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Recent evidence suggests that Pif1p is a catalytic inhibitor of telomerase in *Saccharomyces cerevisiae*. Mean telomere length increases in strains with mutations of *PIF1*, and this is dependent on the RNA template, *TLC1*, of the telomerase reverse transcriptase. It is likely that Pif1p acts as a helicase to inhibit telomerase, as point mutations that knock out helicase activity *in vitro* lead to increased telomere length in vivo. The effect of Pif1p on telomerase is most likely direct, as Pif1p is associated with telomeric chromatin *in vivo*. Here I use chromatin immunoprecipitation (ChIP) to show that the catalytic subunit of telomerase, Est2p, is also telomere-associated *in vivo*. Telomere association appears to be constitutive in the cell cycle, although the intensity of the ChIP signal varies. I plan to ask how this pattern of Est2p telomere association is altered in *PIF1* mutant backgrounds.

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Table of Contents

(4) Introduction

From Dr. Teng's final report:

"*PIF1* is a non-essential *Saccharomyces* gene that encodes a 5' to 3' DNA helicase. Mutations in *PIF1* affect telomeres in three ways. First, telomeres in *pif1* cells are longer than in wild type cells. Second, *de novo* telomere addition on spontaneous or induced double strand breaks occurs much more frequently in *pif1* compared to wild type cells. Third, in *pif1* mutants, new telomeres are often added to sequences with very little resemblance to telomeric DNA whereas wild type cells show a strong preference for forming telomeres at sites with stretches of telomere-like DNA. The *pif1* mutants also exhibit increased loss and decreased recombination of mitochondrial DNA and thus have a high fraction of respiratory deficient (petite) cells."

Telomere lengthening in a *pif1* mutant background is due to telomerase, as it does not occur in a *tlc1Δ pif1* double mutant (Zhou et al). Point mutations that knock out the helicase activity of Pif1p *in vitro* lead to telomere lengthening *in vivo*, suggesting that Pif1p inhibits telomerase through this activity, possibly by unwinding the RNA template of telomerase, *TLC1*, from its single stranded telomeric substrate. Chromatin immunoprecipitation studies showed that Pif1p is telomere-associated *in vivo*, lending indirect support to this model of direct telomerase inhibition. Here, I use chromatin immunoprecipiation (ChIP) to show that the catalytic subunit of telomerase, Est2p, is also telomere-associated *in vivo*. Association is constitutive in the cell cycle, although the intensity of the ChIP signal varies. The reason(s) for these variations in signal is not yet clear, but may represent changes in the conformation of telomerase, possibly from an inactive "resting" state to an actively polymerizing one late in S phase. Using this tool of Est2p ChIP, I intend to ask if the helicase activity of Pif1p does indeed act to inhibit telomerase by inhibiting its telomere association.

(5) Body

A) The role of telomere integrity in cancer.

The hallmark of cancer is uncontrolled and indefinite growth of malignant cells. It is now recognized that the progression of a normal somatic cell to malignancy is a multi-step process, where each step involves a loss or alteration of normal cellular regulatory activities. An initial error in one of these activities may make the chance of further errors more likely and lead to an expanding loss of control that results, eventually, in malignancy. This process may occur over a number of years. It is also clear that the progression to malignancy can occur by any number of different pathways, and is influenced by genetic makeup, environmental factors, and also random mutations that can occur over the course of a lifetime.

While it is true that the mechanism of malignancy is highly complex and varies for different types of cancers, it is possible to make some generalizations about malignant cells. For example, these cells must deactivate the normal controls that regulate cell division, allowing unchecked growth. It is now well established that in order for this unchecked growth to be maintained, malignant cells must find a way to maintain their telomeres.

Telomeres are specialized non-nucleosomal chromatin structures that cap the ends of eukaryotic chromosomes. The DNA of the telomere consists of double stranded simple repeated sequences (in human cells, the telomeres consist of up to 10 kilobases of TTAGGG/AATCCC repeats). In all eukaryotes yet tested, the strand of the telomere running 5' to 3' from the centromere to the chromosome end is always the G-rich strand. A small, single stranded overhang of G-rich DNA forms at chromosome termini at some points in the cell cycle. Addition of telomere repeats is catalyzed by the enzyme telomerase, which is a specialized reverse transcriptase that uses an integral RNA subunit as a template to add repeats to the G-rich overhang. In addition to telomerase, the protein components of the telomere consist of sequence-specific structural factors that bind the repeats (TRF1 and TRF2 in human cells) and a host of peripherally associated factors that are responsible for telomere DNA maintenance and other functions.

With regard to cancer, telomeres have two key roles. The first is to prevent end-to-end fusions of chromosomes that can lead to the large-scale chromosome rearrangements that contribute to many types of human carcinomas. Evidence that telomere dysfunction can lead to chromosomal rearrangements has come from studies of recombinant telomerase-null mice that lack the enzyme's RNA subunit. These mice suffer a higher rate of non-reciprocal chromosome translocations and epithelial cancers (including breast cancers) than age-matched wild type controls (Artandi et al). In addition, individuals with a rare X-linked human disease, dyskeratosis congenita, lack full telomerase function and are predisposed to certain types of skin cancer. The exact mechanism by which telomeres prevent end to end fusions is unknown, however recent findings show that the terminal G-rich overhang of the telomere in mammalian cells folds back and invades the double stranded repeats that are closer to the centromere (Griffith et al). The formation of this "t-loop" may serve to sequester the chromosome's end and so prevent fusions. The other role of the telomere is to ensure complete replication of the chromosome. Because eukaryotic chromosomes are linear, they cannot be fully replicated by DNA polymerase. This is so because DNA polymerases require RNA primers to initiate replication. These primers are removed and filled in after the bulk of replication is complete, however removal of the terminal RNA primer on the lagging strand of the chromosome leaves a gap that cannot be filled in. As a result, chromosomes shorten slightly with each round of replication and cell division; eventually reaching a point where enough genetic information is lost that viability is compromised. Telomerase counters this gradual shortening by adding new telomeric repeats. The bulk of human somatic cells lack telomerase activity, and so the length of the telomere

decreases in these cells as we age. Recent experiments show that telomere shortening is directly responsible for the limited proliferative capacity of primary human cell lines in culture. Human fibroblasts, which lack telomerase activity, will divide in culture only a limited number of times before losing viability (a point termed "crisis"). Strikingly, ectopic expression of telomerase in these cells allows bypass of crisis and indefinite proliferation of the cells (Morales et al.). That telomerase is required for extended numbers of cell divisions is also suggested by the observation that highly proliferative cells such as stem cells and germ line cells are naturally telomerase positive. Indeed, telomerase null mice and dyskeratosis congenita patients show defects in highly proliferative tissues such as testis, bone marrow, and skin.

In cancer, malignant cells proliferate indefinitely. Not surprisingly, the vast majority of cancer cells have telomerase activity (in fact, telomerase activity has been reported to be an effective marker of breast carcinoma in biopsied samples). The normal progenitor cells of these cancers are telomerase negative, so activation of the enzyme likely occurs before or concurrently with oncogenesis. Presumably, malignant cells acquire the ability to divide ceaselessly by activating telomerase. The role of telomerase in oncogenesis is suggested by studies of the telomerase null mouse. When these mice receive doses of a carcinogenic compound under the skin, tumors do not form whereas they do in age-matched wild-type controls (Gonzalez-Suarez et al.). Also, immortal human tumor cell lines will undergo apoptosis if their telomerase activity is inhibited (Herbert et al.). It is perhaps paradoxical that telomerase null mice are resistant to certain forms of cancer, but prone to others. A possible explanation for this may be that there is an alternate pathway for telomere maintenance in a subset of tumor cell lines that are telomerase-negative. This pathway, known as the ALT pathway, relies on a recombinational mechanism to maintain telomere length. It may be that certain cell types are able to activate this pathway in response to loss of telomerase, but other cannot. It is worth noting that even in tumor cell lines that lack telomerase, maintenance of telomere length is still a priority.

A central role for telomere maintenance in oncogenesis is becoming well established. Because the majority of normal somatic cells in humans are telomerase negative, inhibition of this enzyme could be an attractive anti-tumor therapy that might have lower non-specific side effects than currently available treatments. However, the finding that certain types of tumor form in the telomerase null mouse and dyskeratosis congenita patients suggests that some cell types may not require telomerase to become malignant. From the standpoint of breast cancer, while it is true that it is seen in telomerase null mice, it is unclear whether this will be the case in humans. The finding that telomerase activity is an effective marker for malignancy in breast tumor biopsy samples suggests that this may be the main pathway for telomere maintenance in human breast cancer. Because telomere maintenance is so important in oncogenesis, we believe that it is essential to understand its mechanism, by either telomerase or ALT, as thoroughly as possible.

B) Yeast as a model system for telomerase.

I have been using the genetically tractable yeast *Saccharomyces cerevisiae* as a model organism for telomere maintenance in humans. Yeast, like human cancer cells, require either telomerase (the preferred pathway) or telomere recombination (in the absence of telomerase) to proliferate. Numerous genetic screens have been performed in yeast to identify gene products involved in telomere maintenance. To date, over 30 such genes have been identified. Of these, several have close sequence homologs in humans, and in many cases the initial identification in yeast has allowed the cloning and characterization of the human counterpart. For example, work in yeast and another model organism, the ciliate *Tetrahymena*, allowed identification of the catalytic subunit of telomerase (*EST2*) in humans. Other features of the telomerase pathway appear to be shared between yeast and humans. For example, short single stranded overhangs of G-rich telomeric DNA occur in

both organisms. These overhangs are thought to be the substrate for telomerase. Also, the recombinational ALT pathway in yeast results in much longer telomeres than normal, just as in telomerase negative human tumor cells. This conservation at the sequence homology level and also, to at least some degree, the mechanistic level makes yeast a valuable model for telomere maintenance in cancer cells.

C) The telomerase pathway in yeast.

Telomeric DNA repeats in yeast are not as regular as those of vertebrates, and consist of approximately 300 +/- 50 base pairs of TG_{1-3}/AC_{1-3} repeats. The Zakian lab showed that a 30 - 50 nucleotide single stranded G-rich overhang forms at the telomere late in S phase (Wellinger et al.). Single-stranded G-rich DNA is the substrate for telomerase, so it seems likely that telomerase acts late in S phase. However, the technique used to identify the G-rich overhangs cannot detect stretches of single-stranded DNA less than 30 nucleotides, so it is possible that shorter overhangs are present at other times. Therefore, the point in the cell cycle when telomerase acts is yet to be demonstrated with confidence. In mammalian cells, detectable G-rich overhangs are present throughout the cell cycle. The main structural component of yeast telomeres is Rap1p, which binds the double-stranded TG_{1-3}/AC_{1-3} repeats (*RAP1* also has a human homolog that is telomere associated, although it doesn't appear to bind telomeric DNA directly. Li et al.). Various genetic screens have uncovered over 30 gene products that influence telomere maintenance. Of these, four are known to play central roles in the telomerase pathway. Termed *EST* genes (for Ever Shortening Telomeres), deletion of any one of these genes leads to a telomerase-null phenotype. That is, the cells are initially normal, but their telomeres shorten with each cell division to the point where after a finite number of divisions the telomeres are too short and the population dies. In this respect, *EST* deletion yeast resemble telomerase-negative primary human somatic cells in culture.

Telomerase is a specialized reverse transcriptase that uses an integral RNA subunit as a template to add telomere repeats to the chromosome. In yeast, the telomerase catalytic subunit is encoded by *EST2* and the RNA subunit by *TLC1*. In *in vitro* assays, these two gene products alone are sufficient for telomerase activity. The role of the *EST1* gene product is less well understood. Est1p binds both single-stranded G-rich telomeric DNA (but not C-rich or double-stranded) as well as *TLC1* RNA *in vitro*. However, Est1p is dispensable in *in vitro* telomerase assays, and so is not necessary for catalysis. The fact that Est1p binds *TLC1* RNA suggests that it may be a subunit of a telomerase holoenzyme, but this has not been demonstrated. It is known that the interaction between Est1p and *TLC1* RNA is not dependent on Est2p, so it is possible that there are in fact two *TLC1* RNA-containing complexes in yeast - one with Est1p and the other with Est2p. Because Est1p binds both the *TLC1* RNA and the G-rich strand of telomeric DNA, it is thought that this protein may act as a bridge to recruit the catalytic complex to the telomere late in S phase when G-rich overhangs form (presumably such recruitment is not necessary *in vitro*). Less is known about the *EST3* gene product. Again, Est3p is dispensable in *in vitro* assays, so the protein may be involved in recruitment or regulation of the catalytic core.

The fourth *EST* gene is also known as *CDC13*, and is emerging as the central coordinator of telomere maintenance in yeast. Cdc13p has high affinity for single-stranded G-rich telomeric DNA, but unlike Est1p it does not bind RNA. Cdc13p has low affinity for human telomeric G-rich DNA, which suggests that a functional homolog may exist in humans (especially since G-rich overhangs also occur in human cells). Such a homolog has not been identified yet. Like Est1p, Cdc13p is not required in *in vitro* telomerase assays and so is not necessary for catalysis. Sever al lines of evidence point to two separate roles for this protein in telomere replication. First, a temperature sensitive allele, *cdc13-1*, suffers massive degradation of the C-rich strand at non-permissive temperatures, indicating that Cdc13p limits the small amount of degradation that normally creates G-rich overhangs late in S phase. Recent work from the Zakian lab showed that Cdc13p interacts

directly with DNA polymerase alpha (Qi and Zakian). One model for G-rich overhang formation is that Cdc13p recruits DNA polymerase alpha to "fill in" C-strand DNA that is degraded by an as yet unknown nuclease. The competition between "fill-in" and nucleolytic activities results in the G-rich overhang. Presumably, the *cdc13-1* allele loses the ability to recruit DNA polymerase at non-permissive temperatures and massive nucleolytic loss of the C-strand results. In addition to regulating formation of the G-rich overhang, Cdc13p is necessary for telomerase activity *in vivo*. Another mutant allele, *cdc13-2*, has normal G-rich overhangs but acts as a telomerase null. Because Cdc13p is not required for catalysis *in vitro*, it is thought that Cdc13p must recruit the catalytic core. Cdc13p binds Est1p both i*n vitro* and in yeast two-hybrid analysis, and so one model for recruitment is that Cdc13p regulates formation of the G-rich overhang late in S phase by binding the single stranded DNA, and in turn recruits telomerase through contact with the Est1p subunit (Qi and Zakian). Consistent with this model, a fusion between Cdc13p and either Est1p or Est2p results in abnormal lengthening of telomeres *in vivo*. Significantly, the fusion between Cdc13p and Est2p completely bypasses the requirement for Est1p. This bypass is consistent with Est1p acting as a "bridge" between Cdc13p and Est2p/TLC1 RNA (Evans and Lundblad).

From Dr. Teng's final report:

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There are three goals to my current work:

1) Determine if the catalytic subunit of telomerase, Est2p, as well as Est1p and Cdc13p are truly components of telomeric chromatin *in vivo*.
2) If so, whether telomere association occurs only late in S phase as current models predict.
3) Examine the effect of mutations in *PIF1* on telomere association of telomerase components. Dr. Teng and others in lab have determined that there is a human homolog of *PIF1*, so it is likely that understanding Pif1p's effects on telomerase in the yeast system will be relevance to telomere maintenance in human cells.

Est1p, Est2p, and Cdc13p are telomere-associated in vivo.

Chromatin immunoprecipitation (ChIP) is a technique that allows one to determine if a protein is associated with any given DNA sequence in the yeast genome *in vivo*. Growing cells are treated with formaldehyde, which almost immediately induces reversible protein-protein and protein-DNA crosslinks throughout the cell. Excess formaldehyde is then quenched by addition of glycine and the cells are washed extensively. The cells are lysed, and the chromatin is sonicated to shear it into small fragments that will be soluble. The protein of interest is immunoprecipitated, washed extensively, and the sample heat treated to reverse the protein-protein and protein-DNA crosslinks. Co-precipitation of a given DNA sequence with the protein can then be assayed by PCR. I constructed a yeast strain that has a unique sequence tag immediately adjacent to the TG_{1-3}/CA_{1-3} telomeric repeats of chromosome VII-L (figure 1). To assay telomere association, I perform multiplex PCR to simultaneously amplify this telomere tag (TEL) and two control sequences that should not associate with telomeric proteins- one (ADH4) located approximately 6 kb inwards from the marked VII-L telomere and another (ARO1) that is ~700 kb from the nearest telomere. If a given protein is telomere associated *in vivo*, more of the TEL sequence should be co-precipitated than the other two sequences, and this enrichment will be detected in the PCR. I labeled Cdc13p, Est1p, and Est2p with 9X myc epitope tags to allow immunoprecipitation using commercially available anti-myc monoclonal antibodies.

Immunoprecipitation of each of the three proteins - Cdc13p, Est1p, and Est2p - caused enrichment of the TEL sequence as shown by multiplex PCR (figure 2). This enrichment was formaldehyde dependent, which indicates that the proteins were crosslinked to the DNA *in vivo* and did not spuriously bind the TEL sequence in the lysate *in vitro* to create a false-positive signal. For Est1p and Est2p, this is the first demonstration that these proteins are constituents of telomeric chromatin

Cell cycle regulation of Est1p, Est2p, and Cdc13p telomere association

I next asked when in the cell cycle these three proteins bind to telomeres *in vivo*. It is known that single stranded G-rich overhangs form at the telomeres late in S phase. Shorter overhangs may be present throughout the cell cycle, but these would be undetectable using current methods. Both Cdc13p and Est1p have affinity for single stranded G-rich telomeric DNA, so it seems likely that they associate with the telomere late in S phase. Because both proteins are thought to recruit telomerase, it is also likely that Est2p associates with the telomere at this time. To study the cell cycle dependence of telomere association, I arrested strains containing 9X myc tagged Cdc13p, Est1p, or Est2p in G1 phase using alpha factor. Some of these arrested cells were treated with formaldehyde for chromatin immunoprecipitation (ChIP), and the rest were allowed to proceed synchronously through the rest of G1, S, and G2/M by removing the alpha factor. The quality of the synchrony was determined by monitoring the transition from 1C to 2C genomic DNA content by FACS analysis (figures 3-5). Western blots in which an equal number of cell equivalents from each time point were loaded showed that the amount of all three proteins increases gradually as S phase and G2/M progress. This pattern is also seen for the bulk of other proteins in the cell, and represents new protein synthesis to fill the daughter bud that forms and enlarges at this time. Also, I observed that at the beginning of S phase there is an additional induction of Est1p expression (approximately two-fold). This induction is not apparent for Est2p or Cdc13p, and indicates that if Est1p and Est2p are components of a telomerase holoenzyme, the stoichiometry of this complex must be different in G1 phase than in S phase. In terms of the ChIP results, I find that the peak of Est1p telomere association occurs late in S phase - consistent with binding to the G-rich overhang. Significantly, a slight enrichment of the TEL sequence over the no-tag control is apparent at other points in the cell cycle besides late S. It remains to be seen whether these signals reflect *bona fide* telomere association, but the fact that the fold

enrichment at these times is higher than that seen in a 9X myc Est1p *tlc1Δ* strain suggests this is the case (these *tlc1Δ* data will be discussed elsewhere). Therefore, it appears that Est1p is constitutively associated with the telomere. The higher signal of Est1p in late S phase could reflect one of three things - either at this time the protein undergoes a conformational shift that allows more efficient crosslinking, another factor either enters or leaves the telomere chromatin to allow more efficient crosslinking, or extra copies of Est1p in addition to the one(s) already bound associate. At this time, I cannot distinguish between these possibilities, but the fact that Est1p is induced at the beginning of S phase may suggest that additional protein is being recruited to the telomere late in S phase.

The ChIP pattern for Est2p is significantly different than that of Est1p. Peak telomere association in this case occurs in G1 phase, a point at which telomerase is not thought to act. The Est2p ChIP signal decreases for the duration of S phase, and sinks even lower in G2/M. Even at this lowest point, the Est2p ChIP signal is still higher than any Est1p signal, so I conclude that Est2p is also constitutively associated with the telomere (although the possibility remains that it may dissociate and re-associate quickly; this would be missed in the relatively widely spaced 15 minute time points used here). These data show that the current model for telomerase action in which the enzyme is recruited to the telomere only late in S phase must be revised. Just as for Est1p, the variability of the Est2p ChIP signal in the cell cycle can reflect a change in conformation, differential association with other factors, or possibly a change in the number of enzymes bound. Clearly, the pattern is complex and further analysis will be required. As I will discuss below, Est2p ChIP performed in various mutant backgrounds may be the key to understanding these cell cycle data.

Finally, the ChIP data for Cdc13p mirror those of Est1p closely, except for more robust TEL enrichment and a more gradual return to baseline value after the peak in S phase. In summary, these experiments show that the current picture of the telomerase pathway is not accurate. All three proteins appear to be constitutive members of telomeric chromatin. The fact that Est1p and Cdc13p ChIP signals increase late in S phase, concurrently with the known time of G-rich overhang formation, suggests that this may be the time at which telomerase actually adds new telomeric repeats. The fact that Est2p is bound at this time, albeit not at its peak level, supports this idea.

Est2p association in a *pif1* mutant background

The ChIP signal for Est2p varies significantly in the cell cycle, and is at its lowest late in S phase and into G2/M - the time that telomerase is thought to act. At the current time, I cannot say why the signal is changing, but it could represent a change in the conformation of Est2p late in S phase such that the crosslinking reaction to telomeric DNA is less efficient. Perhaps at this time, Est2p is actively polymerizing, and so its interaction with the DNA substrate is more transient and less likely to be crosslinked. Alternatively, the reduction in signal could represent the action of other factors that regulate telomerase. A first step to understanding this relatively complex change in ChIP signals during the cell cycle is to examine Est2p in various mutant backgrounds. Among these will be *pif1*.

It is known that the helicase activity of Pif1p is necessary to inhibit telomerase, as point mutants that knock out this activity *in vitro* lead to long telomeres *in vivo* (Zhou et al). One model for Pif1p action is that it unwinds the RNA template component of telomerase, *TLC1*, from its single stranded telomeric DNA substrate. Inhibition of telomerase by this mechanism would be expected to reduce or eliminate association of telomerase with telomeric DNA. Perhaps the reduction in the Est2p ChIP signal late in S phase and into G2/M (figure 4) is due to this action of Pif1p. I intend to test whether Pif1p is responsible for this reduction in the ChIP signal by

performing a cell cycle synchrony ChIP experiment analogous to that shown in figure 4 in a *pif1* helicase-minus mutant background. If Pif1p is indeed responsible for unwinding *TLC1* from the DNA, I expect that the ChIP signal late in S phase and G2/M will increase in a *pif1* background.

Discussion

Well over 30 genes are known to affect telomere maintenance in yeast, suggesting that this process is complex and probably subject to many layers of regulation. Given that many of these yeast genes have human homologs, it is likely that telomere maintenance in human tumor cells will be at least as complex. I believe that chromatin immunoprecipitation is an excellent technique to begin dissecting the various functions of these yeast gene products. My data indicate that the current model where telomerase recruitment occurs only in late S phase is not accurate, and needs to be revised. A first step in understanding the relatively complex pattern of association seen in the cell cycle is to study association in various mutant backgrounds, of which *pif1* will be a priority. When I start my own research program, I intend to make ChIP experiments my main priority, and hopefully they will help provide a clear picture of the mechanism of telomere maintenance *in vivo*. I believe that knowledge of this mechanism in yeast will be of direct relevance for the study of cancer, including breast cancer, in humans.

MATERIALS AND METHODS

All general yeast procedures were performed as described in Rose *et al.* (1990).

Chromatin Immunoprecipitation

Construction of the reporter strain containing a uniquely tagged telomere on chromosome VII-L was essentially as described in Sandell and Zakian, which a modification of the plasmid to include unique primer sites such that they would occur between the telomere and the *URA3* gene. Labeling of *EST1*, *EST2*, and *CDC13* ORFs with 9Xmyc tags was performed using a technique described by Michaelis et al. Chromatin immunoprecipitation and alpha factor synchrony were as in Aparicio et al.

Key research accomplishments

- Est1p, Est2p, and Cdc13p are telomere-associated in vivo.
- All three proteins are constitutively associated in the cell cycle, although with varying ChIP signals.

Reportable outcomes

None published as yet.

(8) Conclusions

Very few of the over 30 yeast gene products known to affect telomere replication have been shown to be actual constituents of the telomeric chromatin. This work, although in its early stages, has already shown that three of these proteins, Est1p, Est2p, and Cdc13p, are telomere-associated *in vivo*. Contrary to current models for telomerase action, each of these proteins is constitutively associated with the telomere (with the possible exception of Est1p, whose signal is weak at times other than late S). However, the ChIP signals vary. The next

challenge is to understand the source(s) of these variations and what they infer about telomerase regulation. *PIF1* encodes a catalytic inhibitor of telomerase, and I will soon test if Pif1p does indeed inhibit telomere association of Est2p in a manner consistent with our model where the enzyme unwinds *TLC1* from the DNA template.

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(10) Appendices

Curriculum vitae

Andrew K.P. Taggart

Education

1996 to present	Postdoctoral Fellow, Molecular Biology	Princeton University
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Honors and Awards

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1993 - 1994	Penn State University Graduate Fellowship
1991 - 1993	Penn State Eberly College of Science Braddock Scholarship
1987 - 1988	University of British Columbia Undergraduate Scholarship

Publications

Coleman, R. A., Taggart, A. K. P., Burma, S., Chicca II, J. J., and Pugh, B. F. (1999). TFIIA regulates TBP and TFIID dimers. **Molecular Cell** *4(3)*, 451-7.

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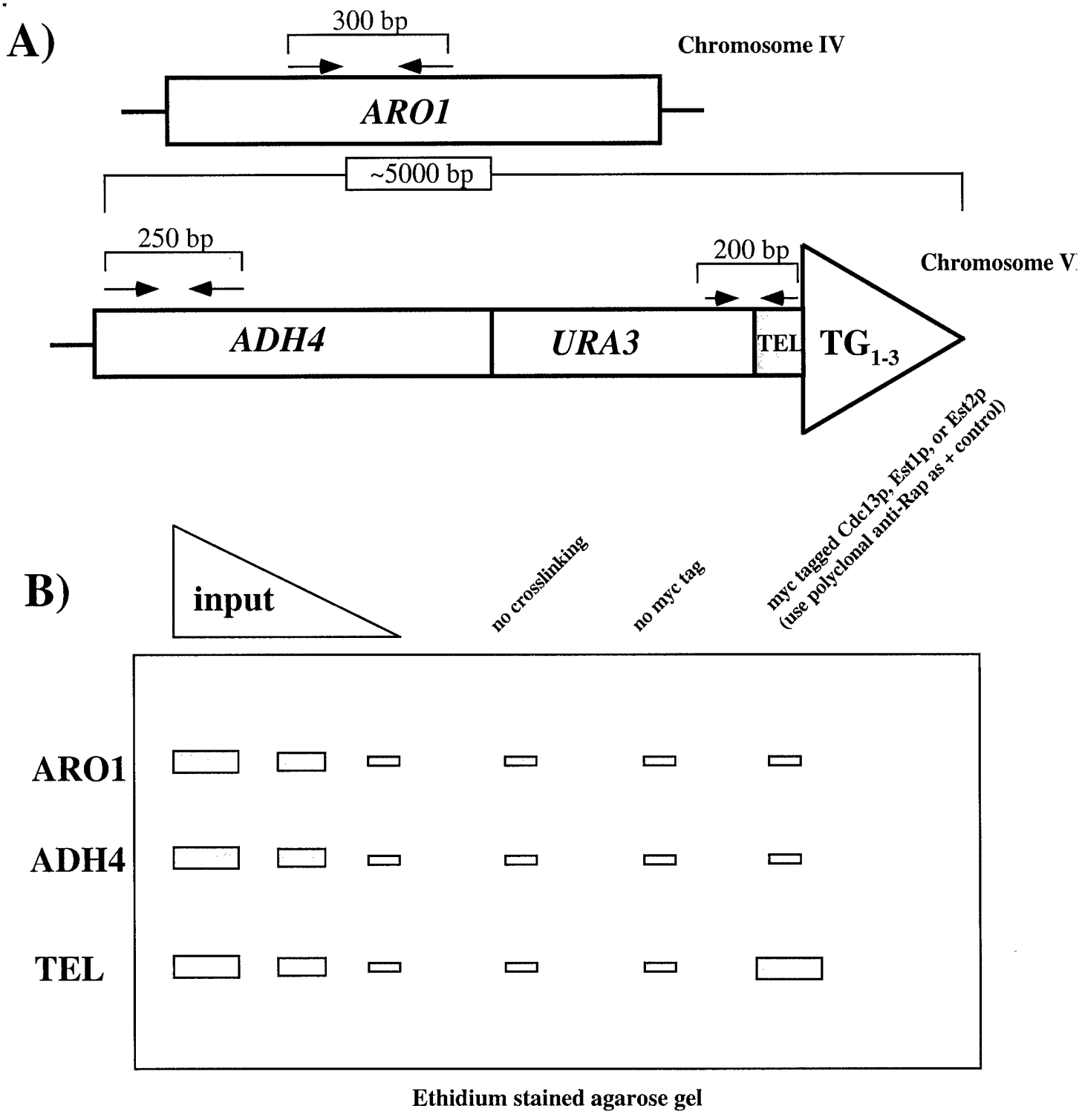
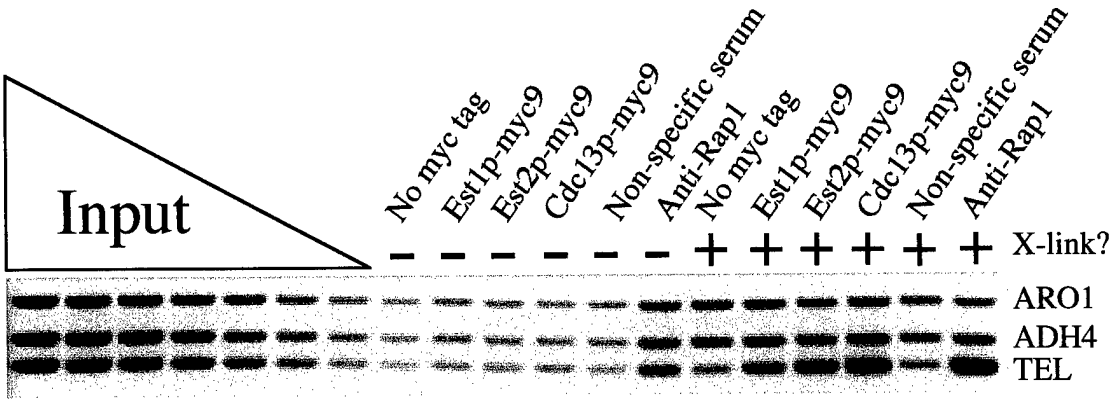


Figure 1. The assay system for chromatin immunoprecipitation (ChIP) analysis of telomere proteins. A) Schematic of the three target sequences amplified in multiplex PCR. Note that the TEL sequence is immediately adjacent to the telomere on chromsome VII. B) Expected results for a protein that is telomere-associated *in vivo*. Input DNA is sampled before the immunoprecipitation and so each target sequence shoul be present in equal amounts. Serial dilutions of input DNA are amplified to show the linear range of the PCF Controls for the ChIP include no crosslinking (any enrichment of a sequence in this control could indicate spurious i*n vitro* binding in the lysate) and also immunoprecipitation in the absence of a 9X myc epitope tag on the proteins of interest. In both controls, there should be some background contamination of genomic DN but the sequences should be present in equal amounts . If a 9X myc tagged protein is truly telomere-associat *in vivo*, an enrichment of the TEL sequence over ADH4 and ARO1 should be apparent (last lane).

A)



B)

Strain	no crosslinking		+ crosslinking	
	Δ ADH4	Δ TEL	Δ ADH4	Δ TEL
No tag	1	1	1	1
Est1p-myc9	1.0	0.9	1.1	2.5
Est2p-myc9	1.0	0.9	1.3	8.8
Cdc13p-myc9	1.0	1.2	1.4	16.5
Non-specific serum	1.0	0.9	1.1	1.2
Anti-Rap1	1.0	1.1	1.5	28.0

Figure 2. Cdc13p, Est1p, and Est2p are constituents of telomeric chromatin *in vivo*.
A) An agarose gel showing results of multiplex PCR from anti-myc immunoprecipitation ChIP analysis of 9X myc tagged Est1p, Est2p, and Cdc13p (note that twofold serial dilutions of the input DNA demonstrate the linear range of the PCR). As a positive control, polyclonal anti-Rap1p serum was used. As negative controls, extracts from the no-tag strain lacking the 9X myc epitope were precipitated with either anti-myc antibodies or a non-specific serum . In each case, enrichment of the TEL sequence is dependent on crosslinking, demonstrating that each protein is telomere-associated *in vivo*. A slightly higher overall background is routinely seen in the anti-Rap1 uncrosslinked sample, but the ratio of the three bands is always the same as the other non-crosslinked samples. B) Quantitation of the gel shown in A. Optical density (OD) of each band was determined using NIH image 1.62. Fold enrichment relative to the no-tag control for each sample is given by ΔADH4, which is defined as $(OD_{ADH4(tagged\ protein)})(OD_{ARO1(no\ tag\ control)}/OD_{ARO1(tagged\ protein)})/OD_{ADH4(no\ tag\ control)}$. ΔTEL is defined similarly. Note that the ratio of bands in the no-tag control is different plus or minus crosslinking, so non-crosslinked samples were only compared to the non-crosslinked no tag control and likewise for crosslinked.

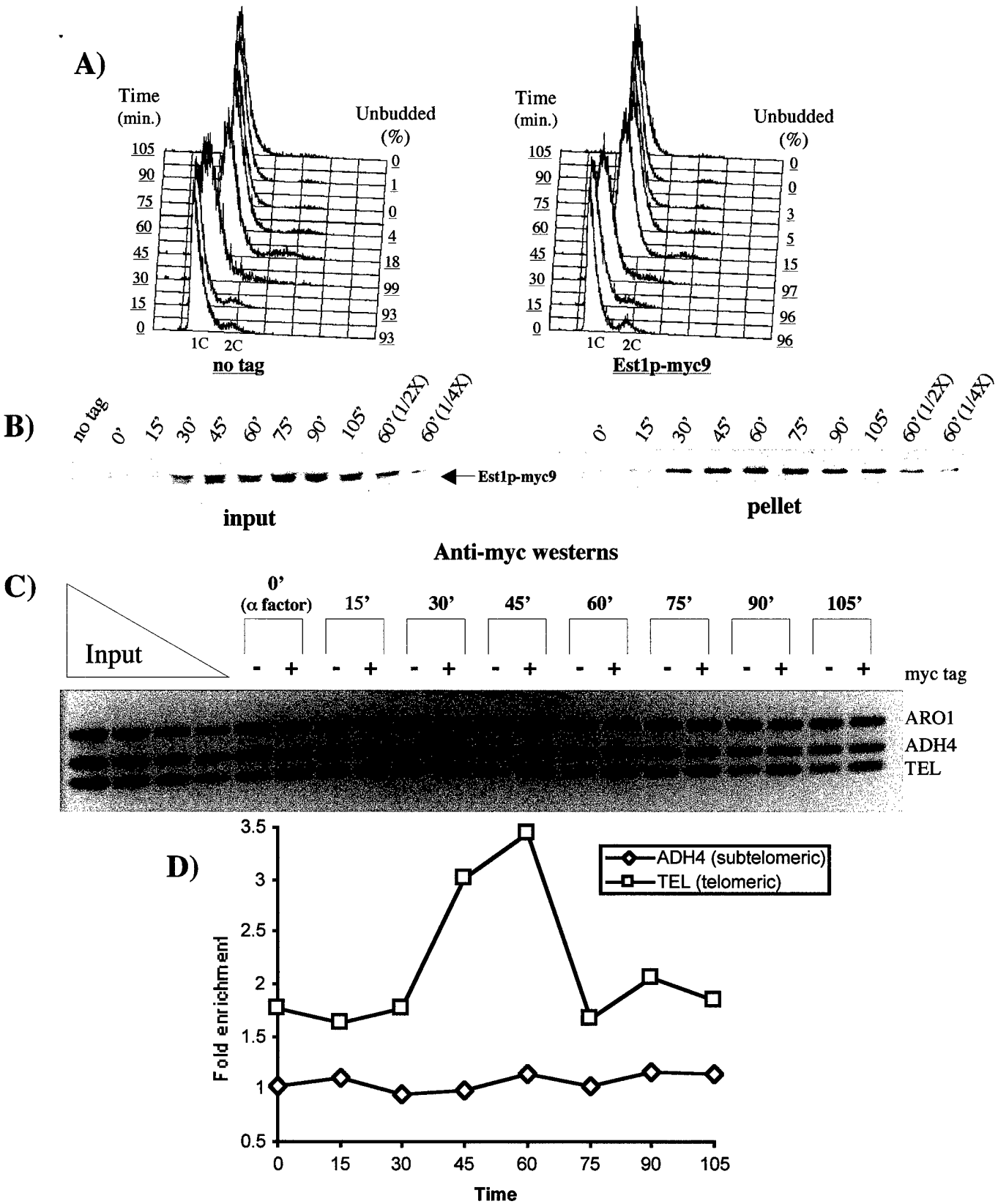


Figure 3. Association of Est1p with the telomere peaks in late S phase.

A) 9X myc Est1p cells were arrested in G1 with alpha factor, and then released to proceed synchronously through S phase to G2 and M. Samples were crosslinked and processed for ChIP analysis starting in alpha factor and every 15 minutes after the release. Quality of the synchrony was monitored by staining the genomic DNA with sytox green and visualizing the progress of DNA replication from 1C to 2C content by FACS analysis. B) Anti-myc Western blots show that the amount of Est1p per cell increases sharply at the onset of S phase and increases gradually thereafter as the daughter bud forms. In each case, the amount of protein that was immunoprecipitated (pellet) matches closely the amount that was in the extract (input). C and D) Multiplex PCR and quantitation (fold enrichment of ADH4 and TEL is relative to the no-tag control at each time point).

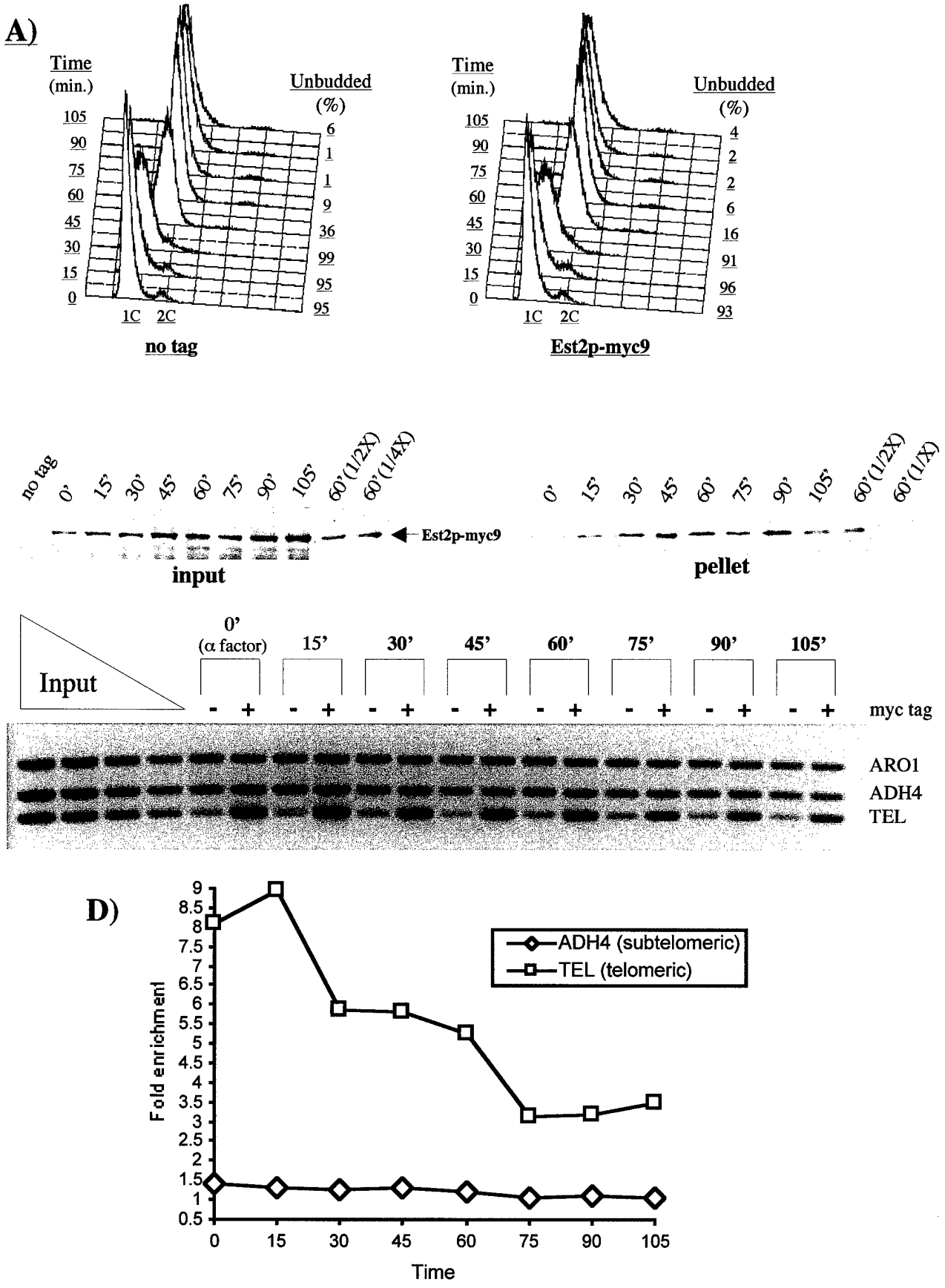


Figure 4. Association of Est2p with the telomere peaks in G1 phase.
This experiment was performed precisely as in figure 3. Note that an induction of Est2p is not seen at the beginning of S phase as it is for Est1p.

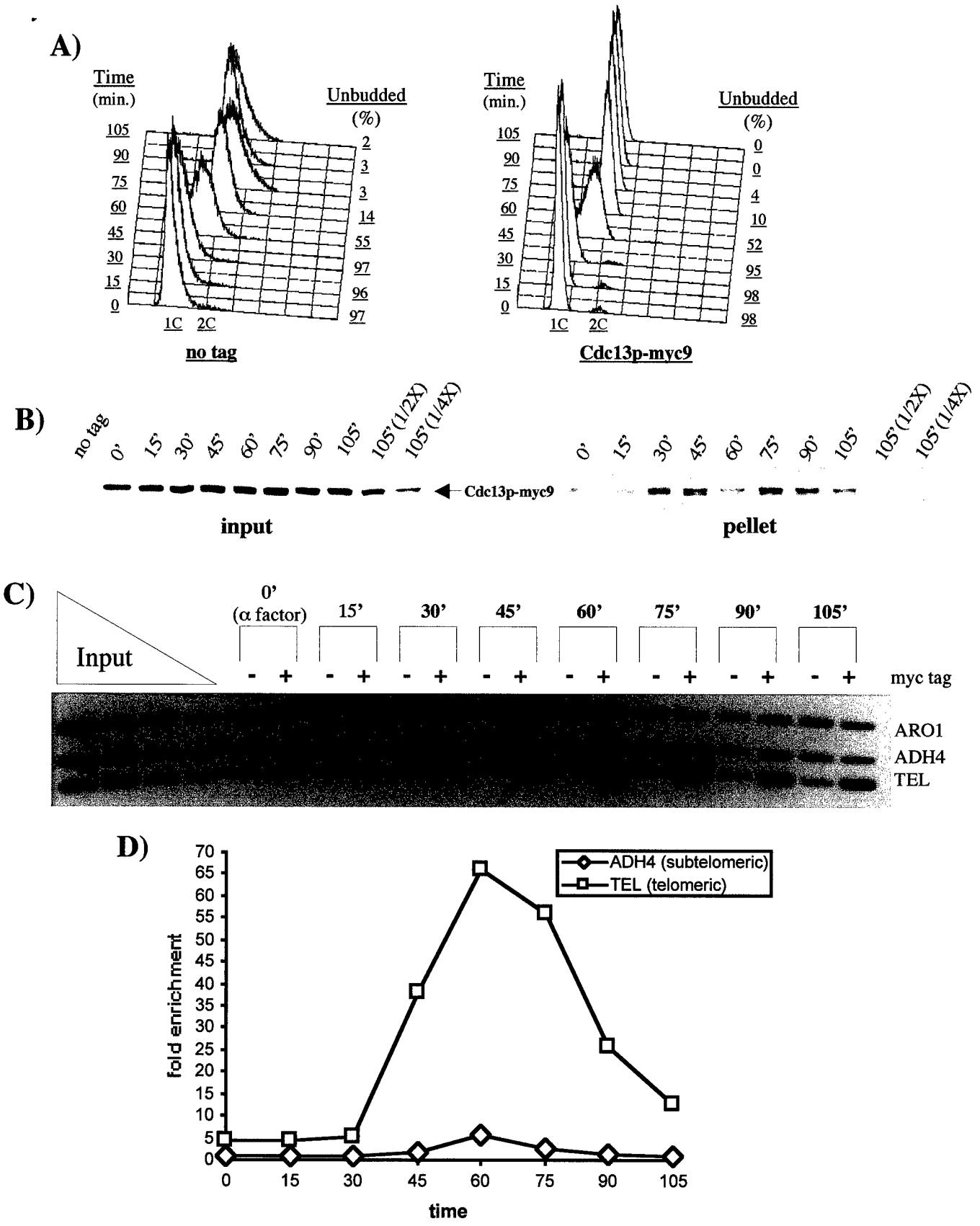


Figure 5. Association of Cdc13p with the telomere peaks late in S phase.
This experiment was done precisely as in figure 3. Note that unlike Est1p, neither Cdc13p nor Est2p are induced at the onset of S phase (panel B, also see figure 5).