copy suppression of *cdc13-1*^{ts} and the ability of the *stn1* alleles to interact with Cdc13p in the two-hybrid assay. This could indicate that the suppression of *cdc13-1* is not necessarily through direct stabilization of the mutant protein by higher levels of Stn1.

To biochemically characterize Stn1p, I have created several different epitope tagged *STN1* constructs. Some of these fusion constructs do disrupt the function of the gene, making subsequent analysis dubious. However, the tagged protein is detectable on westerns from immunoprecipitations and does appear to be modified. Initial crosslinking experiments suggest that Stn1p can crosslink with *CENIII*. It is not yet clear to what extent telomeric DNA will crosslink with Stn1p. Both the genetic and biochemical analysis of *STN1* remain a focus of my research. The following questions regarding the function of Stn1p remain to be more completely addressed:

- What is the nature of the interaction between Cdc13p and Stn1p?

The goal is to determine how interaction of Cdc13p and Stn1p relates to their function in telomere replication and length regulation.

- Can Stn1p be crosslinked to DNA *in vivo*, and if so, does it show a similar or different pattern of DNA association as compared to Cdc13p?
- In addition to Cdc13p, what does Stn1p physically interact with?
  Use the tagged *STN1* strain to look for interaction (by co-IP) with other proteins.
  Identify high copy suppressors of temperature sensitive *stn1* alleles.
- How is *STN1* regulated? (RNA level, protein level, modification state, DNA association, subcellular localization) and is such regulation critical for function?

FIGURE LEGENDS

Table 1. Summary of Est2 two-hybrid screen hits. Approximately 2 x 10^6 library transformants were screened for interaction with full length Est2 in the two hybrid assay. Three different reporters were tested for activation in the strain pJ69-4A. The number of isolates of each clone obtained from the library is indicated in column 2.

Figure 1. Requirement for *CDC13* function varies through the cell cycle. Progression through the cell cycle was ascertained by the budding profile in conjunction with DAPI staining. *cdc13-1^{ts}* haploid cells were grown at permissive temperature (23°C) to log phase, then arrested in G1, S, or G2/M of the cell cycle through the addition of alpha factor, hydroxyurea, or nocodazole, respectively. After arrest at permissive temperature, half of each culture was shifted to restrictive temperature (34°C) for a period of 2.5 hours. Next, each culture was shifted back to 23°C, concomitant with removal of the block to cell cycle progression. The cells were monitored by bud index and DAPI staining to determine the timing with which they proceeded through the next cell cycle. The percent of large budded cells is an approximate indicator of the percent of the population at G2/M. The percent of cells through mitosis reflects the proportion of the cells that are unbudded after having completed mitosis. A delay in cell cycle progression indicates potential activation of the checkpoint response that is sensitive to the presence of DNA damage. A) Cells were arrested in alpha factor (G1) prior to and during the temperature shift. The percent of cells through mitosis indicates the proportion of cells that return to G1 (unbudded) after progression through the remainder of the cell cycle. B) Cells were arrested in S phase through addition of hydroxyurea (HU) prior to and during the temperature shift. The percent of cells through mitosis indicates the proportion of unbudded cells. C) Cells were arrested in nocodazole prior to and during the temperature shift. Nocodazole treatment arrests cells pre-anaphase. The percent of large budded cells indicates the proportion of cells remaining arrested prior to mitosis.

Figure 2. Sensitivity of $Cdc13_{myc18x}$ telomeric association to hydroxyurea (HU) in wild-type and mutant backgrounds. Percent of telomeric DNA in immunoprecipitates from *CDC13*$_{myc18x}$, *yku80-Δ CDC13*$_{myc18x}$, *stn1-13 CDC13*$_{myc18x}$ and untagged strains in extracts from crosslinked cells either progressing through an unperturbed S phase or from crosslinked cells treated with 200 mM HU. The procedure followed for all crosslinking experiments was as published in Aparicio et al. 1997. Data shown was quantified by phosphoimager analysis of dot blots hybridized with a poly d(GT/CA) probe to determine extent of telomeric DNA in immunoprecipitates.

Figure 3. Chromatin IP experiment to examine the effect of the loss of the DNA replication checkpoint on the HU sensitivity of Cdc13p telomeric and centromeric association. The *mec1-21* allele was used to abrogate the checkpoint. Cells were arrested in G1 with alpha factor, and released from the phermone block into media with or without 200 mM hydroxyurea. Formaldehyde was added to the cells for a period of 45 minutes to allow crosslinking. Chromatin immunoprecipitations were performed from extracts of $CDC13_{myc18x}$, *mec1-21* $CDC13_{myc18x}$ and untagged strains. A) Percent of telomeric DNA in immunoprecipitates. B) Percent of *CENIII* locus in immunoprecipitates, as determined by quantitative PCR using primers described in Meluh et al. 1998.

Figure 4. Crosslinking of cdc13-8p with telomeric DNA is similar to Cdc13p with respect to timing and extent of association. Analysis of telomeric chromatin in immunoprecipitates from *cdc13-8* $_{myc18x}$, *CDC13* $_{myc18x}$ or untagged strains through the cell cycle. Cells were arrested in G1 with alpha factor, and released to allow progression through the cell cycle. Cells were subjected to formaldehyde crosslinking for 40 min. The percent of d(GT/CA) in each IP is shown.

Figure 5. Extent of Cdc13p crosslinking with telomeric DNA is increased in *stn1-13* or *ku80-Δ* mutant backgrounds during G1 and S phases of the cell cycle. Calculations of the telomeric association in G1 were made from cells arrested in alpha factor. To obtain cells in S phase, cultures synchronized in G1 were released from the pheromone block and treated with formaldehyde (for crosslinking) at various time points after release. FACs analysis was used to ascertain when the cells were in S phase. Cells indicated as in G2/M were cultures treated with nocodazole. A) Chromatin IP comparison of $CDC13_{myc18x}$ and $CDC13_{myc18x}$ *stn1-13* with telomeric sequences. B) Chromatin IP comparison of $CDC13_{myc18x}$ and $CDC13_{myc18x}$ *yku80-Δ* with telomeric sequences. Data shown was quantified by dot blot hybridization of d(GT/CA) probe to determine extent of telomeric DNA in immunoprecipitates; similar results were obtained by quantitative PCR using primers recognizing Y' sequence adjacent to the telomeric repeats.

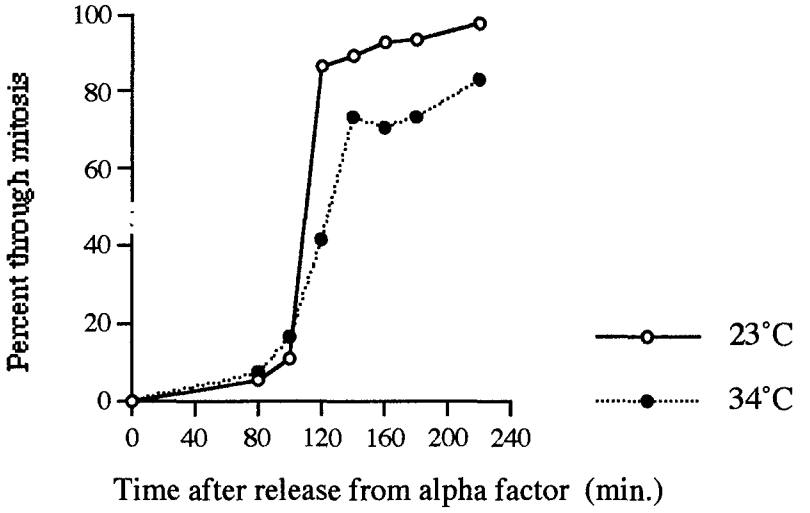
Table 2. Characterization of *STN1* mutant alleles.

Table 1. Est2p two-hybrid interacting clones.

Clone:	# isolates:	Brief information on interacting gene:
High interest:		
CAC1/RLF2	1	Chromatin assembly complex component/ Rap1 localization factor
SDS3	1	Extragenic suppressor of defective (*rap1-12*) silencing.
YDR026c	1	Has c-myb DNA binding domain repeat 2 similarity; similar to *REB1*.
YIL112w	1	123.6 kD. Contains ankyrin repeats.
Could be interesting:		
YLA1	1	Homolog of human La auto-antigen, associates with RNA.
SNU56	1	U1 snRNA associated protein.
TY1	5	Ty retrotransposon. Inserts encode at least part of TYA (gag).
YPR144c	3	Purifies as protein associated with nuclear pore complex.
JNM1	2	Required for nuclear migration during mitosis. Associates with Nip100.
NIP100	1	Mitotic spindle positioning protein. Nuclear import?
NUP85	1	Nuclear pore complex protein. Required for poly(A)+ RNA export.
BUB1	1	Ser/Thr protein kinase required for spindle checkpoint.
Novel ORFS:		
YGR280c	4	31.3 kD, pI=10 Lysine and asparagine rich sequence.
YIR025w	2	42.8 kD; pI=4.6 Poly-serine regions.
YLR387c	1	49.7 kD, pI=7.76 Three zinc finger domains. Proteasome function?
YPR143w	1	28.2 kD, pI=5.6
YKL014c	1	203.3 kD, pI=7.2 Low abundance transcript.
YLR287c	1	40.9 kD, pI=4.95
YDR333c	1	76.4 kD; pI=5.5. Has transmembrane domain.
YEL023c	1	78.3 kD; pI=6.37. Interacts with Cdc7 in two-hybrid.
Odd hits:		
YHL046c	2	Seripauperin (PAU) family member- possible cell wall mannoproteins.
YNL091w	1	141.5 kD. Similar to protein involved in mannosylphosphorylation.
MNN4	1	Regulates mannosyl phosphorylation.
SEC27	1	Vesicle coat protein.
PDC1	1	Pyruvate decarboxylase.
SER2	1	Phosphoserine phosphatase involved in synthesis of serine.
YLR231c	2	ORF neighboring *EST1*, likely active in tryptophan degradation.

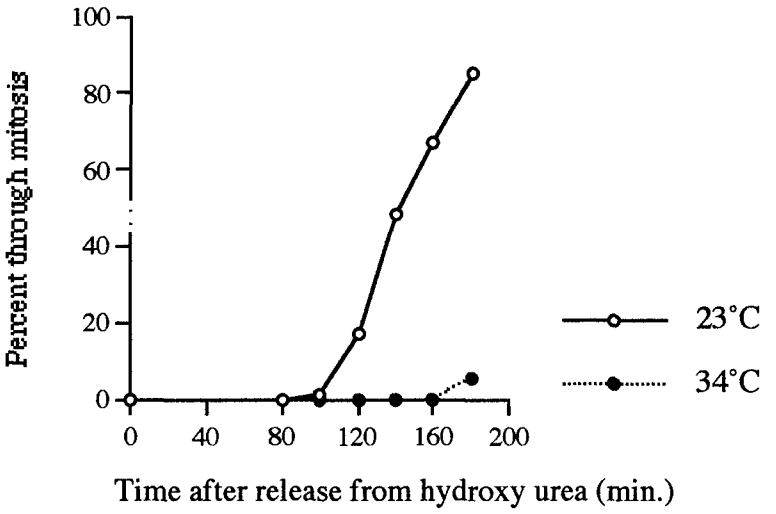
A.

Shift during G1



B.

Shift during S



C.

Shift during G2

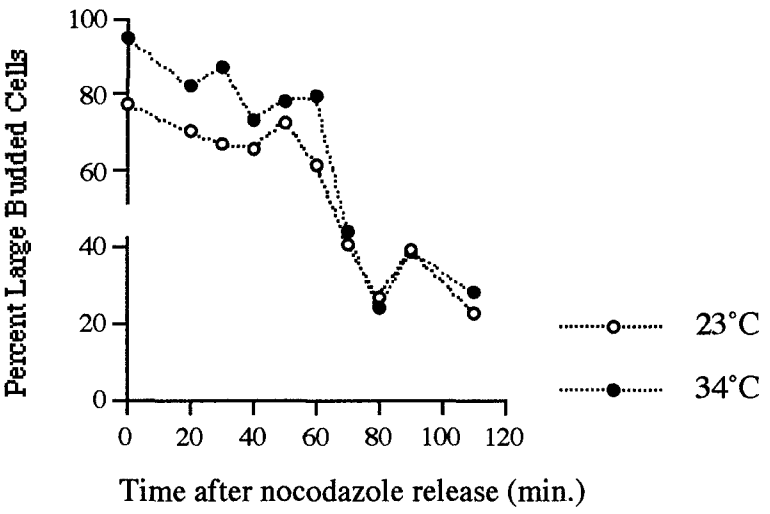


Figure 1. Experiment to determine time of *CDC13* function.

Figure 2.

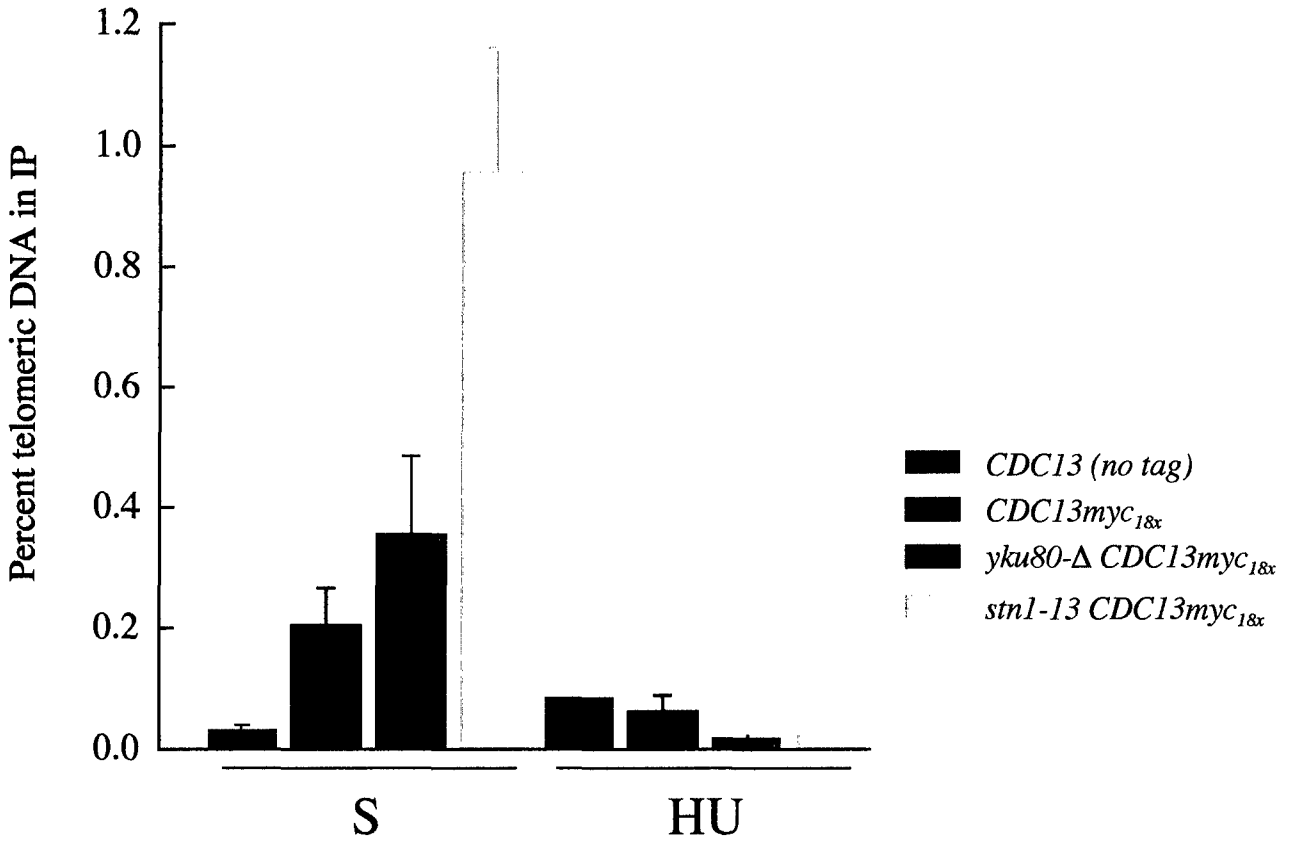

Percent telomeric DNA in IP
1.2
1.0
0.8
0.6
0.4
0.2
0.0
S
HU
CDC13 (no tag)
CDC13myc18x
yku80-Δ CDC13myc18x
stn1-13 CDC13myc18x

Figure 3. Checkpoint dependence of Cdc13p crosslinking sensitivity to HU.

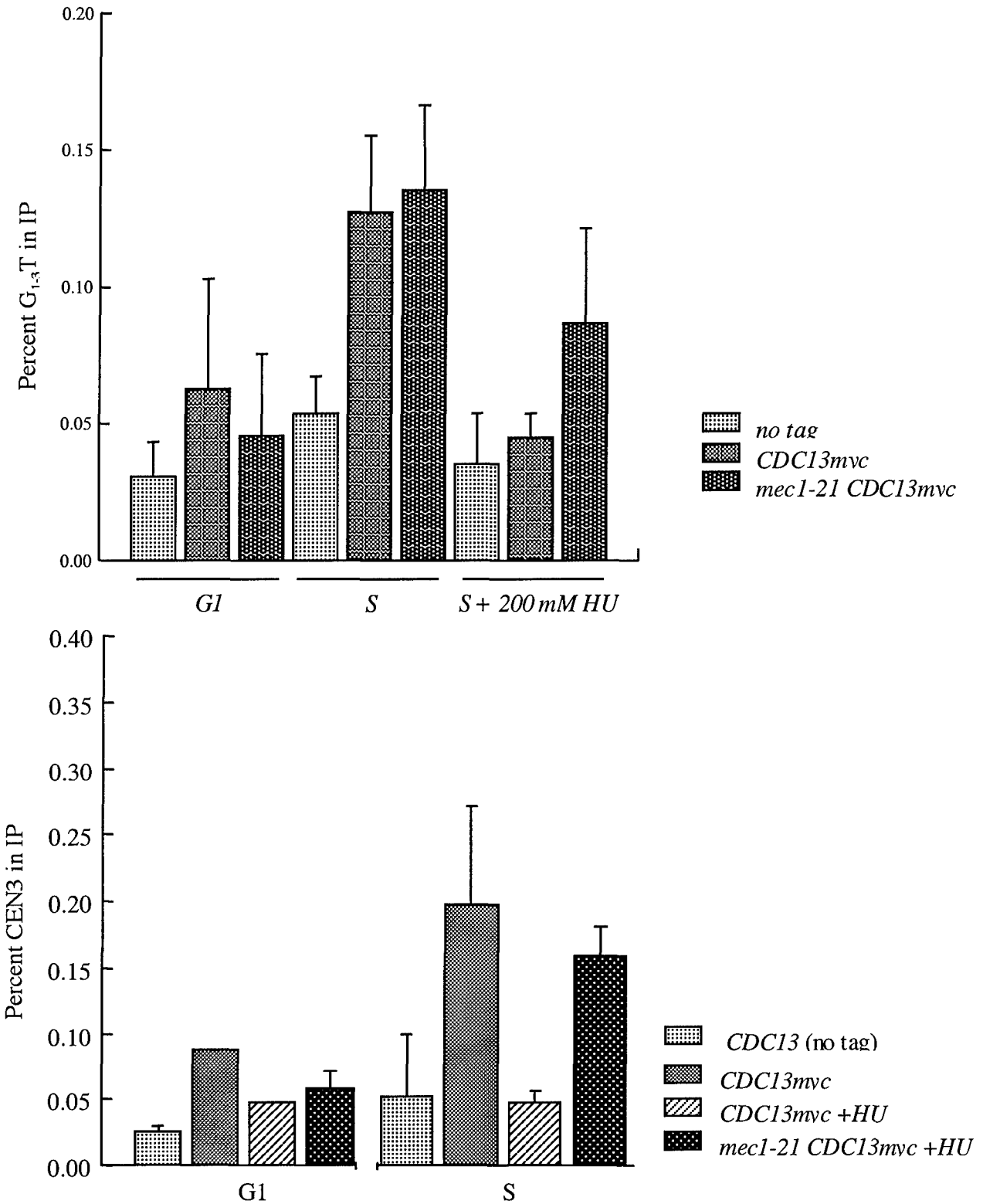


Figure 4. Cdc13p telomeric crosslinking is not altered in mutant allele with short telomeres.

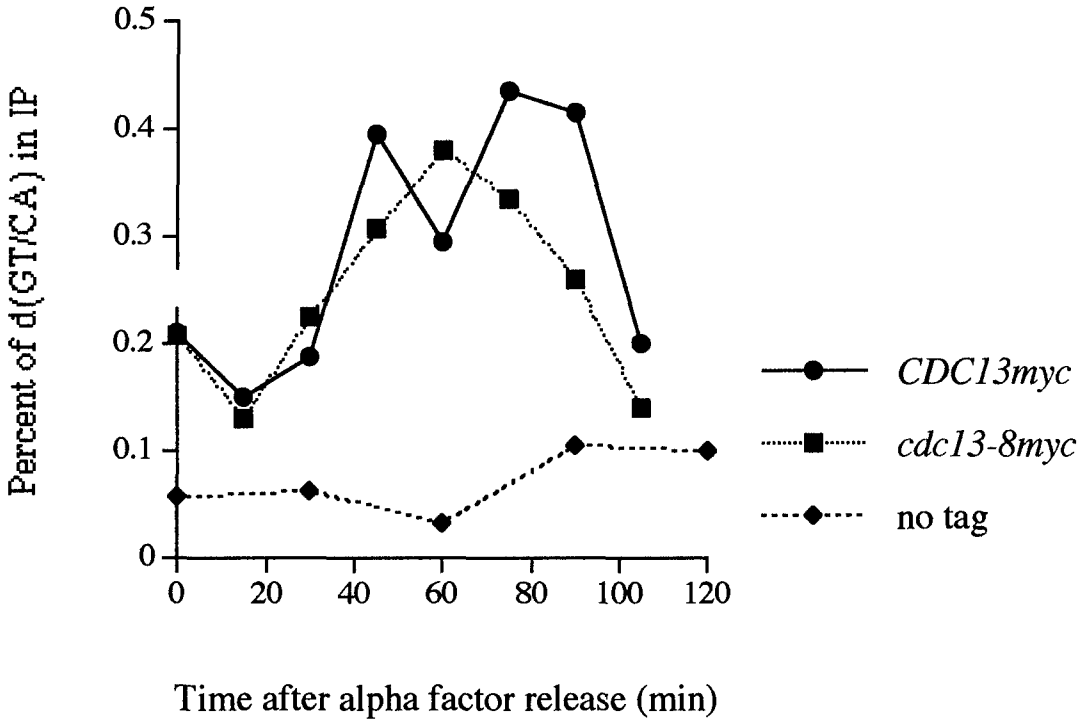
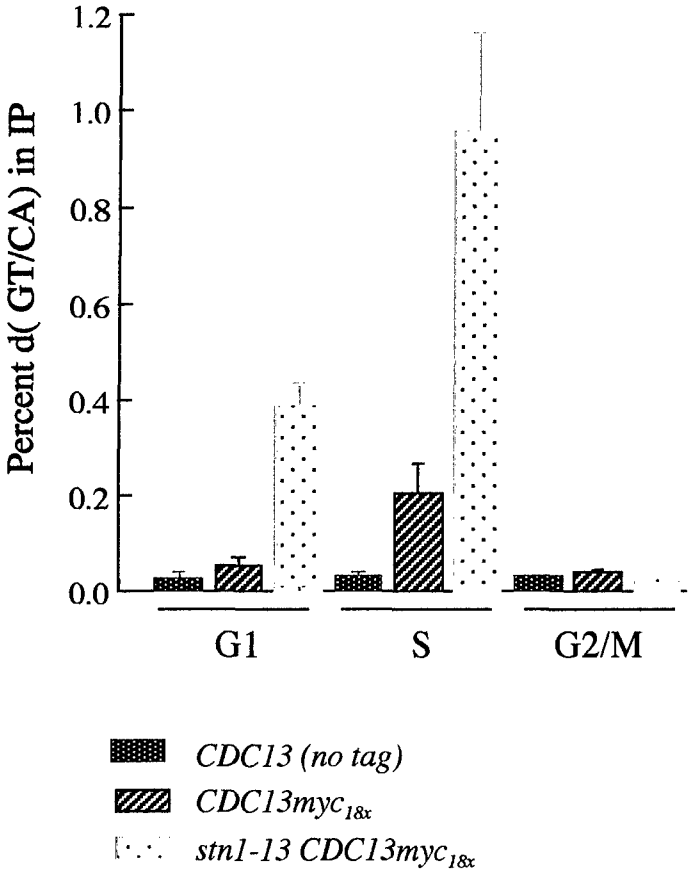


Figure 5.

A.


Percent d(GT/CA) in IP
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G1
S
G2/M
CDC13 (no tag)
CDC13myc18x
stn1-13 CDC13myc18x

B.

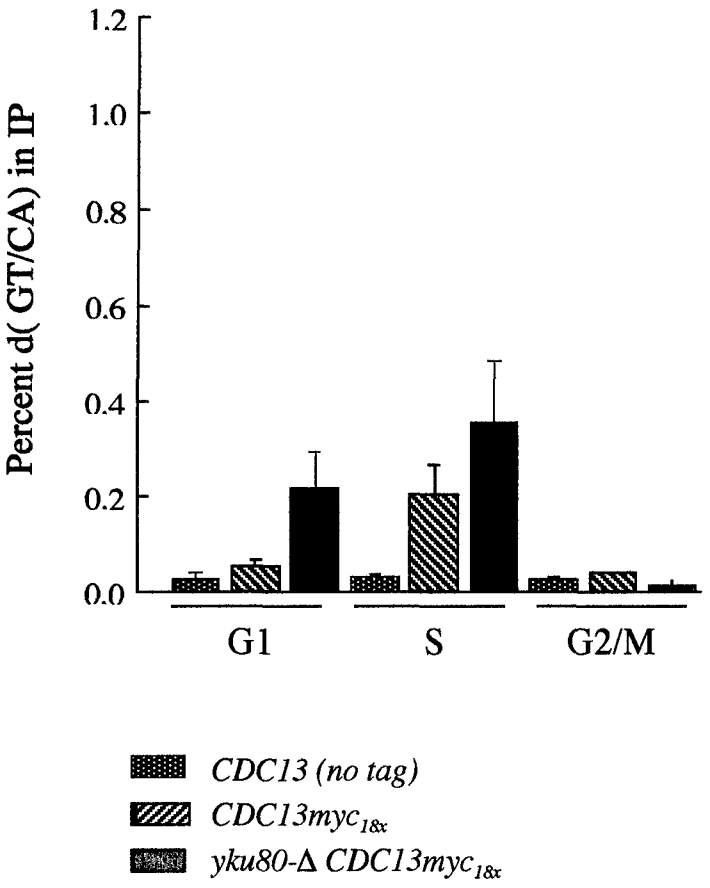

Percent d(GT/CA) in IP
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G1
S
G2/M
CDC13 (no tag)
CDC13myc18x
yku80-Δ CDC13myc18x

Table 2. *STN1* alleles.

Allele	A. Growth	B. Telomeres	C. Two hybrid	D. *cdc13-1*^{ts} Suppression
stn1-31	sick	long	+++	**
stn1-33	sick	long	-+	***
stn1-34	dead	-	-	-
stn1-35	sick	long	-+	*
stn1-36	sick	long	+	*w*
stn1-37	dead	-	-	*w*
stn1-38	dead	-	-	-
stn1-39	sick / t.s.	very long	-	*
stn1-40	sick	long	-	**
stn1-41	w.t.	w.t.	+++	***
stn1-42	w.t.	w.t.	+++	*****
stn1-43	w.t.	long	+++	-
stn1-44	w.t.	w.t.	+++	***
stn1-45	w.t.	long	+++	*
stn1-46	w.t.	w.t.	++	*
stn1-47	sick	long	++	*
stn1-48	w.t.	w.t.	+++	**
stn1-49	w.t.	w.t.	+++	*
stn1-50	sick	very long	-	-
stn1-51	w.t.	slight long	++	*
stn1-52	dead	-	-	-
stn1-53	w.t.	slight long	++	*w*
stn1-54	w.t.	long	++	-
stn1-55	sick / t.s.	very long	-+	-
stn1-56	w.t.	slight long	++	-
stn1-57	w.t.	w.t.	+	-
stn1-58	dead	-	-	-
stn1-59	w.t.	long	+	-
stn1-60	w.t.	slight long	++	*****
stn1-61	dead	-	-+	-
stn1-62	w.t.	w.t.	+++	*
stn1-63	t.s.	very long	+-	*
stn1-64	dead	-	-	-

A. Growth phenotype of mutant strain at 23°C. "w.t." indicates growth similar to that of wild-type strains. "sick" indicates growth slower or with more inviability than in wild-type strains. "t.s." indicates that the growth phenotype is worse at high temperatures (36°C). "dead" indicates no growth, i.e. a null allele (*STN1* is an esssential gene).
B. Length of telomeres as compared to wild-type strains was assessed by southern blot analysis. "w.t." indicates that the telomeres appeared similar to the wild-type strain. Elongated telomeres observed in these mutant strains tended to be fairly heterogeneous in length.

C. The mutant alleles of *STN1* were subcloned into pACT2 and tested for two-hybrid interaction with *CDC13*, using the bait pVL705, which encodes *CDC13* with an in-frame deletion of its DNA binding domain. The relative strength of the interaction indicates the transcriptional activation of the adenine and histidine reporters in the strain pJ69-4A.
D. Extent of suppression of the temperature sensitive growth phenotype of *cdc13-1*^{ts} at 32.5°C by high copy expression of *stn1* mutant alleles from an ADH promoter. The maximum permissive temperature of *cdc13-1*^{ts} is 26°C; each "*" in the column indicates a log of growth at 32.5°C. "-" indicates no suppression of the growth phenotype. "*w*" indicates weak suppression of *cdc13-1*^{ts} growth at 28°C.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that *MRE11*, *RAD50* (and *XRS2*), genes that encode proteins involved in the DNA repair process of non-homologous end joining, are also important for telomere replication.

- Found that the Ku complex is required for telomere maintenance in a role distinct from those provided by either telomerase or Cdc13p, protein that binds single-stranded DNA comprising telomere repeats *in vitro.*

- Identified a number of proteins that can associate with the catalytic component of telomerase in the two-hybrid assay. These are potential regulators of telomerase activity.

- Discovered that Cdc13p is phosphorylated in a cell cycle dependent manner.

- Found that Cdc13p is associated with telomeres *in vivo*, and that the association is cell cycle dependent and correlates with the requirement for *CDC13* function during S phase.

- Determined that Cdc13p may not function exclusively at telomeres; Cdc13p can associate with some centromeric regions with timing similar to its telomeric association.

- Observed that the Cdc13p chromatin association is sensitive to hydroxyurea, suggesting a function during late replication. This inhibition is mediated through checkpoint genes.

- Identified Stn1p as a binding partner for Cdc13p. A mutant *cdc13* allele that displays a phenotype similar to deficiency for telomerase is defective for two hybrid interaction with Stn1p. Thus, the *cdc13-2^{est}* allele may define a domain of interaction with Stn1p that is required for positive regulation of telomere replication.

- Created panel of *stn1* mutant alleles useful for analysis of *STN1* function. The phenotypes of these mutant alleles indicate that *STN1* is required for negative regulation of telomere length, as well as for allowing either complete replication or protection of the chromosome ends.

- Found that *YKU80* and *STN1* are negative regulators of the Cdc13p telomeric association.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Presented poster at DoD Era of Hope Breast Cancer Research meeting, June 2000

Presented talk at MD Anderson Information Exchange Seminar, October 1999

Presented talk at interdepartmental seminar at Baylor College of Medicine, April 1999

Presented poster at Cold Spring Harbor Telomeres and Telomerase meeting, March 1999

Presented poster at Molecular and Human Genetics departmental retreat, February 1999

Published paper: Evans, S.K. et al. *Chromosoma* **107**: 352-358 (1998).

Published paper: Nugent, C.I. et al. *Curr. Biol.* **8**, 657-660 (1998).

Presented poster at FASEB meeting on Yeast Chromosome Structure, Replication and Segregation, August 1998

Presented talk at interdepartmental seminar at Baylor College of Medicine, April 1998

Presented talk at regional seminar series at Baylor College of Medicine, March 1998

Published paper: Nugent, C. I. and V. Lundblad, *Genes Dev.* **12**, 1073-1085 (1998).

Presented poster at EMBO meeting on Chromatin and Epigenitic Regulation, October 1997

Employment received based on research experience supported by this grant:

Interviewed for jobs, February - May 2000.
Accepted job as Assistant Professor at University of California, Riverside. (Position will start January 1, 2001)

Funding:

This work will form the basis of a future grant application I will submit to the NIH.

Personnel receiving salary from the research effort: Dr. Constance Nugent.

CONCLUSIONS

Multiple factors act to regulate cell proliferation, and one factor that can determine the proliferative potential of cells is the ability to maintain functional telomeres. The goal of my research is to understand how telomere length and integrity are regulated and maintained. I chose to use the yeast *S. cerevisiae* as a model system to identify and characterize genes that impact telomere function. Ultimately, the results from this research may help provide the underpinnings for insight into how telomeres afford protection to chromosome ends and how telomerase can be mis-regulated to allow unlimited proliferation of cells, contributing to cancer.

This work has contributed to the identification of both genetic pathways and genes important for proper telomere function. These data suggest that the Ku heterodimer and telomerase participate in distinct pathways required for telomere function. My data, together with recent data from other labs, suggests that the Ku heterodimer plays an important role in protecting chromosome ends. *RAD50*, *MRE11* and *XRS2*, members of a complex that has typically been thought to be primarily involved in DNA double-strand break repair with the Ku complex, are also required to maintain telomeres at their proper length, but their role is required in the telomerase genetic pathway. A yeast two-hybrid screen was conducted to find additional genes that impact telomere function through interaction with the protein catalytic component of telomerase. Several of the *EST2* interacting proteins identified in this screen may be components or regulators of the telomerase holoenzyme; further characterization of these proteins is necessary to understand the nature of these interactions.

CDC13 and *STN1* appear to function in an independent pathway that contributes not only to maintaining telomere integrity, but also to telomere replication. Alterations of either *CDC13* or *STN1* can confer telomere shortening or lengthening phenotypes, suggesting these gene products may be critical targets for telomere length regulation. A second approach taken to investigate regulation of telomere replication has been to assess the association of these proteins with chromosome ends throughout the cell cycle. Consistent with a regulated role in DNA replication, I have found that Cdc13p appears to be enriched at telomeres during active DNA replication and is phosphorylated in a cell-cycle dependent manner. The timing of the association of Cdc13p with telomeres correlates with the time during the cell cycle that *CDC13* function is required. One unexpected observation from these experiments is that Cdc13p may not be associated solely with telomeres, but may also localize in a cell cycle dependent manner to centromeric regions. Thus, *CDC13* potentially functions at multiple chromosomal regions. Further experiments will be necessary to elucidate its role at these regions.

Funding of this research grant has provided an opportunity to conduct basic research aimed at further understanding a fundamental cellular problem. Investigation of telomere function and replication by telomerase is important for cancer biology for two reasons. The first is that it is important to understand how telomeres function such that they protect chromosome ends from events that lead to genomic instability, such as fusion with other chromosome ends. The second reason is that telomere function appears to be a factor in determination of the proliferative potential of cells. Inappropriate telomerase activity has not only been detected in the majority of breast cancers, but also has been shown to lead to the extension of the life span of normal mammalian cells (Bodnar et al. 1998). Thus, an understanding of factors that regulate telomerase activity could potentially lead to ideas for means to limit the lifespan of cells through inhibition of telomerase activity.

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Holt, S.E., Aisner, D.L., Baur, J., Tesmer, V.M., Dy, M., Ouellette, M., Trager, J.B., Morin, G.B., Toft, D.O., Shay, J.W., Wright, W.E. and M.A.White. 1999. Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev.* **13**:817-826.

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

Nugent, C.I., Bosco, G., Ross, L., Evans, S.K., Salinger, A.P., Moore, J.K., Haber, J.E., and V. Lundblad. 1998. Telomere maintenance is dependent on activities required for end repair of double strand breaks. *Curr. Biol.* **8**: 657-660.

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APPENDICES

Abstract of poster presented at FASEB meeting, August 1998:

THE ROLE OF *CDC13* IN *S. CEREVISIAE* TELOMERE REPLICATION

Constance Nugent and Victoria Lundblad
Baylor College of Medicine, Houston, Texas 77030

Telomere length in many immortal eukaryotic cell populations is maintained at least in part through the action of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. However, telomerase alone is not responsible for maintaining the length and integrity of chromosome ends. In the yeast *S. cerevisiae*, the *CDC13* gene has been implicated in both the telomerase-mediated pathway for telomere replication and in maintaining telomere integrity. One current model for Cdc13p function is that it associates with telomeres as a single-stranded binding protein and facilitates complete replication of the telomeres by both protecting the chromosome end and regulating access of telomerase to the chromosome terminus.

To further explore the role of *CDC13* in telomere replication, we identified two *CDC13*-interacting proteins through a yeast two-hybrid screen. One of these proteins interacts in the two-hybrid assay not only with *CDC13*, but also with *EST2*, the catalytic subunit of telomerase. Although deletion of this novel gene does not lead to perturbations of telomere length, preliminary analysis indicates that there may be some genetic interactions with *CDC13*; further characterization of the function of this gene may reveal some insight into the regulation of telomere replication. The second *CDC13*-interacting protein identified was *STN1*, a gene isolated by M. Charbonneau's lab as a suppressor of the *cdc13-1*^{ts} mutant. In order to understand the role of the Cdc13p/Stn1p complex in telomere replication, experiments are in progress to assess the regulation of these proteins throughout the cell cycle as well as the association of these proteins with chromosome ends *in vivo*. Current data suggests that Cdc13-2^{est}p may be unable to interact with Stn1p, suggesting that the *cdc13*^{est} alleles define a domain of interaction with Stn1p that is required for positive regulation of telomere replication. We are also isolating dosage suppressors of temperature-sensitive *stn1* alleles to further elucidate the role of *STN1* at telomeres.

Poster abstract presented at Molecular and Human Genetics departmental retreat, February 1999.

THE ROLE OF *CDC13* IN *S. CEREVISIAE* TELOMERE REPLICATION

Constance Nugent and Victoria Lundblad
Dept. of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030.

Telomere length in many immortal eukaryotic cells is maintained in part through the action of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. However, telomerase alone is not responsible for maintaining the length and integrity of chromosome ends. In the yeast *S. cerevisiae*, the *CDC13* gene has been implicated in both the telomerase-mediated pathway for telomere replication and in maintaining telomere integrity. *CDC13* may be a critical target for telomere length regulation, as mutant alleles have been identified that confer telomere shortening or lengthening phenotypes. We have evidence that Cdc13p is regulated; it becomes phosphorylated in a cell-cycle dependent manner. Experiments are progress to ascertain both the phosphorylated residues and the functional significance of this phosphorylation.

One model for Cdc13p function is that it associates with telomeres as a single-stranded DNA binding protein and facilitates complete replication of the telomeres, thereby both protecting the chromosome end and regulating access of telomerase to the chromosome terminus. To further test this model, we are analyzing the *in vivo* association of Cdc13p with chromatin to determine both the cell-cycle timing and sequence specificity of its binding.

A two-hybrid screen identified *STN1*, a gene isolated by M. Charbonneau's lab as a suppressor of the *cdc13-1* ^{ts} mutant, as a *CDC13*-interacting protein. The inability of the senescent *cdc13-2*^{est} allele to interact with *STN1* in two-hybrid suggests that Stn1p may be critical for proper Cdc13p function in maintaining telomere length.

Abstract presented at Cold Spring Harbor Telomeres and Telomerase meeting, March 1999.

S. CEREVISIAE CDC13 ENCODES A CELL-CYCLE REGULATED PHOSPHOPROTEIN IMPORTANT FOR TELOMERE REPLICATION

Constance Nugent and Victoria Lundblad
Dept. of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030.

Telomere length in many immortal eukaryotic cells is maintained in part through the action of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. However, telomerase alone is not responsible for maintaining the length and integrity of chromosome ends. In the yeast *S. cerevisiae*, the *CDC13* gene has been implicated both in the telomerase-mediated pathway for telomere replication and in maintaining telomere integrity. One model for Cdc13p function is that it associates with telomeres as a single-stranded DNA binding protein and facilitates complete replication of the telomeres, thereby both protecting the chromosome end and regulating access of telomerase to the chromosome terminus. To further test this model, I am analyzing the *in vivo* association of Cdc13p with chromatin to determine both the cell-cycle timing and sequence specificity of its binding.

CDC13 may be a critical target for telomere length regulation, as mutant alleles have been identified that confer telomere shortening or lengthening phenotypes. I have shown that Cdc13p is phosphorylated in a cell-cycle dependent manner, with phosphorylated protein appearing during S phase and accumulating prior to completion of mitosis. Experiments are progress to ascertain both the phosphorylated residue(s) and the functional significance of this phosphorylation.

Using a two-hybrid screen, I identified *STN1* as a *CDC13*-interacting protein, a gene also isolated by M. Charbonneau's lab as a high-copy suppressor of the *cdc13-1*^{ts} mutant. The inability of the senescent *cdc13-2*^{est} allele to interact with *STN1* in the two-hybrid assay suggests that Stn1p may be critical for proper Cdc13p function in telomere replication. I am characterizing additional *stn1* mutant alleles, as well as isolating dosage suppressors of temperature-sensitive stn1 alleles to further elucidate the role of *STN1* at telomeres.

Abstract of poster presented at DoD Era of Hope Breast Cancer Research meeting, June 2000.

ANALYSIS OF TELOMERE LENGTH REGULATION IN *S. CEREVISIAE*

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Telomeres, specialized structures at the ends of chromosomes, help maintain the stability of the genome and are essential for continued cell proliferation. The enzymes that replicate the bulk of the genome are not capable of fully duplicating the ends of the chromosomes; thus a special mechanism is required to replenish these sequences. In the yeast *S. cerevisiae*, telomere length is maintained through the aid of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. In order to fully replicate the chromosome end, telomerase activity is likely to be coordinated with the conventional DNA replication machinery. The goal of my research has been to understand the processes involved in maintaining the length and integrity of chromosome ends using the yeast *S. cerevisiae* as a model system.

A number of genes have now been implicated in telomere length regulation in *S. cerevisiae*. My research has focussed on *CDC13*, a gene involved in both the telomerase-mediated pathway for telomere replication and in maintaining genome integrity. One model for the role of Cdc13p at telomeres is that it functions as a single-stranded DNA binding protein that facilitates complete replication of the telomeres. It could regulate the ability of telomerase and the replication machinery to function at the chromosome terminus and thereby aid in maintaining the integrity of the chromosome end. Cdc13p, and its binding partner, Stn1p, may be critical targets for telomere length regulation, as I have found that alterations of either gene product can confer telomere shortening or lengthening phenotypes. In addition, the inability of a senescent *cdc13-2^{est}* allele to interact with *STN1* in the two-hybrid assay suggests that interaction with Stn1p may be critical for proper Cdc13p function. In order to understand the role of Cdc13p and Stn1p in telomere replication, I am assessing the *in vivo* modification and association of these proteins with chromosome ends throughout the cell cycle. Consistent with a regulated role in DNA replication, I have found that Cdc13p is phosphorylated in a cell-cycle dependent manner and appears to be enriched at telomeres during active DNA replication.

The U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7126 supported this work.

Curriculum Vitae

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Education:

1995 Ph.D.	University of Colorado, Boulder (Molecular, Cellular & Developmental Biology)
1987 B.S.	University of Wisconsin, Madison (Molecular Biology, with Honors)

Research Experience:

4/95-present	Postdoctoral Fellow in Dr. Victoria Lundblad's laboratory
8/87-3/95	Graduate student, University of Colorado. Advisor: Dr. Karla Kirkegaard Thesis: Analysis of Poliovirus Assembly and Genome Encapsidation
1/85-5/87	Undergraduate Research Assistant in Dr. Judith Kimble's laboratory University of Wisconsin, Madison

Teaching Experience:

10/99–12/99	Faculty for graduate level "Genetics B" course at Baylor College of Medicine
1989-1992	Taught math and verbal skills to high school students for The Princeton Review
8/88-5/89	Teaching Assistant, University of Colorado, Boulder Introductory Biology, Developmental Biology

Fellowships, Awards, Activities and Honors:

1997-2000	U.S. Army Materiel Command Breast Cancer Research Postdoctoral Fellowship
1996-1997	NIH Postdoctoral Training Grant
1990-1991	Colorado Institute for Research in Biotechnology Fellowship
1987-1988	Colorado Merit Fellowship
1987-1988	NIH Training Grant
1986	Trewartha Undergraduate Research Award, University of Wisconsin, Madison
1986	Genetics Society of America Summer Research Grant

Manuscript in preparation:

Nugent, C.I., and V. Lundblad. Cdc13p telomere binding *in vivo* is S-phase specific and sensitive to replication origin firing status.

Publications:

Nugent, C.I., Johnson, K.L., Sarnow, P. and K. Kirkegaard (1999). Functional coupling between replication and packaging of poliovirus replicon RNA. *J. Virol*: **73**: 427-435.

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Invited Talks:

10/99 "Telomere length regulation in *S. cerevisiae*". MD Anderson Information Exchange Seminar.

2/96 "Telomeres, Telomerase and Cancer." Rotary International meeting, Frankfort, Michigan.

5/96: Speaker, Cold Spring Harbor Cell Cycle meeting
"A dual role for yeast Cdc13 protein in telomere maintenance"

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

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 the technical reports listed at enclosure. Request the limited distribution statement for these reports 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

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be Downgraded to Unlimited Distribution

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