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DEVELOPMENT AND CHARACTERIZATION
OF A NEW ASSAY TO EXAMINE TELOMERE-
PROTEIN INTERACTIONS *IN VIVO*

by
Brenda Bourns

A dissertation submitted in partial fulfillment of
the requirements for the degree of

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D.F. Bourns-Pope
Chairperson of Supervisory Committee

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to Offer Degree _____

Department of Pathology

Date _____

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Abstract

DEVELOPMENT AND CHARACTERIZATION
OF A NEW ASSAY TO EXAMINE TELOMERE-
PROTEIN INTERACTIONS *IN VIVO*

by Brenda Bourns

Chairperson of the Supervisory Committee: Professor Dan Bowen-Pope

Department of Pathology

An *in vivo* telomere binding assay using one-hybrid methodology was devised. A reporter gene, *lacZ*, was integrated immediately adjacent to a chromosomal telomere in *Saccharomyces cerevisiae*. If a telomere binding protein fused to a transcriptional activation domain were expressed in such a strain, the telomere binding portion should bind the telomere, thus bringing the activation domain within reach of the reporter gene and allowing activation. To test the system, the behavior of Rap1p, which is known to bind telomeres, was analyzed. As expected, Rap1 fused to an activation domain activated *lacZ* when it was near a telomere, but not when the reporter gene was located internally on a chromosome. These results confirmed the utility of a one-hybrid approach to assay telomere binding *in vivo*. Three other proteins implicated in telomere biology, Rif1p, Sir4p, and Sir3p tested positive in the system. A more sensitive assay was developed in which a minimally active *HIS3* allele was used as a reporter. Each of the fusion proteins that tested positive in the *lacZ* assay, as well as a Sir2 fusion protein, activated *HIS3* when it was located adjacent to a telomere. Additionally, in an even more sensitive telomere one-hybrid assay lacking the repression of genes near telomeres, Cdc13p tested positive for telomere association. These results demonstrated that Rif1p,

Sir2p, Sir3p, Sir4p, and Cdc13p associate with a *bona fide* chromosomal telomere *in vivo*, and provide a new assay for protein-telomere interaction. When *HIS3* plus adjacent telomeric sequence was integrated at an internal position on the chromosome, Rap1, Rif1, Sir2, Sir3, and Sir4 fusion proteins were still associated. In contrast, Cdc13p did not bind the internal telomeric sequence. These results strengthen the idea that Cdc13p binds telomeric sequence preferentially when it is located at chromosomal ends and imply that this assay is able to determine whether a protein has increased affinity for actual telomeric ends as opposed to internal telomeric sequence. Finally, the system was used to screen fusion libraries to obtain more information about proteins associated with chromosomal telomeres, as well as to attempt to identify novel telomere binding proteins.

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GLOSSARY

Telomere Position Effect. Reversible transcriptional repression of genes located near telomeres

LIST OF ABBREVIATIONS

3-AT. 3-amino-1,2,4-triazole

C-terminus. carboxyl-terminus

DNA. deoxyribonucleic acid

FOA. 5-flouoroorotic acid

N-terminus. amino-terminus

TPE. telomere position effect

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INTRODUCTION

BACKGROUND

Even before chromosomes were understood to carry the heritable material of cells, researchers were aware that the ends of these cytologically defined structures were somehow special. It was in the late 1930s that H. J. Muller first recognized that the ends were different from the rest of the chromosome. He noticed that flies subjected to X-irradiation showed all types of chromosomal abnormalities including deletions, inversions, and translocations, but that these events never involved a chromosomal end. He used the term "telomeres" to describe what he reasoned must be a specialized structure present at chromosome ends that was essential for chromosome integrity (Muller, 1941; Muller, 1938). Around the same time, Barbara McClintock was studying broken chromosomes in corn and noticed that ends resulting from a chromosomal break could fuse with other broken ends. This event often led to the formation of a chromosome with two centromeres that was then pulled apart in the next mitosis, ultimately resulting in the loss or the duplication of parts of chromosomes. The fact that natural ends did not undergo such fusion events prompted her to theorize that the essential function of telomeres is to protect against the end to end fusions that lead to chromosome instability (McClintock, 1939; McClintock, 1941).

TELOMERIC DNA

With the advent of molecular biology came the ability to define the DNA component of telomeres. The sequence and its arrangement were found to be largely

conserved among organisms; most chromosomes end in tandem arrays of simple, repeated G/C rich sequence (rev. in (Zakian, 1996)). These repeats are biased such that the G-rich strand is the strand whose 3' end is at the end of a molecule. The telomeres of many organisms, including vertebrates, consist of short, regular repeats. However, in the yeast *Saccharomyces cerevisiae*, telomeres consist of a variable number of irregular repeats of $(TG)_{1-6}TG_{2-3}$, often abbreviated TG_{1-3} (Shampay et al., 1984; Wang and Zakian, 1990).

Adjacent to the telomeric repeats, most organisms harbor telomere associated elements just internal to the G/C rich sequence. In yeast, these elements are of two major types, X and Y' (Chan and Tye, 1983). An individual telomere can contain 0-4 copies of the 6.7 kb Y' element which, when present, are found immediately adjacent to the TG repeats. In addition, most telomeres contain an ~560 bp X element (or a derivative thereof) internal to any Y' sequence (Louis et al., 1994). Often, stretches of ~50-130 bp of telomeric sequence are found between tandem Y' elements or between X and Y' (Walmsley et al., 1984).

Telomeres are dynamic structures. In yeast for instance, telomeres are not of a precise length, but vary between 225 and 375 base pairs with the average length being ~300 base pairs (Wright et al., 1992). This length varies from telomere to telomere within the same strain as well as between strains. Despite the lax constraint on precise telomere length, the average length appears to be well-regulated because several proteins are implicated in the process. For example, mutations in Tel1p and Tel2p cause telomeres to be respectively 250 and 150 bp shorter than wild type (Lustig and Petes, 1986). The precise function of these proteins is not yet known, however, they have been postulated to function in telomere length regulation (Greenwell et al., 1995; Runge and Zakian, 1996). Although telomere length appears to be well regulated, yeast are able to generate and survive

considerable telomere length changes. For example, telomeres with lengths up to 4 kb have been detected in cells in which the telomere binding protein Rap1 is missing the C-terminal approximately 150 amino acids (Kyrion et al., 1992). Interestingly, these telomeres are very unstable, shortening by up to 3 kb or more within only a few generations.

Not only is the telomere sequence and its arrangement conserved, but the structure of the telomere end is as well. In diverse organisms, the G-rich 3' strand extends beyond the end of the duplex region to form a single-stranded overhang. In the macronuclear DNA of ciliated protozoa, the G-rich strand extends 12-16 bases beyond the end of the chromosome (Henderson and Blackburn, 1989; Klobutcher et al., 1981; Oka et al., 1980), whereas in mammalian chromosomes the overhangs may be as long as 45 bases (McElligott and Wellinger, 1997). While this presumably constitutive G overhang has not yet been detected in yeast, methods used to analyze the yeast telomere to this point would not have been able to detect an overhang shorter than 30bp (Wellinger et al., 1993). Considering that other features of telomeres are so well conserved, it seems probable that yeast telomeres have a short constitutive G overhang as well.

TELOMERE REPLICATION

In yeast, a different kind of 3' overhang has been detected. A transient overhang of greater than 30 bases is detected only in late S phase (Wellinger et al., 1993; Wellinger et al., 1993). Because this is the time during which telomeric DNA is replicated, this transient extension is thought to be a telomere replication intermediate.

Telomeres replicate by a method that is distinct from the rest of the chromatin. This unique method is thought to be necessary due to what has been dubbed "the end dilemma." Due to the polarity of DNA polymerase activity, the conventional

replication machinery would leave a gap where the last primer was removed at one end of each chromosome with each round of replication (Figure 1.1, Steps A-C) (Watson, 1972; Wellinger et al., 1996)]. Left unchecked, this would result in a gradual shortening of telomeres, eventually leading to the loss of essential genes.

In multicellular organisms this process of telomere shortening has been postulated to function as a "molecular clock" (Harley, 1991). Due to successive rounds of replication, older cells have shorter telomeres than younger cells (Harley et al., 1990). By monitoring the length of telomeres, cells could theoretically determine when to stop dividing as the telomeres reached a critical short length. This process would provide cells with a natural protection against cancer. Indeed, many tumor cells seem to have circumvented this natural protection, for tumor cells are often found to have telomeres that no longer shorten with successive cell divisions (rev. in (Harley, 1991)).

In unicellular organisms like yeast, or in other "immortal" cells, telomeres have a special function in replication. They solve the end dilemma by serving as a substrate for a ribonucleoprotein complex, telomerase ((Greider and Blackburn, 1985), rev. in (Blackburn, 1992)). This enzyme recognizes the single-stranded telomeric overhang and adds G-strand telomeric repeats to the end, thus compensating for the loss inherent in conventional replication. To accomplish this function, telomerase copies its RNA template, which contains the telomere consensus sequence, into DNA (Figure 1.1, Step D, left branch). Thus telomerase lengthens the G-rich overhang, allowing conventional replication machinery to fill in. In this way, no net loss of telomeric DNA need happen.

Recently, the transient G-rich overhang in yeast was demonstrated to be present on both ends of a linear DNA molecule (Wellinger et al., 1996). Because the

established model for telomere replication results in each daughter molecule having an overhang on only one end (Figure 1.1, Step D, left branch), Wellinger et al., reasoned that they were observing an overhang that resulted from a new step in telomere replication. They postulated that a C-strand specific exonuclease acts on telomeres at this time to make both ends a single-stranded overhang (Figure 1.1, Step D, right branch); therefore, both ends could be acted on by telomerase (Figure 1.1, Step E). Alternatively, or in addition, it might be necessary to process both ends to a single-stranded G-rich overhang so that they can form the shorter constitutive overhang postulated to be the normal structure of telomeres (Wellinger et al., 1996).

Because the single-stranded overhangs are present only in late S phase, the protein responsible for governing this degradation must be regulated in a cell cycle dependent manner. The protein Cdc13p is a good candidate for this regulator. Based on phenotypes of the *cdc13-1* strain, Wellinger et al. postulated that Cdc13p may be the protein that regulates the putative C-strand degradation they had identified (Wellinger et al., 1996). The temperature-sensitive *cdc13-1* cells were previously shown to accumulate single-stranded DNA at semi-permissive temperature (Garvik et al., 1995). This single-stranded DNA has a strand bias that is consistent with defective regulation of a C-strand specific exonuclease. Additionally, *cdc13-1* cells, at non-permissive temperature, arrest at the G2/S boundary in the cell cycle — near the time telomeres are replicated (Hartwell, 1973; McCarroll and Fangman, 1988). In support of this hypothesis, Cdc13p binds single-stranded telomeric DNA *in vitro* (Lin and Zakian, 1996; Nugent et al., 1996). Another mutant allele, *cdc13-2*, is defective in the telomerase pathway (Nugent et al., 1996), therefore, Cdc13p may have a second regulatory

function: to recruit telomerase to the telomere. Both of these models assume that Cdc13p binds to actual chromosomal telomeres *in vivo*.

TELOMERIC PROTEINS

Although the DNA component of yeast telomeres has been well characterized, the protein components are less so. Yeast telomeres are known to be organized in a special non-nucleosomal protein-DNA complex, termed a telosome. This complex is quite large, encompassing approximately 2 1/2 times the amount of DNA in a nucleosome. The abundant, sequence specific, DNA binding protein Rap1p is its major component (Wright et al., 1992), binding to approximately 20 sites per telomere (Gilson et al., 1993; Wang and Zakian, 1990). In addition to binding to telomeres, Rap1p can act as either a transcriptional repressor or transcriptional activator depending upon the chromosomal context of its binding site (rev. in (Shore, 1994)).

Other proteins are thought to associate with telomeres via their binding to Rap1p. The proteins, Rif1p and Rif2p, were isolated in a screen for Rap1p interacting factors. Although little is known about how these proteins function, they appear to be involved in telomere length regulation because strains deleted for either possess longer than normal telomeres (Hardy et al., 1992; Wotton and Shore, 1997).

In addition, the Sir proteins are thought to associate structurally with the telomere. These proteins were originally identified as proteins required to repress transcription at the silent mating type loci (Rine and Herskowitz, 1987). Subsequently, three of the Sir proteins, Sir2, Sir3, and Sir4 were found to be essential for a phenomenon known as telomere position effect (Aparicio et al., 1991).

Telomere Position Effect

Telomere position effect (TPE) is the reversible repression of transcription of genes located near a telomere (rev. in (Grunstein, 1997)). This effect is thought to result from a specialized "heterochromatin-like" chromatin structure that excludes access by basal transcription factors (Aparicio et al., 1991; Gottschling, 1992; Gottschling et al., 1990). Several lines of evidence indicate that chromatin structure plays a role in this localized repression of genes. First, mutations in the N-termini of histones H3 and H4 eliminate TPE (Aparicio et al., 1991; Hecht et al., 1995; Thompson et al., 1994). Second, the DNA adjacent to telomeres resists modification by the *E. coli* protein *dam* methylase when it is expressed in yeast (Gottschling, 1992). This resistance is interpreted as being due to a "closed" chromatin structure that is eliminated under conditions where TPE is abolished. Finally, telomere adjacent chromatin contains histones that are hypoacetylated relative to the rest of the chromatin, and this acetylation state is eliminated when TPE is abolished (Braunstein et al., 1993).

One model to explain TPE posits that Rap1p recruits a complex of Sir proteins to the telomere (rev. in (Grunstein, 1997)). Sir3p and Sir4p then oligomerize, interacting with histones and each other to spread inward along the chromosome, setting up a "closed" chromatin structure as they go. This model stems from genetic data implicating interactions of each of these proteins with the others. For example, two hybrid analysis indicates that both Sir3p and Sir4p interact with Rap1p, as well as with each other and themselves (Moretti et al., 1994). Furthermore, overproduction of Sir3p suppresses the loss of TPE seen in certain point mutants in the carboxyl terminus of *RAP1*, whereas deletion of the carboxyl region of Rap1p does not allow this suppression (Liu et al., 1994). The model

is further supported by *in vitro* (Hecht et al., 1995) and genetic (Johnson et al., 1990) evidence that Sir3p and Sir4p interact with histones H3 and H4. Moreover, immunoprecipitation experiments and protein affinity chromatography analysis indicate that Sir4, Sir3, and Rap1 proteins are present in a complex that is associated with DNA near the telomere (Hecht et al., 1996; Moazed and Johnson, 1996; Strahl-Bolsinger et al., 1997). Finally, Sir3p overexpression causes silencing to extend farther inward (Renauld et al., 1993), and is accompanied by Sir3 and Sir4 protein presence farther along the chromatin (Hecht et al., 1996; Strahl-Bolsinger et al., 1997).

Although it is less clear that Sir2p is also a structural component of the heterochromatin-like DNA, one recent study shows that Sir2p can associate with sub-telomeric DNA (Strahl-Bolsinger et al., 1997). In addition, overexpression of Sir2p leads to histone H4 deacetylation (Braunstein et al., 1993), one of the hallmarks of silenced DNA. These results led to the hypothesis that one function of Sir2p may be to regulate histone acetylation in silenced domains. This could be accomplished either through a catalytic activity of the enzyme itself, or by inhibiting acetylation by a histone acetyltransferase (Braunstein et al., 1996). If it is an inhibitor, Sir2p could function by specifically inactivating an acetyltransferase enzyme or by associating with nucleosomes and restricting access of the enzyme to the histones.

It is also believed that the association of telomeres with the nuclear periphery is important for TPE (Gotta et al., 1996; Palladino et al., 1993). Telomeres from a variety of organisms are known to associate both with each other and with the nuclear periphery (rev. in (Gilson et al., 1993)). Immunofluorescence and *in situ* hybridization has shown that yeast telomeres are often clustered in a limited number of discrete foci that

appear to be near the nuclear periphery (Cockell et al., 1995; Gotta et al., 1996; Palladino et al., 1993). Apparently, the Sir proteins are involved in this property of telomeres because deletions in *SIR3* and *SIR4* cause a dispersion of the clustered telomeres (Cockell et al., 1995; Gotta et al., 1996; Palladino et al., 1993).

Structural proteins

Recently, molecular techniques in yeast have made it possible to demonstrate what was postulated over 50 years earlier — that telomeres are essential for chromosome stability (McClintock, 1939; McClintock, 1941; Muller, 1941; Muller, 1938). These experiments demonstrated that it is the TG_{1-3} sequences and their associated proteins that are necessary for chromosome stability (Sandell and Zakian, 1993). In these experiments, removal of telomeric repeats from the end of a chromosome usually led to loss of that chromosome, presumably by degradation. It seems likely that this crucial function of telomeres is mediated by structural proteins that associate with the telomeric repeats. Indeed, *Oxytricha* terminus binding proteins have been identified that bind tenaciously to the single-stranded overhang, and protect DNA from exonuclease digestion *in vitro* (Gottschling and Zakian, 1986; Price, 1990; Price and Cech, 1987).

Another structural protein, Rap1p, has been implicated in the maintenance of yeast telomeres. A temperature-sensitive allele of Rap1p exhibits a shortening of telomeres (Conrad et al., 1990). This has been interpreted to indicate that Rap1p protects telomeres from degradation. *RAP1* is an essential gene. Because the DNA binding domain is in the region that is required, it is possible that the essential function of Rap1p is to protect telomeres from degradation (Lustig et al., 1990). Furthermore, another putative structural protein, Cdc13p, appears to protect the chromosome from strand-specific degradation that

originates at the telomere (Garvik et al., 1995). Additionally, this protein seems to function in a regulatory capacity, limiting or facilitating access of telomere replication machinery to telomeres (Nugent et al., 1996). Knowing whether this protein associates with telomeres *in vivo* is critical to the understanding of how it functions.

Existing assays for determining whether a structural protein is located in the vicinity of a telomere involve *in vitro* techniques (Cockell et al., 1995; Palladino et al., 1993; Strahl-Bolsinger et al., 1997). While these methods clearly generate valuable information, some interactions detected *in vitro* do not seem to be relevant *in vivo*. As an example, *in vitro* binding to single-stranded telomeric G-strand sequence was used to search for an end-binding factor in yeast (Lin and Zakian, 1994). Some of the proteins identified in this way did not appear to function at telomeres and may be RNA binding proteins *in vivo*. Ideally, one would be able to monitor protein-telomere associations *in vivo*, thereby increasing the likelihood that detected interactions are biologically significant.

Because telomeres are critical to the stability of chromosomes, it is important to gain an accurate understanding of telomere-protein interactions *in vivo*. Therefore, I set out to develop a strategy to determine whether a given protein was associated with the telomere in living yeast cells, as well as to characterize detected interactions. Furthermore, because it is possible that not all proteins that act at telomeres have been identified, I used the telomere one-hybrid system to screen for new interactions and to learn more about previously known interactions.

SUMMARY

AN *IN VIVO* ASSAY TO STUDY STRUCTURAL PROTEINS ASSOCIATED WITH A TELOMERE

One-hybrid systems were developed as a means to screen for DNA binding proteins (Dalton and Treisman, 1992; Li and Herskowitz, 1993; Wang and Reed, 1993). In these screens, the binding site of interest is placed immediately upstream of a promoter-compromised reporter. Then, a fusion library that encodes protein fragments fused to an acidic transcriptional activation domain is introduced. If a peptide binds to the binding site, it brings the activation domain into proximity of the reporter, and activates it. Any plasmid that activates transcription encodes a peptide that is potentially a sequence-specific DNA binding protein, or is associated with one.

I conceived of the idea of using one-hybrid methodology slightly differently. It seemed that the technique could be readily adapted for use as an *in vivo* telomere binding assay for *Saccharomyces cerevisiae*. In the past, one-hybrid systems have taken DNA sequences out of their normal context to assay for binding proteins. If, instead, the DNA binding site were in its normal, functioning context, it could be monitored by a one-hybrid method to determine whether a protein actually associates with that structure in a living cell.

To develop a telomere one-hybrid system, I constructed a strain that contained a reporter gene, *lacZ*, immediately adjacent to a chromosomal telomere (Figure 1.2). Upon binding to the telomere, a telomere binding protein fused to a transcriptional activation domain would bring the activation domain within reach of the reporter gene and cause activation of that gene (Figure 1.2, left side). Furthermore, expressing the fusion

protein in a strain with the reporter gene integrated at an internal position on the chromosome should have no effect on the reporter gene expression (Figure 1.2, right side).

To test the system, I expressed a fusion of the known telomere binding protein Rap1p fused to a transcriptional activation domain in the strain with *lacZ* at a telomere. The system behaved as predicted, thus validating the use of a telomere one-hybrid system as a new tool to assay telomere binding *in vivo*. The telomere one-hybrid system was made more sensitive by using the *HIS3* gene as a reporter. This HISTEL strain was then employed as a means to test whether specific proteins were associated with a chromosomal telomere. The system was able to detect the association of several proteins, and furthermore, allowed the first demonstration in living cells of telomere interaction with a protein that binds single-stranded telomeric DNA *in vitro*.

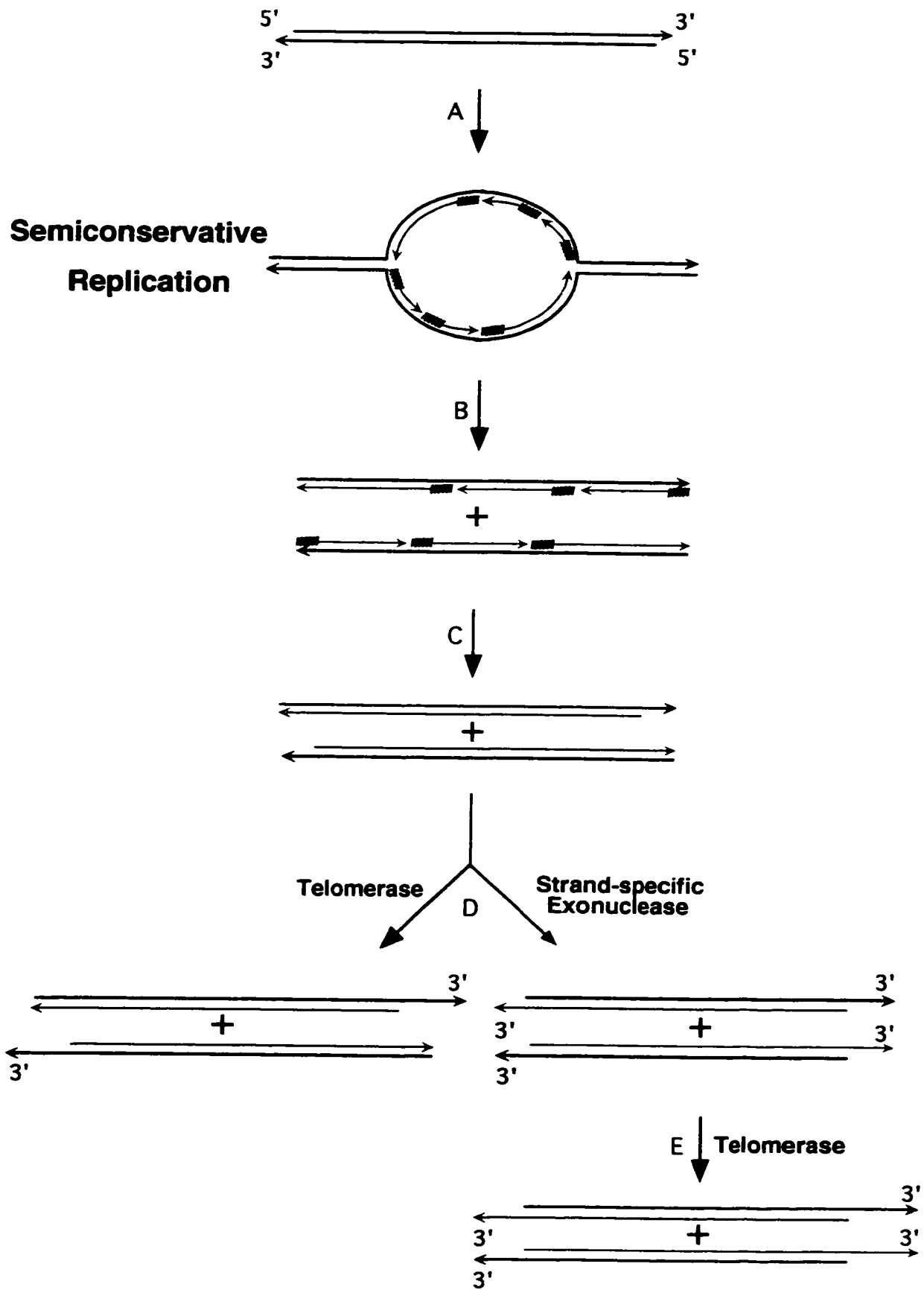
CHARACTERIZATION OF PROTEIN INTERACTIONS WITH A TELOMERE USING ONE-HYBRID METHODOLOGY

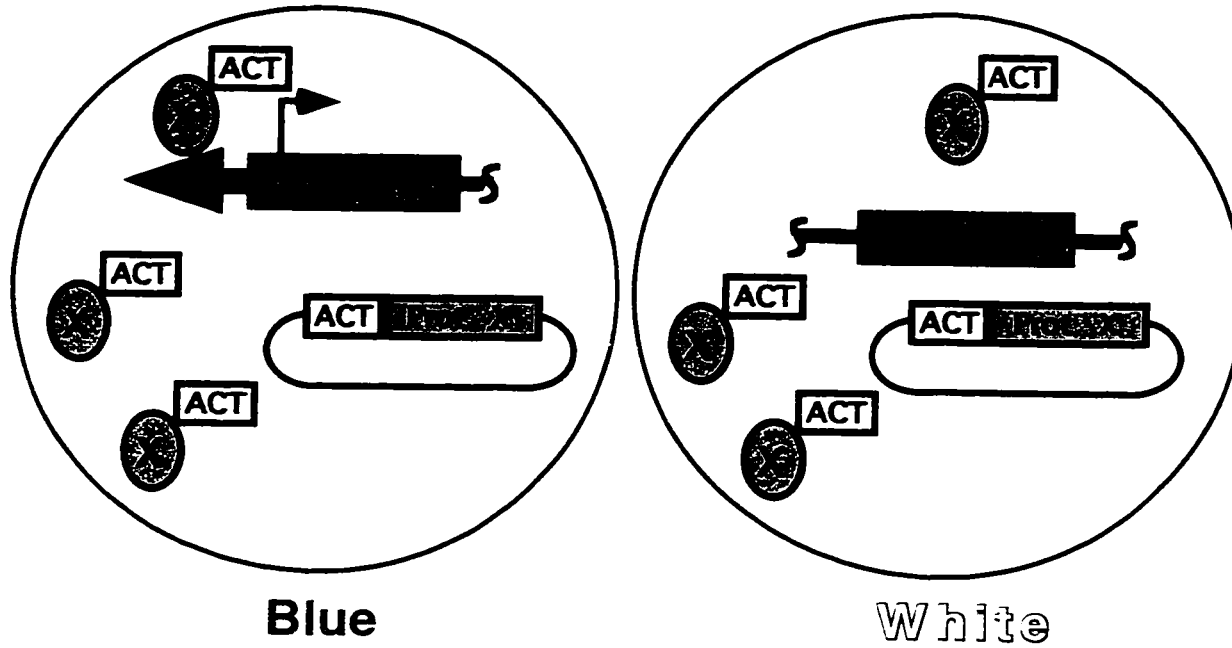
Given that the telomere one hybrid system was useful for assaying protein interactions with a telomere, it seemed that it might also be used to characterize further those interactions. Therefore, the telomere one-hybrid system was modified so that it could be used for this purpose.

A TELOMERE ONE-HYBRID SCREEN TO IDENTIFY TELOMERE ASSOCIATED PROTEINS

Finally, this work describes the screening of a library for novel telomere associated proteins. Using the telomere one-hybrid system as a screen for novel proteins, two Sir4 peptides and Rif1p were identified. Several other genes await further characterization to determine whether they encode true telomere binding proteins.

Figure 1.1 The end dilemma. The replication of linear molecules of DNA presents eukaryotic cells with a unique problem. Because DNA polymerase acts in the 5' to 3' direction, a gap will be left at the end of each molecule when the last primer is removed (C) at the end of S phase. This would lead to gradual shortening of the chromosome with successive rounds of replication. To compensate for this loss, eukaryotic cells have evolved a unique enzyme to replicate telomeres. The ribonucleoprotein complex telomerase, recognizes these single stranded 3' overhangs, and adds telomeric sequence to them (D, left branch). Recently, an additional step in telomere replication has been postulated (D, right branch). A strand-specific exonuclease is thought to process ends such that both ends of a molecule have single-stranded 3' overhangs at the end of S phase. This would allow telomerase to act on both ends of each molecule (E, right branch).





Candidate Telomere Associating Protein



Transcriptional Activation Domain

Figure 1.2 Schematic of the One-Hybrid System. In both cases, the plasmid expresses protein X fused to the activation domain. Only when Protein X interacts with a telomere adjacent to the *lacZ* gene (left), does the colony turn blue when assayed for β -galactosidase. When the *lacZ* gene is instead internal in a chromosome (right), the colony remains white.

CHAPTER 2: A TELOMERE ONE-HYBRID ASSAY CAN BE USED TO DETERMINE TELOMERE-PROTEIN INTERACTIONS *IN VIVO*

SUMMARY

An *in vivo* telomere binding assay was devised using one-hybrid methodology. A reporter gene, either *lacZ* or *HIS3* was integrated immediately adjacent to a chromosomal telomere. When a telomere binding protein fused to a transcriptional activation domain was expressed in such a strain, the telomere binding portion bound the telomere and allowed the activation domain to activate transcription of the adjacent gene. A known telomere binding protein, Rap1p, as well as three candidate telomere binding proteins, Rif1p, Sir3p, and Sir4p, tested positive in both one-hybrid systems. Additionally, Sir2p, and Cdc13p as well as Rap1p, Rif1p, Sir3p, and Sir4p tested positive in the more sensitive *HIS3* assay. For each fusion protein, it was shown that expression from the reporter gene was due to its transcriptional activation, not to the alleviation of telomere position effect. None of the fusion proteins activated *lacZ* or *HIS3* transcription when either gene was integrated at an internal chromosomal position. The finding that Cdc13p binds telomeres is the first demonstration in living cells of an interaction that is likely to occur via the single-stranded overhang found at telomere ends. These results demonstrate that Rif1p, Sir2p, Sir3p, Sir4p, and Cdc13p associate with a *bona fide* chromosomal telomere *in vivo*, and provide a new assay for protein-telomere interactions.

INTRODUCTION

BACKGROUND

Telomeres are the protein-DNA complexes at the ends of eukaryotic chromosomes. Telomeric DNA typically consists of simple, repeated sequence with the strand running 5'-3' towards the end of the molecule being rich in G residues. In at least some organisms, this G rich strand extends beyond the end of the molecule (reviewed in (Zakian, 1995)). Because these extensions are found in organisms from single-celled eukaryotes (Wellinger et al., 1993) to vertebrates (McElligott and Wellinger, 1997), it is probable that they are a universal feature of eukaryotic chromosomes. In the yeast *Saccharomyces cerevisiae*, telomeres consist of a variable number of repeats of the sequence TG₁₋₃ (Shampay et al., 1984). The ~300 bp of yeast telomeric DNA is organized in a large, non-nucleosomal, protein-DNA complex, the telosome (Wright et al., 1992). Genetic (Conrad et al., 1990; Lustig et al., 1990), biochemical (Wright et al., 1992), and cytological (Gotta et al., 1996) data show that the sequence-specific duplex DNA binding protein Rap1p is the major protein in the yeast telosome. In addition to binding to telomeres, Rap1p can act as either a transcriptional repressor or transcriptional activator depending upon the chromosomal context of its binding site (rev. in (Zakian, 1989)). Typically, a yeast telomere contains ~20 Rap1p binding sites (Gilson et al., 1993; Wang and Zakian, 1990).

Besides Rap1p, there exist other candidate telosomal proteins. Three of these candidates, *SIR2*, *SIR3* and *SIR4*, are known to be essential for telomere position effect (TPE) (Aparicio et al., 1991). TPE is the reversible silencing of telomeric genes thought to result from a specialized chromatin structure that excludes access by basal transcription factors (Gottschling, 1992, Aparicio et al., 1991). The association of these

other candidate proteins most likely occurs, not by binding directly to the DNA, but via protein-protein interactions. In one model for a mechanism for TPE, Rap1p at the telomere nucleates a complex of Sir3 and Sir4 proteins that then oligomerize inward via their interactions with histones, silencing genes in adjacent chromatin (rev. in (Grunstein, 1997)). The mechanism by which silencing occurs may be via the recruitment of Sir2p by Sir3p and Sir4p (Braunstein et al., 1996). Silenced genes are associated with hypoacetylated histones, and the role of Sir2p may be to facilitate this hypoacetylation, because overexpression of Sir2p has been shown to correlate with a general hypoacetylation of histones (Braunstein et al., 1993).

Although neither Sir3p nor Sir4p have been shown directly to bind telomeres, other evidence implicating the presence of Sir3p and Sir4p at the telosome includes the localization of both proteins to the vicinity of telomeres by immunofluorescence analysis (Cockell et al., 1995; Palladino et al., 1993), as well as the ability of antibodies against Sir3p and Sir4p to immunoprecipitate sub-telomeric chromatin (Hecht et al., 1996; Strahl-Bolsinger et al., 1997). Deleting *SIR3* or *SIR4* causes a slight decrease in telomere length as well as a change in the clustering of telomeres at the nuclear periphery (Palladino et al., 1993). Whereas less is known about the role of Sir2p at a telomere, recent evidence suggests that it, too, might be associated with sub-telomeric DNA (Hecht et al., 1996). Although the Sir proteins have previously been localized to the telomeric vicinity (Cockell et al., 1995; Palladino et al., 1993; Strahl-Bolsinger et al., 1997), interaction with a *bona fide* telomere in living cells has never been demonstrated.

A fourth candidate telosomal protein, Rif1p, was isolated in a screen for Rap1p interacting factors. While little is known about the function of this protein, *rif1* Δ strains have long telomeres (Hardy et al., 1992) and an increase in TPE (Kyrion et al.,

1993; Wiley and Zakian, 1995). In addition, several proteins that bind to single-stranded (ss) TG₁₋₃ DNA *in vitro* have been identified (Lin and Zakian, 1994; Lin and Zakian, 1996; Nugent et al., 1996; Lendvay, 1996 #1243), although none has yet been shown to be associated with telomeres *in vivo*.

Telomere replication

One function of telomeres is to allow the complete replication of chromosomes. Due to intrinsic properties of the replication machinery, ends of chromosomes are expected to shorten 8-12 bases with each round of replication (rev. in (Zakian, 1995)). To compensate for this loss, one function of telomeres is to recruit telomerase, a ribonucleoprotein that adds telomeric repeats specifically to the ends of chromosomes. Recent work has shown that a single-stranded G-rich overhang of >30 bases appears on telomeres during the time telomeres are replicated (Wellinger et al., 1993; Wellinger et al., 1993; Wellinger et al., 1993). This overhang is detected in cells lacking *TLC1*, the RNA component of telomerase (Dionne and Wellinger, 1996). This, along with the finding that the overhang occurs on both ends of a newly replicated DNA molecule, supports the hypothesis that there exists a hitherto unknown step in telomere replication (Wellinger et al., 1996). In this model, a strand-specific exonuclease acts on both ends of a linear molecule to create the substrate on which telomerase acts: a single-stranded G-rich overhang (rev. in (Blackburn, 1992)). Because the cell division cycle mutant *cdc13-1* suffers extensive degradation of the C-rich strand, it was postulated that Cdc13p acts to block or regulate the C-strand degradation (Wellinger et al., 1996). Evidence in support of that notion is the finding that Cdc13p binds single-stranded telomeric G-strand DNA *in vitro* (Lin and Zakian, 1996; Nugent et al., 1996). In addition, another important role in telomere replication has been suggested for Cdc13p.

The fact that a different mutant allele, *cdc13-2*, is defective in the telomerase pathway (Nugent et al., 1996), led to the hypothesis that Cdc13p has a second regulatory function: to recruit telomerase to the telomere. Both of these models depend upon the ability of Cdc13p to bind a telomere *in vivo*. Because many proteins have been shown to bind telomeric DNA *in vitro*, but fail to affect telomeres *in vivo* (Lin and Zakian, 1994), demonstration of Cdc13p binding to telomeres *in vivo* is important.

Interest in telomeric structural proteins like Cdc13p is increasing as it becomes clear that in diverse organisms, they are responsible for protecting against degradation (Conrad et al., 1990; Garvik et al., 1995; Gottschling and Zakian, 1986), and regulating both positively and negatively access to telomeres during replication (Nugent et al., 1996; Wellinger et al., 1996). A surprisingly large number of proteins affect telomere length and biology. It is unlikely that all of them would be directly associated with a telomere and function as structural components. In addition, several proteins shown to have specific ss TG₁₋₃ binding activity *in vitro* have no effect on telomeres when deleted or overexpressed and are probably RNA binding proteins *in vivo*. Therefore, development of a means to assay telomere binding in living cells is critical to the understanding of how this process takes place.

To address whether the Rif1, Cdc13, and Sir proteins are functioning via direct association with a telomere, I developed a one-hybrid system to assay protein-telomere interactions in living cells. Because the binding site is functioning in its normal capacity, all proteins normally associated with the telosome are likely to be present, and any interactions detected by the system have a high probability of being functionally relevant.

MATERIALS AND METHODS

YEAST MEDIA, STRAINS, AND PLASMIDS

Strains were grown in standard yeast media (Zakian and Scott, 1982). pH4TZ was used to target the *lacZ* gene to *ADH4* on chromosome VII-L in such a way that the ~20 kb distal to *ADH4* was deleted and a new telomere was formed immediately adjacent to the *lacZ* gene. The pH4TZ fragment transformed into cells contained, from 5' to 3', 270 bp of TG₁₋₃ sequence, the *CYC1-lacZ* gene (from pLGSD5 (Guarente et al., 1982)), the 1.7 kb *HIS3* gene, and the 3' end of *ADH4* (from MscI site to gene end). To construct ZTEL, YM701 (*Mat a ura3-52 his3-200 ade2-101 lys2-801 trp1-901 tyr1*) was transformed with SmaI-SalI digested pH4TZ as described (Hill et al., 1991), except that single-stranded carrier DNA was added (Schiestl and Gietz, 1989). A strain with *lacZ* at an internal site on chromosome VII was constructed by transforming SpeI-SalI digested pYA4-2Z into YM701. pYA4-2Z was constructed by ligating a cassette containing *CYC1-lacZ* with *HIS3* into the MscI site in the *ADH4* gene in pYA4-2 (Paquin and Williamson, 1986). The HISTEL strain was constructed by transforming YM701 with PvuII-XmnI digested pYAHISTEL. pYAHISTEL contained 71 bp of TG₁₋₃ immediately 5' to the *HIS3* allele in a 3.6 kb fragment that also contained URA3 (obtained from p601 (Alexandre et al., 1993)); this was followed by the 3' end of *ADH4* (from XbaI site in pYA4-2). The INTHISA strain was constructed by digesting the plasmid pYAHU with PvuII and transforming YM701. pYAHU was constructed by inserting the ~3.7 kb PvuII-SmaI fragment of p601 into the MscI digested pYA4-2. The INTHISL strain was made by cutting the plasmid pTDHU with AatII and transforming YM701. pTDHU was made by inserting the PvuII-SmaI fragment of p601 into pTD27 digested with StuI.

To construct plasmids for expressing fusion proteins (Figure 2.1), fragments of genes were inserted in frame into the EcoRI cloning site in pJG4-5 (Gyuris et al., 1993) for fusion to the B42 activation domain and pRF4-6NL (a kind gift of Russ Finley) for proteins without a fused activation domain. RAP1A was a 1.7 kb NruI-BglII fragment; RAP1B was a 1.4 kb NruI-BglII fragment. The *RIF1* constructs contained a 2.7 kb XmnI-HindIII fragment from pCH450 (Hardy et al., 1992). *SIR3* constructs contained a 1.6 kb EcoRI fragment from pKL3 (a puc19-based plasmid from R. Sternglanz containing *SIR3*). The entire open reading frame of *SIR4* was PCR amplified from genomic W303 (*MAT a leu2-3, 112 trp1 ade2-1 ura3-1*) DNA using Vent Polymerase (New England Biolabs, Beverly, MA). EcoRI and XhoI sites engineered into the PCR product allowed insertion of *SIR4* into an EcoRI-XhoI digested pJG4-5. The EcoRI-XhoI fragment was subsequently excised and cloned into pRF4-6NL. For each fusion protein, two independent clones from *E. coli* were assayed. *CDC13* was assayed as three separate polypeptides (amino acids 1-754, 755-1525, and 1526-2941) or the complete protein. The 2.1 kb HincII fragment of *EST1*, encompassing almost the entire protein, as well as the carboxyl-terminal portion from the EcoRI site to the protein end were assayed as fusions. The *TEL2* construct expressed amino acids 27-688 of Tel2p. Expression of proteins made without activation domains, and Est1 and Tel2 fusion proteins were verified by Western analysis using a monoclonal mouse anti-HA antibody (Boehringer Mannheim) which detected the expressed proteins. *sir3::LYS2* strains were constructed by transforming with EcoRI digested pJR317 (Kimmerly and Rine, 1987). After transformation, all integrations were verified by Southern analysis.

REPORTER GENE EXPRESSION ASSAYS

β -galactosidase assays

β -galactosidase assays were performed using a filter-lift assay essentially as (Breedon and Nasmyth, 1985), except that cells were picked from fresh growing colonies into 20 μ l of water and spotted onto nitrocellulose filters atop plates selecting for plasmid maintenance. Galactose plates were incubated two days and glucose plates one day at 30°. The β -galactosidase reaction dishes contained 2.5 ml of Z Buffer (Breedon and Nasmyth, 1985) with 0.75 mg of X-GAL (Sigma, St. Louis, MO). Reactions were allowed to proceed 2 days at 30°. Liquid β -galactosidase assays were performed as described (Guarante, 1983). The values reported in Table 1 were from reactions allowed to proceed for ~7.25 hours. Each experiment was performed at least twice with similar results. In addition, similar results in filter-lift as well as liquid β -galactosidase assays were found when RAP1-A was expressed as a fusion with the GAL4 activation domain.

HIS3 expression growth assays

Cells were grown to stationary phase at 30° (~2 days) in liquid medium lacking tryptophan and containing 3% raffinose to eliminate glucose repression of the expression of the fusion proteins. 5 μ l of cells were then spotted in ten-fold serial dilutions onto Gal-his plates containing 3-amino-1,2,4-triazole (3-AT)(Sigma, St. Louis, MO) or control plates. The concentration of 3-AT was determined empirically for each experiment because different lots of 3-AT had varying strength. Therefore growth can be compared within one experiment at a particular concentration of 3-AT, but sometimes less accurately between experiments.

Gel mobility shift assays

Gel mobility shift assays were performed as described (Lin and Zakian, 1994; Lin and Zakian, 1996).

RESULTS

A TELOMERE ONE-HYBRID SYSTEM TO DETECT *IN VIVO* TELOMERE BINDING

The goal of this study was to exploit one-hybrid screening methodology as an *in vivo* assay for telomere binding. The overall design involved integrating a reporter gene with low transcriptional activity immediately adjacent to a chromosomal telomere. Then a hybrid protein consisting of a candidate telomere binding protein fused to a transcriptional activation domain was expressed. The transcriptional activation domain used was an *E. coli* sequence, B42, that activates transcription in yeast (Ma and Ptashne, 1987). In the telomere one-hybrid assay described here, two reporter genes were used. The first was the *lacZ* gene which, when transcribed, confers blue color to colonies assayed for its gene product, β -galactosidase. The second was a promoter-defective *HIS3* allele that allows growth on plates lacking histidine when the gene is expressed. In both cases, the reporter genes were integrated adjacent to the left telomere of chromosome VII.

Protein fusions that specifically bind to the telomere should activate the telomeric reporter gene, but not the same gene when it is integrated at a non-telomeric location. Additionally, neither half of the fusion protein alone should activate the telomeric reporter gene. Since the fusion proteins were expressed under the control of a galactose-inducible promoter, activation should occur on medium containing galactose but not on glucose medium, which represses the galactose-inducible promoter.

To determine if a telomere one-hybrid system would be an effective way to assay whether a protein associates with telomeres *in vivo*, we first examined the behavior of the known telomere binding protein, Rap1p. The strain ZTEL bore the *lacZ* gene immediately adjacent to the telomere at chromosome VII-L, such that its promoter region was approximately 160 base pairs from the TG₁₋₃ sequence. When it contained the vector alone, thus expressing only the activation domain (ACT), it produced white colonies, indicating that there was no activation of the *lacZ* reporter gene (Figure 2.2, top row). However, expression of a protein fusion of the DNA binding region of Rap1 (Figure 2.1, region B) to the transcriptional activation domain (RapBActp) (Figure 2.2, RAP1 panel, row 2), resulted in blue colony color, indicating transcription from the telomeric *lacZ* gene. In contrast, when RapBActp was expressed in a strain in which the *lacZ* gene was ~20 kb from a telomere (Figure 2.2, RAP1 panel, row 3), no signal was observed, demonstrating that the reporter must be close to telomeric sequence to be activated by the fusion protein. Cells expressing only the Rap1p DNA binding domain produced white colonies (Figure 2.2, RAP1 panel, bottom row). In addition, the ZTEL strain containing the RAPBACT vector produced white colonies when grown on glucose plates, indicating that the signal required expression of the fusion protein (data not shown). Taken together, these results indicate that a protein known to bind telomeres *in vivo*, Rap1p, behaves as predicted in this one-hybrid system, thus defining a new *in vivo* telomere binding assay for *S. cerevisiae*.

RIF1P AND SIR4P FUSIONS ARE TELOMERE ASSOCIATED

To determine whether other proteins implicated in telomere metabolism are also directly associated with a chromosomal telomere, regions of Rif1p or Sir3p or the complete Sir4p were subjected to telomere one-hybrid analysis. RifActp, an ACT

fusion containing the 367 C-terminal amino acids of Rif1p (Figure 2.1), activated the *lacZ* gene adjacent to a telomere (Figure 2.2, RIF1 panel, row 2), but not when the *lacZ* gene was integrated internally (Figure 2.2, row 3). The Rif1 polypeptide did not activate *lacZ* when it was expressed without an activation domain (Figure 2.2, row 4). Likewise, Sir4Actp, a fusion protein containing the entire Sir4 open reading frame, activated the *lacZ* gene when it was adjacent to a chromosomal telomere (Figure 2.2, SIR4 panel, row 2), but not when *lacZ* was internal (Figure 2.2, row 3). Again there was no detectable *lacZ* transcription when Sir4p was expressed without an activation domain (Figure 2.2, row 4). Activation by both the Rif1 and Sir4 fusion proteins occurred only on galactose medium (data not shown). Although the signal was too weak to be reproduced for publication, a 541 amino acid polypeptide (Figure 2.1) of the Sir3 protein also tested positive in the *lacZ* system. Taken together, the ZTEL experiments demonstrate that Rif1 and Sir4 proteins can associate with a chromosomal telomere, and suggest that Sir3p may as well (see next section).

Liquid β -galactosidase assays, which allow quantitative comparisons of expression of *lacZ*, were performed on some *lacZ* containing strains (Figure 2.3). In this assay, a larger region of Rap1p was expressed (Figure 2.1, region A), which included the endogenous transcriptional activation domain of Rap1p. In agreement with the previous assay, RapAActp in ZTEL showed > 50-100 fold increase in β -galactosidase levels relative to vector alone (Figure 2.3, RAP1 column, compare row 2 with row 1) or when the *lacZ* gene was internal (compare row 2 with row 3). The effect was protein dependent as no β -galactosidase activity was detected in cells grown in glucose medium (glucose row).

Another way transcription from a telomeric gene can be increased is by decreasing TPE. Overexpression of Sir4p (stated in (Singer and Gottschling, 1994)) or

the carboxyl-terminus of Rap1p (Wiley and Zakian, 1995) is known to decrease TPE, presumably because the overexpressed protein titrates factors important for TPE away from telomeres. Thus, it was important to ensure that the activation of *lacZ* observed in strains expressing candidate fusion proteins was not due merely to an effect on TPE. Deletion of *SIR3* eliminates TPE (Aparicio et al., 1991). Therefore if the *lacZ* transcription seen when a Rap1 fusion protein was expressed were due only to decreasing TPE, *lacZ* levels should not increase in a *sir3::LYS2* strain when it expresses the fusion protein. Since there was detectable transcription of the telomeric *lacZ* gene in a *sir3::LYS2* strain in the absence of any fusion protein (Figure 2.3, row 4), this gene in ZTEL was somewhat repressed by TPE. However, the amount of β -galactosidase activity was higher when RapAActp was expressed in a wild type strain than in a *sir3::LYS2* strain with no fusion protein (Figure 2.3, compare row 2 to row 4). Furthermore, expressing RapAActp in the *sir3::LYS2* strain (Figure 2.3, row 5) led to even higher levels of β -galactosidase expression, suggesting an additive effect. These results demonstrated that RapAActp had an effect beyond simply relieving TPE, consistent with the interpretation that RapAActp activated *lacZ* by virtue of its association with the telomere.

Similarly, expression of RifActp in ZTEL caused a 3-6 fold increase in *lacZ* expression relative to vector alone (Figure 2.3, RIF1 column, compare rows 1 and 2), and a 3-5 fold increase relative to expression from the *lacZ* gene at an internal location (Figure 2.3, row 3). The effect was protein dependent (glucose row), and was not due simply to effects on TPE (Figure 2.3, compare row 4 to rows 2 and 5).

A MORE SENSITIVE TELOMERE ONE-HYBRID ASSAY DETECTS SIR2P AND SIR3P ASSOCIATION WITH TELOMERES

Because the activation of ZTEL in cells expressing Sir3Actp was difficult to detect (data not shown), a more sensitive telomere one-hybrid assay was developed. *HIS3* was chosen as a reporter gene because a competitive inhibitor of His3p, 3-amino triazole (3-AT), can be added to plates to distinguish between cells expressing different levels of His3p: more 3-AT requires expression of more His3p for growth (Alexandre et al., 1993). A *HIS3* allele with a very low basal level of transcriptional activity (Alexandre et al., 1993) was integrated adjacent to the telomere of chromosome VII-L, such that its promoter was approximately 50 base pairs from the TG₁₋₃ sequence (Figure 2.4, A). This strain, called HISTEL, grew poorly on 5mM 3-AT galactose plates lacking histidine (hereafter called test plate). If a HISTEL one-hybrid assay detects telomere-protein interactions, expressing a telomere binding protein fused to ACT should activate *HIS3* and allow growth on test plates (Figure 2.4, B). Furthermore, the His⁺ signal should depend upon the proximity of a telomere (Figure 2.4, C), as well as the fused activation domain (Figure 2.4, D).

HISTEL strains containing the same fusion vectors tested in the *lacZ* strain were plated in 10-fold serial dilutions on test plates. In all experiments, the same dilutions were also plated on control (i.e. galactose plates selecting for plasmid maintenance) plates to demonstrate that similar numbers of cells were plated for each spot. Each experiment was performed on two different identical clones (where available), or on at least two transformants. Each experiment (in which 3 or more plates were spotted for each transformant) was repeated at least once and a representative plate is shown; experiments that showed less than 1000-fold colony forming ability when compared with vector alone were repeated several times. When HISTEL

contained vector alone (Figure 2.5, left panel, top row), it generated colonies out to the first dilution. However, when RapBActp, RifActp, or Sir4Actp was expressed in the same strain, colonies were generated out to much higher dilutions (Figure 2.5, middle 3 rows), indicating that expression of the fusion proteins caused activation of the telomere adjacent *HIS3* gene. RapBActp allowed an ~100-fold increase in colony forming ability compared to vector alone, RifActp ~10,000-fold, and Sir4Actp allowed ~1000-fold increase over vector alone. Using this assay, a fusion protein containing 541 amino acids from the carboxyl terminus of Sir3p (Sir3Actp) caused an ~100-fold increase in plating efficiency (Figure 2.5, row 5). These results confirmed that Rif1p and Sir4p, and demonstrated for the first time that Sir3p, associate with a chromosomal telomere *in vivo*.

Besides activation by a candidate fusion protein, another circumstance causes transcription from a telomeric gene to be increased; perturbation of TPE. Overexpression of Sir4p (stated in Singer and Gottschling, 1994) or the carboxyl-terminus of Rap1p (Wiley and Zakian, 1995) is known to decrease TPE, presumably because the overexpressed protein titrates factors important for TPE away from telomeres. Thus, it was critical to ensure that the transcription of *HIS3* observed in strains expressing candidate fusion proteins was not due merely to an effect on TPE. If a protein were having the effect of decreasing TPE, rather than activating the *HIS3* gene, the ability to transcribe *HIS3* should not require fusion with the activation domain. Therefore, it was important to show that the improved growth was dependent upon the fused activation domain. When the polypeptides were expressed with no activation domain, Rap1p, Rif1p, Sir4p, and Sir3p expressing cells did not show as great an increase in colony-forming ability as did their counterparts fused with the activation domain (Figure 2.6, bottom plate, compare rows 2-6 to top plate rows 2-6). These

results demonstrated that improved growth on test plates of cells expressing candidate telomere binding fusion proteins is due to an effect of the activation domain (local) rather than effects on TPE (away from the telomere).

When HISTEL cells expressing full-length Sir2p fused to ACT (Sir2Actp) were assayed at the same level of 3-AT (10mM) that Rap1, Rif1, Sir3, and Sir4 proteins were, they were unable to form colonies (data not shown). However, when 5mM 3AT plates were allowed to remain in the incubator longer, slow growing colonies appeared in the Sir2Actp rows ~100X more frequently than in vector alone (Figure 2.7, 2nd row). As with the other strains, this growth was dependent on the fused ACT, since cells expressing Sir2p had limited ability to form colonies (Figure 2.7, row 3). These results indicate that Sir2p does interact with a telomere in living cells. Sir2Actp may have a weaker interaction than the other fusion proteins since it grows slowly at low levels of 3-AT and cannot form colonies at 10mM 3-AT. However, overexpressing either protein seemed to have a toxic effect on cells since the colonies grew slowly even when being selected only for plasmid maintenance (Figure 2.7, right panel), so this toxicity may make it more difficult for these strains to grow on test plates.

To ensure that the activation of *HIS3* was specific to the proteins assayed, a random collection of 20 genomic fragments fused to the transcriptional activation sequence was surveyed in the HISTEL strain. Each was indistinguishable from vector alone (data not shown). Furthermore, RNA polymerase II subunit B4 (Khazak et al., 1995), which is not suspected to have any telomere function, was negative in the one-hybrid assay (data not shown). Other proteins important for telomere length maintenance, Est1p (Lundblad and Szostak, 1989) and Tel2p (Lustig and Petes, 1986), were also negative in this system (A. Smith, B. Bourns, and V. Zakian unpublished observations).

To determine whether the interactions with the *HIS3* gene required an adjacent telomere, the fusion proteins were expressed in a strain in which the same *HIS3* allele present in HISTEL was integrated at two sites away from the telomere. The internal *HIS3* bearing cells expressing the various fusion peptides were unable to generate colonies, even on Gal-his plates with no 3-AT (Figure 2.8, rows 2-6, both panels). Therefore, as expected for a genuine telomere binding protein, the ability to generate colonies for strains expressing these fusion proteins was dependent upon the presence of a nearby telomere. Taken together, these results indicate that Rif1Actp, Sir4Actp, Sir3Actp, and Sir2Actp associate with a functioning telomere *in vivo*.

THE TELOMERE ONE-HYBRID SYSTEM DETECTS TELOMERE INTERACTION WITH CDC13P, WHICH MAY TAKE PLACE VIA THE SINGLE-STRANDED G OVERHANG

Cdc13p binds single-stranded G-strand telomeric DNA *in vitro* and has been postulated to function at telomeres *in vivo* via this interaction (Lin and Zakian, 1996; Nugent et al., 1996). Therefore, using the one-hybrid system, I set out to determine whether the Cdc13 protein interacts with a telomere *in vivo*. HISTEL cells carrying Cdc13Actp, were able to generate colonies on test plates better than vector alone (Figure 2.9, compare CDC13ACT rows to Vector rows). However, when Cdc13p was expressed without an activation domain, the cells were still able to generate colonies on test plates, although somewhat less frequently (Figure 2.9, top panel, CDC13 row). This indicated that the His⁺ signal was partly due to an effect of overexpressing the protein (perhaps a decrease in TPE), as well as to activation by the fusion protein. Interestingly, the His⁺ phenotype was lessened when a fusion protein made from the temperature-sensitive allele *cdc13-1*, was assayed (Figure 2.9, bottom panel, compare rows 3 and 5 to rows 2 and 4). Therefore, one of the effects (either the ACT-dependent, or the ACT-

independent effect) is defective in the temperature-sensitive protein. Westerns probed for the fusion proteins indicated that the stability of the temperature-sensitive version was not dramatically decreased relative to wild type fusion proteins (data not shown). This is consistent with either interaction with a telomere or interaction with a protein important for telomere position effect being defective in the *cdc13-1* allele.

Notably, colonies from strains overexpressing Cdc13p often grew more slowly on plates that induce fusion protein expression but only select for maintenance of the plasmid (Figure 2.9, right side). This toxicity may cause the signal indicating telomere interaction (i.e. growth on test plates) to underestimate a stronger interaction because relative to vector alone, the CDC13ACT cells are often seen to grow very slowly.

In an attempt to find a domain whose overexpression was not toxic, regions of Cdc13p were assayed rather than the full-length protein. Regions of the CDC13 N-terminus (251 amino acids), middle (M, 257 amino acids) or C-terminus (667 amino acids) were subjected to one-hybrid analysis. HISTEL cells carrying the fusion of the Cdc13 N-terminus with an activation domain (13NACT) were able to generate colonies 100-1000 fold better than vector alone (Figure 2.10, top panel, compare row 4 to row 1). Additionally, in this assay the C-terminal region may display some activity (Figure 2.10, top panel, compare row 2 to row 1), however, the signal is sufficiently weak (approximately 10 fold) that interpretation of a positive signal at this time would be premature. In contrast to the full-length Cdc13p, the N-terminal effect on HISTEL did require the fused activation domain (Figure 2.10, bottom panel compared to vector alone). Additionally, neither full-length nor the N-terminal region of CDC13 activated HIS3 at an internal location (Figure 2.11, bottom two rows of each panel). Taken together, these results indicate that a domain of Cdc13p, its N-terminus, associates with

a telomere *in vivo*. Furthermore, these results suggest that the telomere one-hybrid system may be able to detect protein interactions with the single-stranded G-rich extension found at telomere ends.

Another aspect of Cdc13p function that has been investigated is its ability to bind single-stranded telomeric DNA *in vitro*. To test whether the fusion proteins I have assayed in the one-hybrid system correlate with this *in vitro* activity, I performed a gel mobility shift assay. As expected, the full-length fusion protein (Figure 2.12, lane 5) displayed the ability to bind to a single-stranded telomeric oligonucleotide similar to Cdc13p without a fused activation domain (Figure 2.13, lane 3). Both were supershifted by addition of an anti-HA antibody (Figure 2.13, lanes 4 and 6), although the background of endogenous Cdc13p is seen in the ACT fusion protein lane whereas it is missing in lane 4 because that strain has no endogenous Cdc13p. No additional bands are detected in lanes of extract from cells expressing Cdc13M, or N fusions with the activation domain (Figure 2.13, lanes 7 and 8). Because conditions were suitable for detecting full-length Cdc13Actp binding, this may suggest that neither the M nor the N-regions contain the DNA binding region. However, it is possible that they bind with lower affinity than full-length protein does and therefore were undetectable under these conditions.

DISCUSSION

A TELOMERE ONE-HYBRID ASSAY DEMONSTRATES INTERACTION OF RIF1, SIR2, SIR3, SIR4, AND CDC13 WITH A TELOMERE

Experiments presented here demonstrate that a one-hybrid system using a functioning chromosomal telomere can be successfully employed to assay proteins binding to a yeast telomere *in vivo*. The first step was to test the system by assaying a known telomere binding protein, Rap1p. Once Rap1p analysis indicated that the system behaved as predicted, it was then used to determine if other proteins that affect telomeres are directly associated with a chromosomal telomere. *RIF1*, *SIR2*, *SIR3*, and *SIR4* all affect telomere length and TPE (Aparicio et al., 1991; Hardy et al., 1992; Kyrion et al., 1993; Palladino et al., 1993). Additionally the Sir proteins are involved in mediating telomere localization in the nucleus (Palladino et al., 1993). Because these effects could be indirect, it was of interest to determine whether these proteins physically associate with a telomere. By showing that Sir4 and Sir3 proteins were associated with a chromosomal telomere *in vivo*, our results provide direct demonstration of an interaction that was previously inferred by considerable genetic and cytological data. To our knowledge, these experiments are the first indication that Sir2p, Rif1p and Cdc13p are physically associated with a telomere.

Potential impediments to using the telomere one-hybrid system

Several problems can be imagined that might have impeded our ability to assay telomere binding by one-hybrid analysis. Given that telomeres transcriptionally repress nearby genes, it is perhaps surprising that transcription can be used to monitor telomere binding. However, since TPE affects basal, not activated transcription (Gottschling et al., 1990; Renauld et al., 1993), the activating nature of the fusion

proteins was predicted to overcome the repressive effects of TPE. In addition, to minimize any repressive effects of the telomere on transcription of the reporter gene, the TATA of the gene was positioned in the nuclease hypersensitive region that occurs ~50-100 bp from the telomeric sequence (Wright et al., 1992). Since this region is accessible *in vivo* to site specific nucleases (Sandell and Zakian, 1993) and DNA modifying enzymes (Wright et al., 1992), we reasoned that it might also be more accessible to transcription factors. Despite these considerations, both *lacZ* and *HIS3* transcription was somewhat repressed by TPE as demonstrated by increased expression of the reporter gene in the *sir3::LYS2* ZTEL strains (Figure 2.3), and inferred from the His⁺ signal resulting from overexpression of Cdc13p (Figure 2.9). However, as predicted, the activating nature of Rap1, Rif1, Sir3, Sir2, Sir4 and Cdc13 N-region fusion proteins was able to overcome the repressing effects of TPE.

A second potential problem with a telomere one-hybrid approach is that short tracts of TG₁₋₃ DNA can act as a transcriptional activator when inserted upstream of a gene (Runge and Zakian, 1990). Such an intrinsic activation might have caused too much reporter signal in the starting strain and thereby interfered with our ability to detect the telomere interaction of a candidate fusion. Fortunately, the *lacZ* strain did not demonstrate this effect since no β-galactosidase was detectable in the absence of a telomere binding fusion protein (Figures 2.2 and 2.3). For the *HIS3* strain, some growth occurred on medium lacking histidine in cells carrying vector alone. However, this low level growth was reduced by plating cells on medium containing 3-AT. In this case, cells did not grow well unless the telomere-binding fusion proteins were expressed (Figures 2.5, 2.6, 2.7, and 2.10).

A third potential problem with a telomere one-hybrid assay is that positive signals might result from changes in TPE rather than activation per se. Because a reduction in TPE would have the same effect (transcription of the telomeric reporter gene) as a positive signal in a one-hybrid assay, it was important to ensure that we were not assaying relief of TPE. Indeed, overexpressing certain telomere binding proteins does cause a reduction in TPE (Singer and Gottschling, 1994; Wiley and Zakian, 1995). Two lines of evidence indicate that the positive signals in the one-hybrid system were not due to alleviation of TPE. First, expressing RapAActp or RifActp in *sir3::LYS2* ZTEL led to a β -galactosidase signal greater than deletion of *SIR3* alone. Because deleting *SIR3* completely eliminates TPE (Aparicio et al., 1991), the protein fusions must be responsible for an effect beyond simply alleviating TPE. Interestingly, the RifActp signal in *sir3::LYS2* ZTEL was higher than would be expected for simply an additive effect of the deletion alone plus RifActp expression alone (Figure 2.3). This result is consistent with a model in which Sir3p and Rif1p have opposing functions at the telomere due to competition for binding similar regions of Rap1p (Moretti et al., 1994). If this were the case, eliminating Sir3p would allow more RifActp access to the telomere complex, which would be expected to increase the signal, precisely what is observed. We cannot, however, rule out that Sir3p and Rif1p are simultaneously bound to the complex because the same effect would be seen if elimination of Sir3p simply allowed better accessibility of the activation domain of RifActp to *lacZ*.

The second type of experiment that ruled out the concern that reporter signals resulted from effects on TPE, addresses the more general issue of whether the signal is dependent upon the activation domain. The decrease in TPE observed when a fragment of Rap1p is overexpressed is thought to result from titration away from telomeres of factors required for TPE (Wiley and Zakian, 1995). If that were occurring

with the fusions used in this study, transcription of the reporter gene should not require the activation domain in the fusion protein. When peptides lacking the transcriptional activation domain were expressed, little or no increase in reporter signal was detected (Figures 2.2, 2.6, 2.7 and 2.10).

Cdc13 effects

The full-length Cdc13p fused to ACT seemed to have two effects on *HIS3* transcription, an increase in colony-forming ability that did require the fused activation domain, in addition to an effect that did not (Figure 2.9). This result is consistent with Cdc13Actp having two effects at the telomere, activation of *HIS3* and relief of TPE, perhaps by interaction with some protein required for TPE such that overexpression causes titration of that protein away from telomeres. The His⁺ signal was reduced but not eliminated when a fusion of the temperature-sensitive Cdc13 protein was subjected to one-hybrid analysis. This decrease could be due to a failing in Cdc13p either to interact with the telomere or with the factor it is titrating.

When the protein was assayed as smaller regions, the N-terminal region was found to generate a positive signal that did depend on the fused activation domain (Figure 2.10). This finding lends support to the model that Cdc13p performs its telomere regulatory functions by interacting directly with a telomere *in vivo*. Moreover, these experiments are the first demonstration in living cells of a protein-telomere interaction likely to take place via the single-stranded G overhang.

Strength of signal in the telomere one-hybrid assay

Different fusion proteins produced signals of different magnitude in both the *lacZ* and the *HIS3* reporter strains. For example, Sir4 and Sir3 fusion proteins activated the reporter genes in a smaller fraction of cells than RifActp (Figure 2.5).

There are several possible explanations for these differences. Most obvious is that the

assayed protein has a function at the telomere that might counteract the transcriptional activation function required for a positive signal in the one-hybrid assay. Perhaps the normal repressing characteristics of the Sir proteins interfered with the activation effect, resulting in a weaker signal than RifActp containing cells. A weak signal might also be due to instability or improper folding of the fusion protein or to an inability of the fusion protein to compete well with the endogenous protein for binding. Given these considerations, it is clear that the negative results reported for *EST1*, and *TEL2* do not mean that these proteins are not telomere associated.

Of the Sir proteins, the strength of activation was hardest to detect for Sir2Actp containing cells. While it is tempting to speculate that this reflects the potential catalytic nature of the protein, relative to the more structural nature of the other Sirs, Rap1, Rif1, and Cdc13p, we cannot rule out that because of its location in the complex it is less well able to activate *HIS3* or compete with the endogenous protein.

Although relative differences in growth patterns (colony size or colony forming frequency) seen between proteins were consistent from experiment to experiment in the HISTEL strain, it is not clear whether these differences indicate relative affinity for the telomere complex, or number of molecules bound per telomere. Variations in growth pattern could reflect any of the previously discussed reasons for weakness of signal, which are independent of telomere binding affinity. Alternatively, different proteins may have different access to the reporter TATA depending upon their position in the complex. Cells expressing RapActp grew faster than cells expressing the other fusion proteins, but did not generate colonies as frequently as cells expressing RifActp or Sir4Actp. The slower growth rate might be due to some toxic effect of overexpressing RifActp or Sir4Actp as cells expressing these proteins grew slowly even under conditions that did not select for telomere binding (data not shown). Additionally,

strains expressing RapActp may exhibit an overall increase in their metabolism due to effects of the fusion protein on some of Rap1p's other functions. Paradoxically, the strains expressing RifActp were the slowest growing yet generated colonies to the highest dilution. Perhaps a slower cell cycle increases the probability that a fusion protein will associate with the telosome at some point during the cell cycle. Given the caveats concerning activity or abundance of the fusion proteins, it is not clear that any conclusions about binding affinity or abundance in the complex can be drawn from the magnitude of the signal in a one-hybrid system.

Selecting for expression of amino acid biosynthesis enzymes is thought to be a very sensitive means of detecting reporter gene expression. The fact that the *lacZ* signal in cells expressing Sir3Actp was barely detectable whereas HISTEL expressing Sir3Actp had a 100-fold growth enhancement (data not shown and Figure 2.5), implies that selecting for telomeric *HIS3* expression is indeed more sensitive than assaying the telomeric *lacZ* gene.

CONCLUSION

In conclusion, we have overcome the unique transcriptional properties of telomeres to develop a novel telomere one-hybrid system. We used it to demonstrate that Rap1, Rif1, Sir2, Sir3, Sir4, and Cdc13 proteins bind to a chromosomal telomere *in vivo*. These results imply that the telomere one-hybrid system can detect both duplex and single-stranded DNA-protein interactions as well as indirect associations with a telomeric complex.

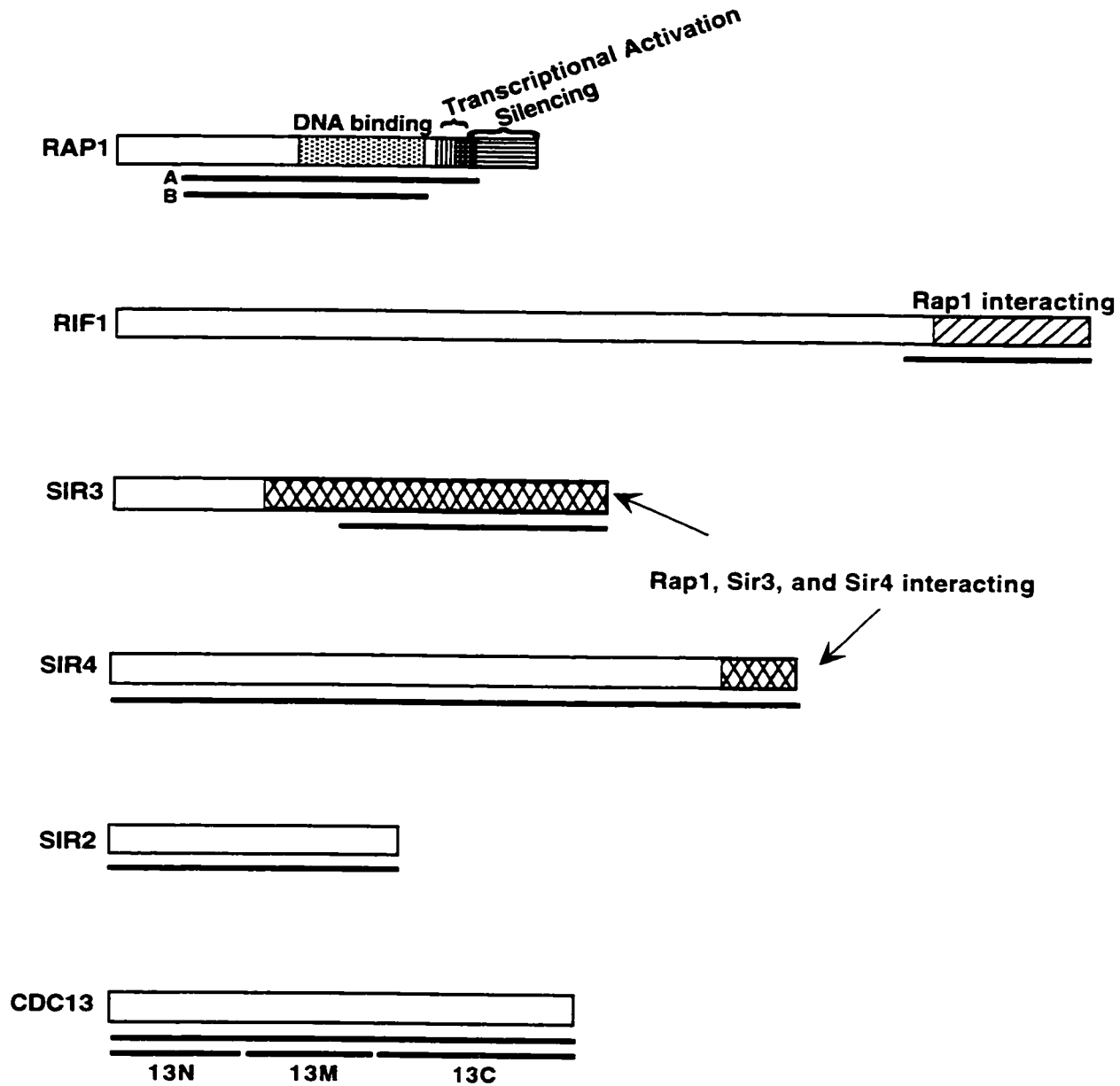


Figure 2.1 Schematic representation of proteins tested for *in vivo* telomere binding using the telomere one-hybrid assay. Previously defined qualities of the proteins are indicated by shading. Bars below define regions of the proteins tested in this study.

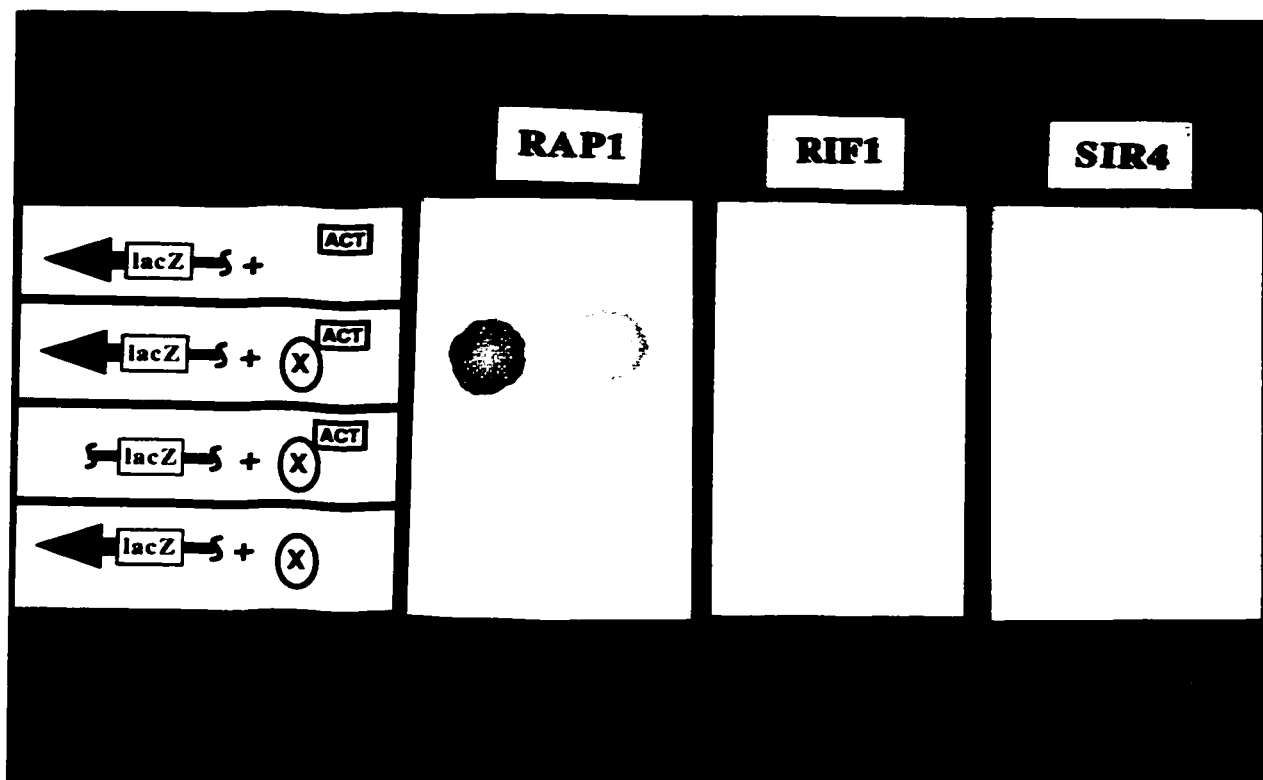


Figure 2.2 Rap1, Rif1, and Sir4 fused to ACT cause expression from the telomeric *lacZ* gene. Strains containing the *lacZ* gene and expressing various proteins were suspended in water and spotted onto nitrocellulose paper on top of Gal-Trp plates. After incubation for 3 days, spots of cells were tested for β -galactosidase activity. Two transformants each of cells expressing protein X, where X is Rap1-B fragment (left), Rif1 (center), or Sir4 (right), were tested. Rows 1, 2, and 4 are strains with *lacZ* integrated at a chromosomal telomere, whereas row 3 has the *lacZ* gene integrated internally.

		β -gal Activity ($\times 10^{-3}$ nmol/min $\times 10^7$ cells)	
		<u>RAP1</u>	<u>RIF1</u>
← lacZ — ζ + [ACT]		<1	9 +/- 0.00
		<1	8 +/- 0.00
← lacZ — ζ + (X) [ACT]		99 +/- 4.50	48 +/- 0.47
		56 +/- 2.05	34 +/- 0.94
	glucose	<1	10 +/- 0.47
		<1	4 +/- 0.47
ζ — lacZ — ζ + (X) [ACT]		2 +/- 0.94	11 +/- 1.25
		<1	10 +/- 0.00
← lacZ — ζ sir3::LYS2		14 +/- 0.00	17 +/- 0.00
		3 +/- 0.00	35 +/- 0.00
← lacZ — ζ sir3::LYS2 + (X) [ACT]		161 +/- 3.40	134 +/- 3.86
		75 +/- 3.86	124 +/- 1.25

Figure 2.3 β -galactosidase activities for strains containing an integrated *lacZ* gene, and plasmids expressing Rap1 or Rif1 fragments fused to ACT. Activities shown are two independent transformants in the case of ACT or *sir3::LYS2*, or two independent identical clones in the case of the fusion proteins. Logarithmically growing cultures were divided into 3 samples to be assayed. Each number represents the mean of the three samples followed by the standard deviation. The RAP1 column represents data from a fragment of Rap1 that includes the DNA binding domain as well as its endogenous transcriptional activation domain, but lacks the silencing domain (Bar A in Figure 2.2). The value "<1" indicates that spectrophotometric measurements were slightly negative relative to blanks containing no cells.

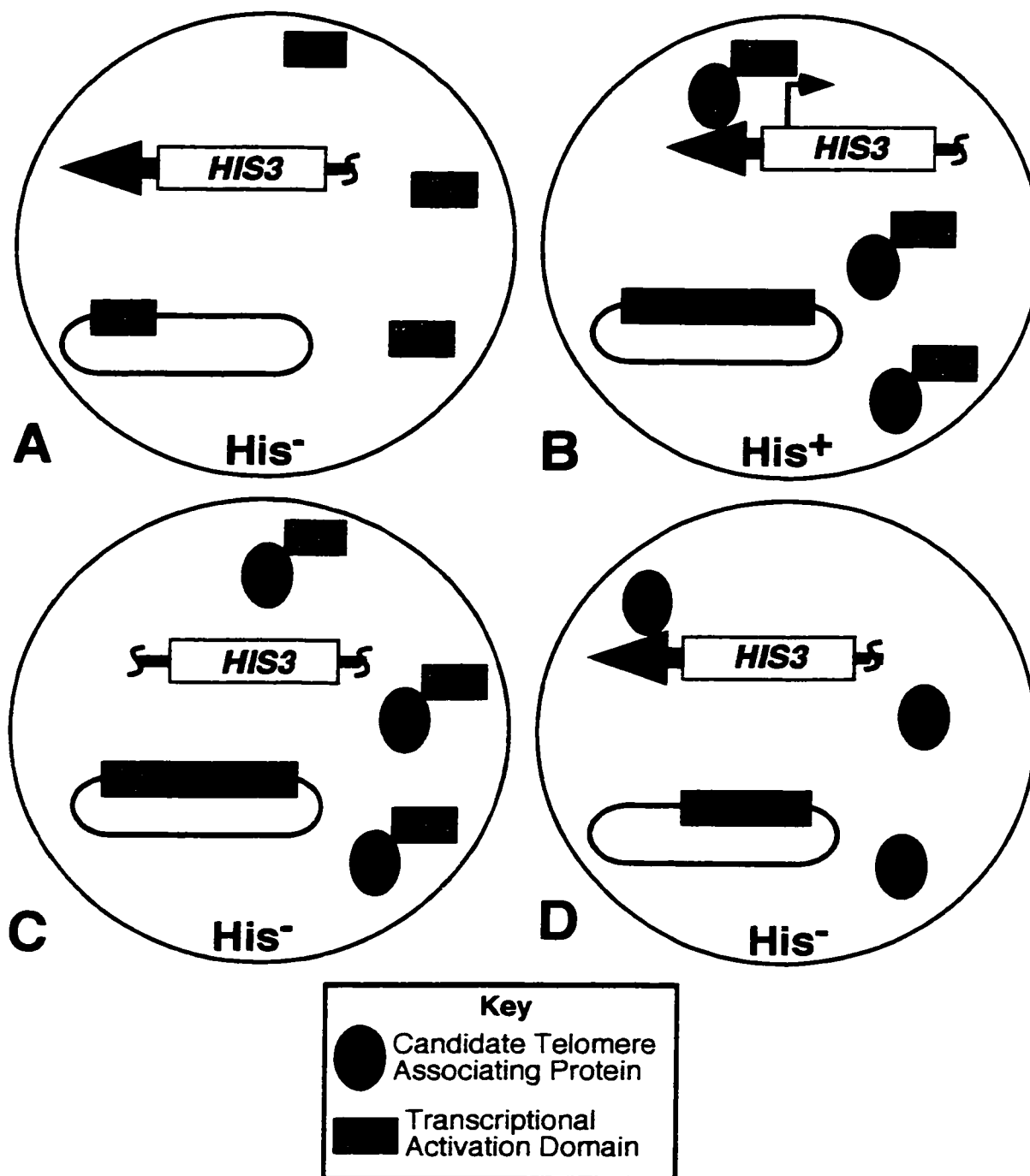


Figure 2.4 Schematic of the *HIS3* reporter one hybrid system. A HISTEL strain without or with the vector alone (A) will not grow on plates lacking histidine. If protein X interacts with the telomere, it brings the transcriptional activation domain into proximity with the *HIS3* reporter gene, causing activation (B). If the *HIS3* gene is located at an internal site away from a telomere (C), the fusion protein will not be able to activate it. If protein X is expressed without the fused activation domain (D), no expression of *HIS3* will occur.

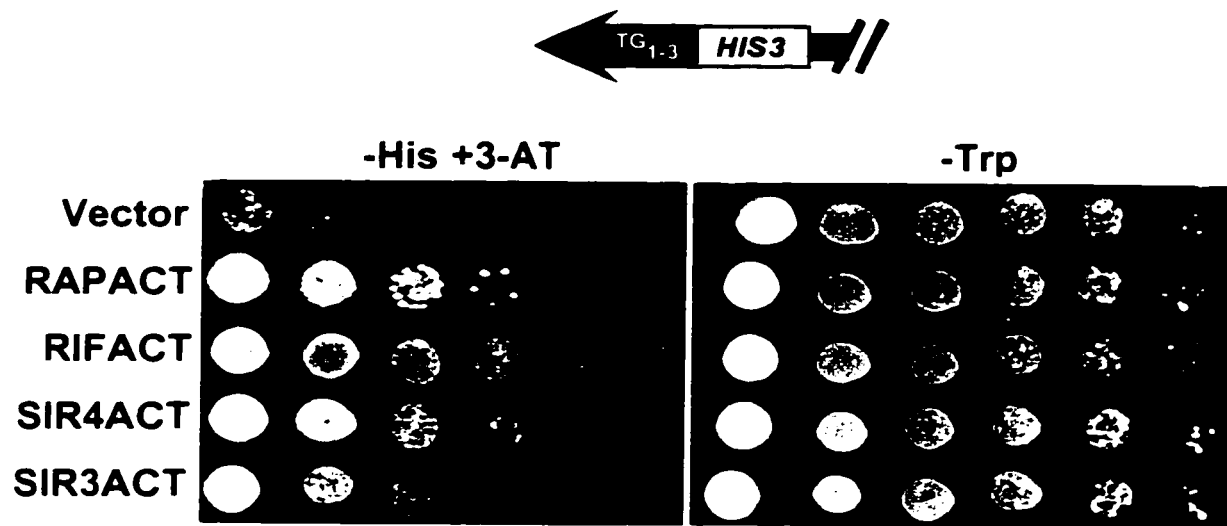


Figure 2.5 Rap1, Rif1, Sir4, and Sir3 fused to ACT activate the telomeric *HIS3* gene. Strains containing the low-activity *HIS3* allele integrated adjacent to a chromosomal telomere and expressing fusions of various proteins to the transcriptional activation domain were grown to stationary phase. They were then serially diluted in 10-fold increments. Dilutions were spotted onto galactose plates selecting for the telomere interaction (-His + 20mM 3-AT, left panel) or for plasmid-containing cells (right panel) and allowed to grow for 6 and 4 days, respectively.

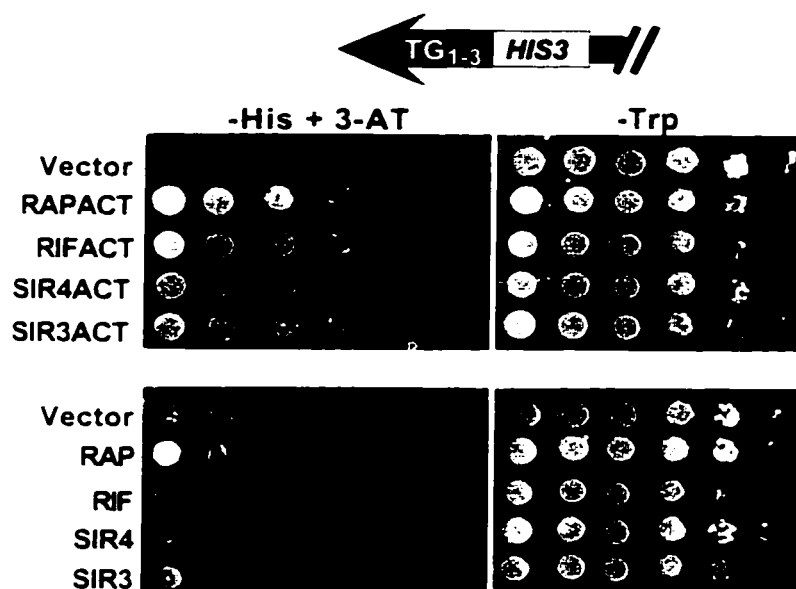


Figure 2.6 Activation of telomeric *HIS3* requires the fused activation domain. HISTEL cells expressing various proteins fused to the activation domain (top panel) or without a fused activation domain (bottom panel) were grown to stationary phase. Ten-fold serial dilutions were plated on galactose plates selecting for the telomere interaction (left, Gal-His + 10mM 3-AT) or for the presence of the plasmid (right). Plates were incubated until colonies grew up, 7 days for the left plate, and 4 days for the right plate.

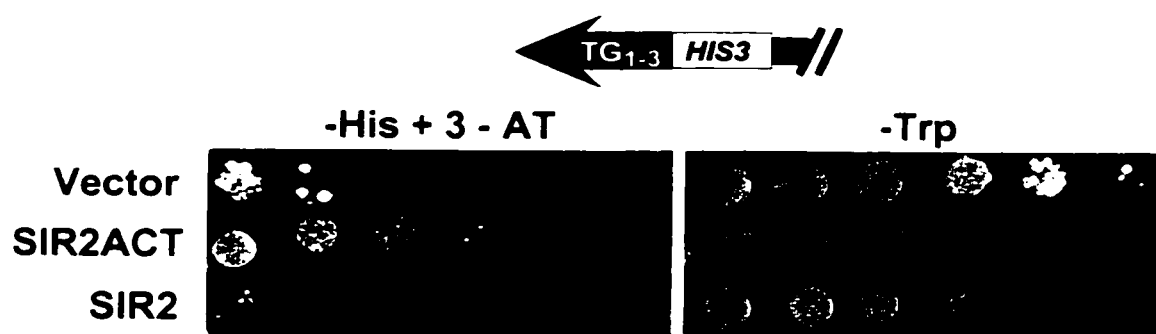


Figure 2.7 A Sir2p fusion protein activates the telomeric *HIS3* gene. Each strain was grown to stationary phase and serially diluted in 10-fold increments onto galactose plates selecting either for the telomere interaction (left, Gal - His + 5mM 3-AT), or selecting for the presence of the plasmid (right panel). Plates were incubated for 8 days and 4 days, respectively.

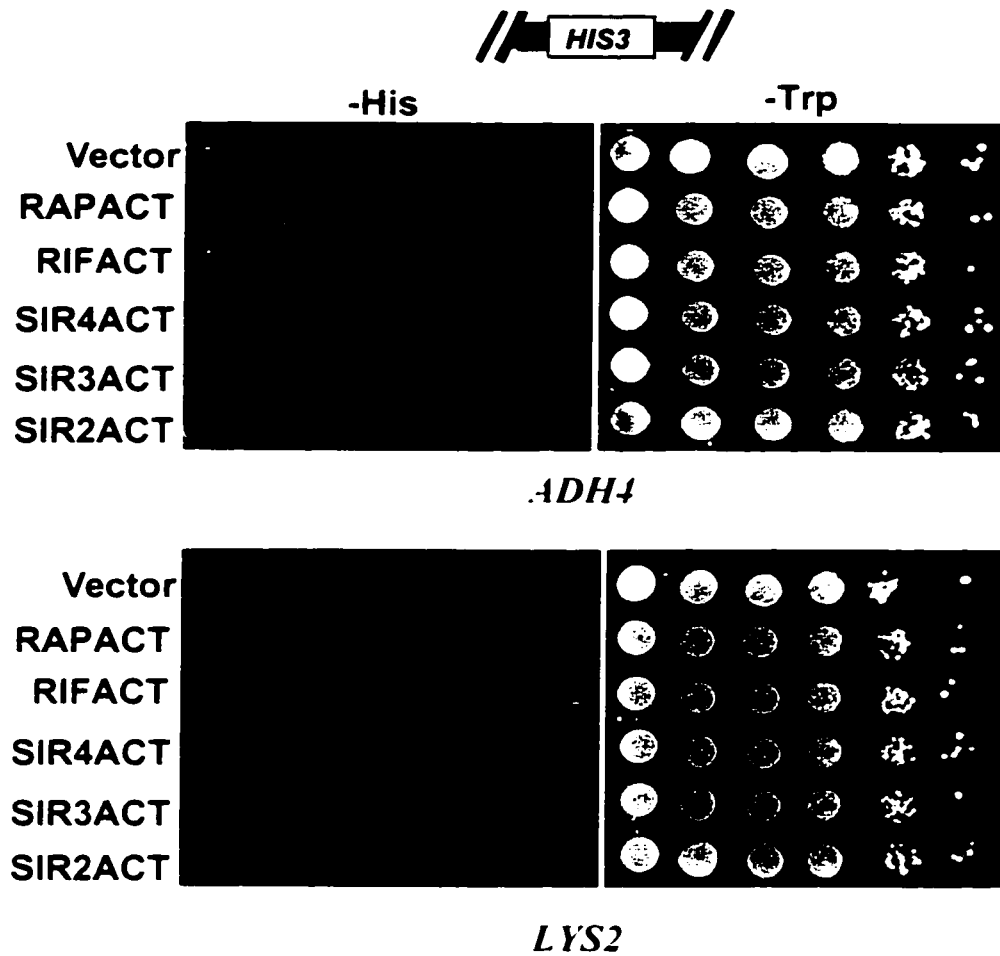


Figure 2.8 Rap1, Rif1, Sir4, Sir3, and Sir2 fusion proteins do not activate a *HIS3* gene integrated internally on a chromosome. Strains containing the promoter-defective allele of *HIS3* integrated internally either at the *ADH4* locus (top panel) or the *LYS2* locus (bottom panel), that were expressing each of the fusion proteins, were grown to stationary phase. Ten-fold serial dilutions were spotted onto plates selecting either for the activation of *HIS3* (left side), or for the presence of the plasmid (right side). Plates were incubated for 6 and 4 days, respectively.

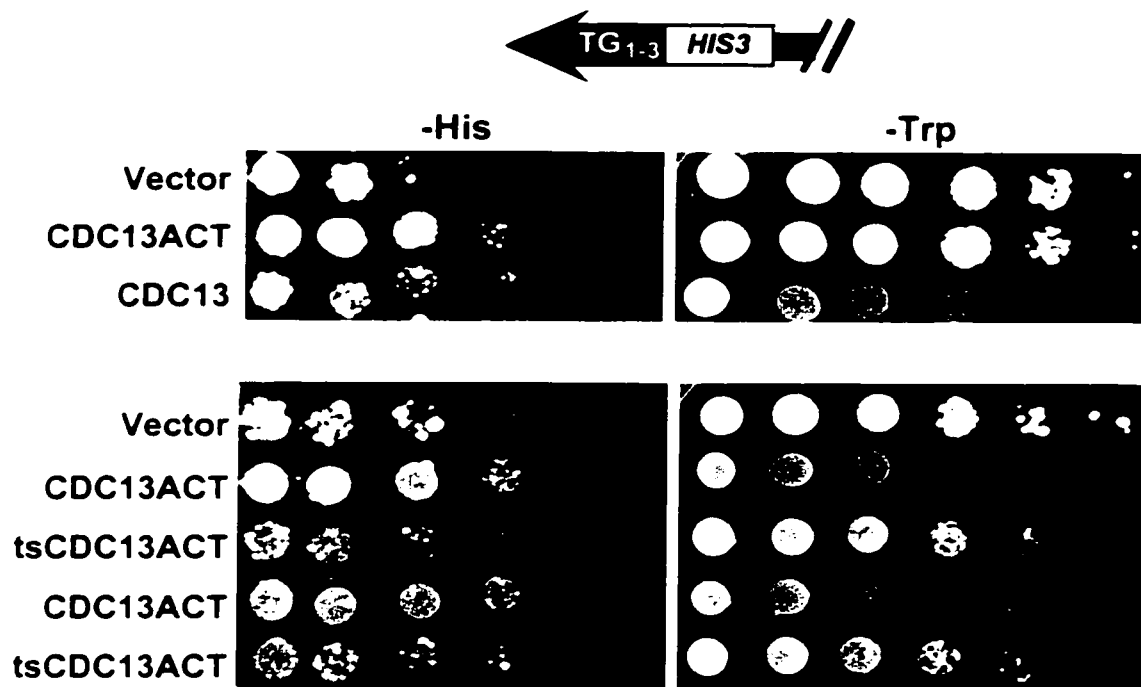


Figure 2.9 Overexpression of Cdc13p causes a decrease in TPE that is not present in the temperature-sensitive protein. HISTEL strains containing the promoter-defective allele of *HIS3* adjacent to a telomere and expressing either Cdc13p or the temperature sensitive Cdc13-1p were grown to stationary phase, serially diluted in 10-fold increments, and spotted onto galactose plates selecting either for the activation of *HIS3* (Gal-His, left), or for the presence of the plasmid (right). Plates were incubated for 6 and 4 days, respectively.

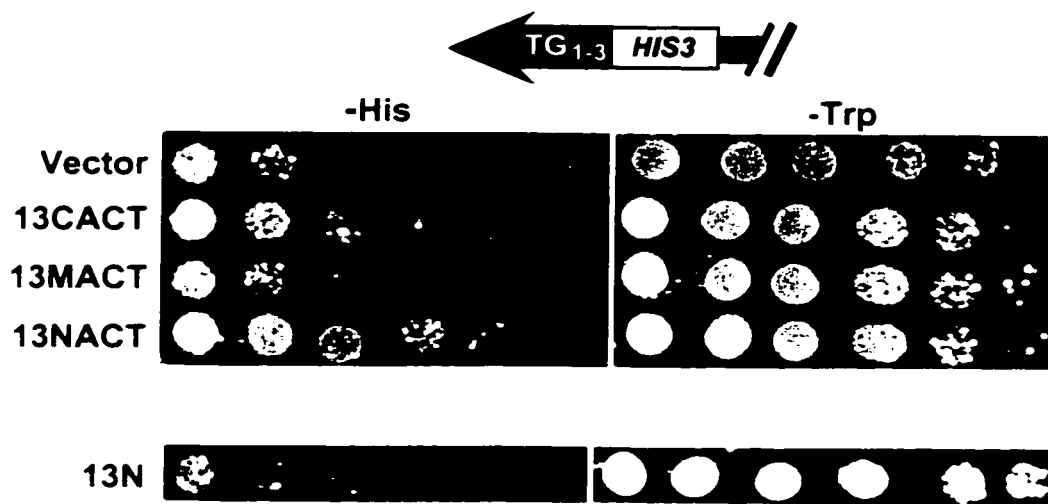


Figure 2.10 The Cdc13-N fusion protein tests positive in the telomere one-hybrid assay. Strains containing a promoter-defective allele of *HIS3* integrated adjacent to a telomere expressed regions of the Cdc13 protein. Ten-fold serial dilutions of Cdc13 C, M, and N, fusion proteins (top panel) were spotted onto galactose plates selecting either for the activation of *HIS3* (Gal-His, left), or for the presence of the plasmid (right). Cells expressing the Cdc13 N region without the fused activation domain (bottom panel) were plated on the same plates. Plates were incubated for 6 and 4 days, respectively.

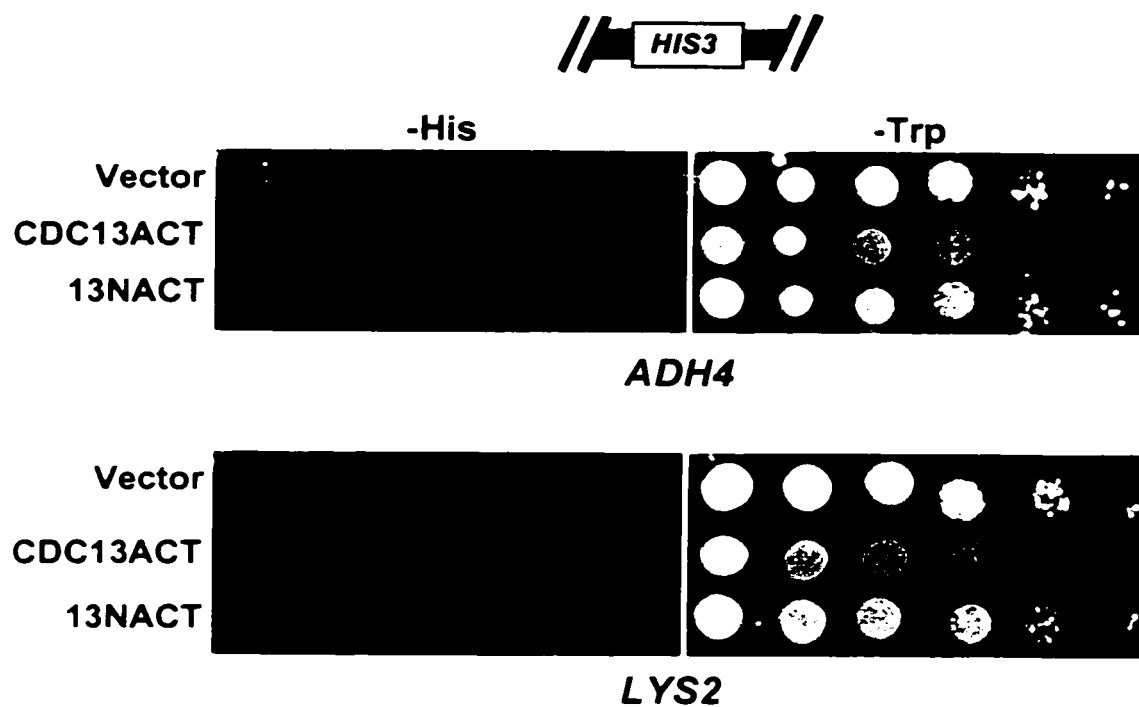


Figure 2.11 Cdc13 fusion proteins do not activate a *HIS3* gene integrated internally on a chromosome. Strains containing the promoter-defective allele of *HIS3* integrated at the *ADH4* locus (top panel) or at *LYS2* (bottom panel) and expressing protein fusions, were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for activation of *HIS3* (Gal-His, left) or for plasmid-bearing cells (right) and allowed to grow for 6 and 4 days respectively.

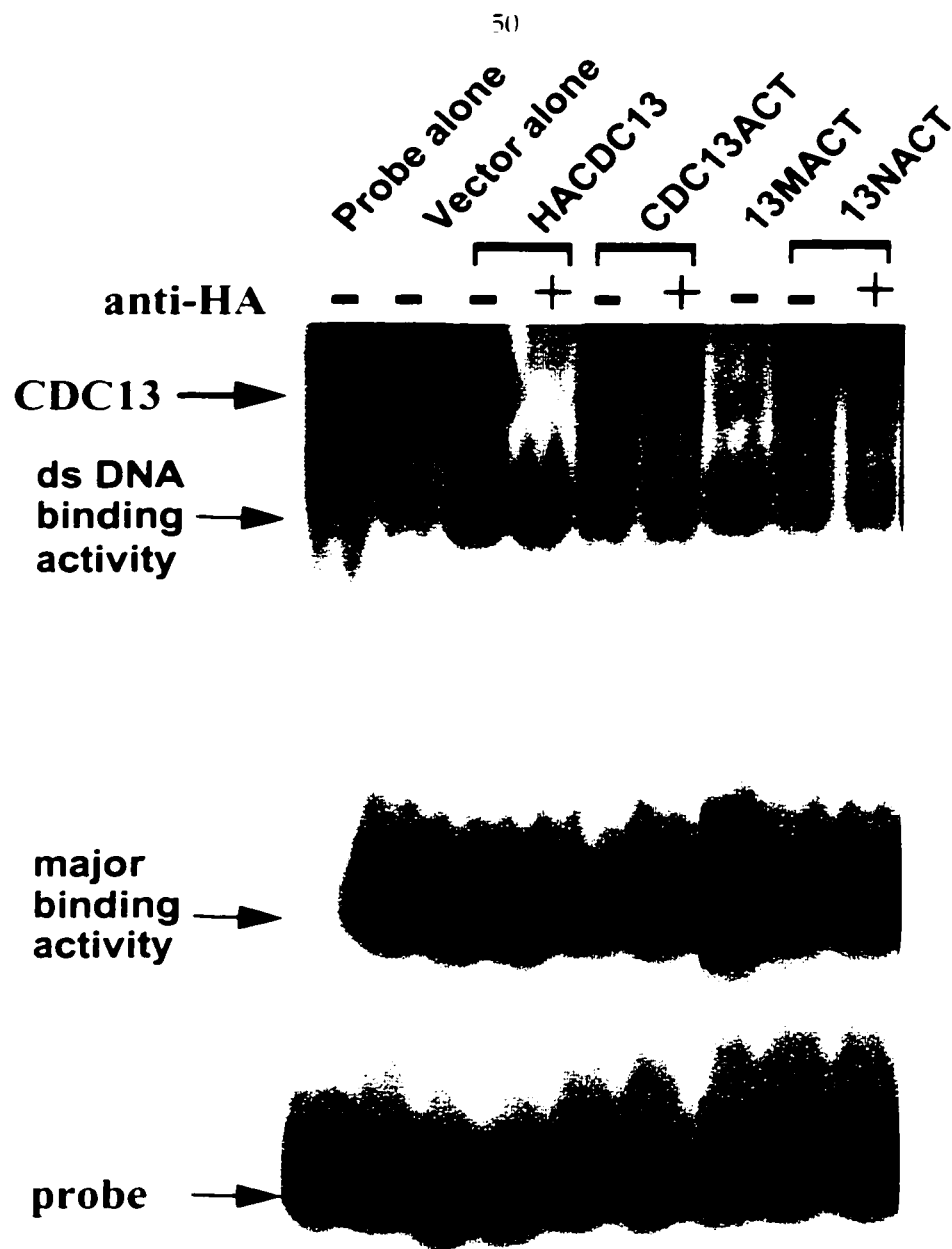


Figure 2.12 Cdc13Actp binds single-stranded G-rich oligonucleotides *in vitro*. Extracts made from strains containing vector alone, HA-tagged Cdc13p, or Cdc13 fusion proteins were subjected to gel mobility shift analysis in the absence (-) or presence (+) of HA antibodies. A double-stranded DNA binding activity of unknown origin, the binding activity of Tif51-A, and free probe bands are indicated.

CHAPTER 3: CHARACTERIZATION OF PROTEIN-DNA INTERACTIONS USING AN *IN VIVO* SYSTEM

SUMMARY

In this chapter, the telomere one hybrid approach was used to characterize protein interactions with a *bona fide* telomere, as well as with internal telomeric sequence. It was found that the C-terminus of Sir3p, but not Rap1p, Rif1p, or Sir4p, require the presence of endogenous Sir3p to interact with a telomere. In addition, Rap1p, Rif1p, Sir4p, Sir3p, and Sir2p were shown to interact with an internal tract of telomeric sequence; for Sir3p and Sir4p this binding required endogenous Sir3p. A telomere one-hybrid system with increased sensitivity was able to detect the interaction of two regions of Cdc13p with a telomere, including the previously demonstrated N-terminus as well as a new interaction with the middle region. Moreover, the binding of the middle region was decreased when the fusion protein was made from the temperature-sensitive allele. Finally, because the N-terminal region of Cdc13p was not able to associate with an internal tract of telomeric sequence, it appears that this protein binds specifically to telomeric sequence at the end of a chromosome, and further, that this assay was capable of distinguishing between a protein interaction with any telomeric sequence and one that required an actual end.

INTRODUCTION

BACKGROUND

Given that a telomere one-hybrid approach is an effective means of examining protein interactions with a telomere (Chapter 2), a similar approach might be used to learn more about these interactions. Several aspects of protein-telomere interactions lend themselves to further analysis using this assay system. First, the system could be easily used to determine which components of the telosome are recruited by which others and thus, how the structure forms. Performing the telomere one-hybrid assay in a strain that is defective for any of the components in the telosome, should allow the determination of which proteins are required for the presence of which other proteins in the complex. In this way, the roles of individual components in the formation of the complex might be elucidated.

TELOMERE POSITION EFFECT SPREADING

Second, the one-hybrid system might be used to investigate how the spreading of telomere position effect is mediated. One model for the mechanism of telomere position effect (TPE), posits that Rap1p interacts with the telomeric sequence and thereby recruits Sir3 and Sir4 proteins to the telomere proper (rev. in (Grunstein, 1997)). This recruitment by Rap1p increases the local concentration of Sir proteins thus nucleating a chromatin complex. The Sir3/Sir4 seed then oligomerizes inward toward the interior of the chromosome via interactions between Sir3p and Sir4p themselves, as well as with the N-termini of histones H3 and H4, forming a closed chromatin structure as they go. In wild type cells, TPE and therefore likely this spreading, may persist for up to (4-5) kb away from a telomere (Gottschling et al., 1990). Sir3p is thought to be the limiting protein in the process, because when this Sir protein, and not Sir2p and Sir4p, is

overexpressed, the spreading propagates much farther into the chromosome such that genes as far as 17 kb from a telomere are repressed (Hecht et al., 1996; Renauld et al., 1993).

Although the precise function of Sir2p is far from understood, it has been incorporated into the Sir3/Sir4 oligomerization model. According to this model, Sir3/Sir4p recruit Sir2p to the heterochromatin-like structure allowing it to modify the adjacent histone by deacetylation (Braunstein et al., 1996). This modification might facilitate further Sir3p and Sir4p binding, thus spreading the silencing complex along the chromatin by Sir3p and Sir4p interactions with histones. In this model, Sir2p may function at the telomere both structurally and catalytically to induce silencing.

Interestingly, integrating a telomeric tract at a locus that is not normally subject to TPE, causes repression near the tract. This effect is stronger the closer to a telomere the tract is integrated (Stavenhagen and Zakian, 1994). It may be that mechanism behind this effect is that the 270 bp tract of telomeric sequence is able to nucleate a silencing seed, thereby repressing genes in surrounding chromatin. In this model, the proximity of a telomere functions to bring a pool of silencing proteins nearer to the tract, thus increasing the frequency of silencing at this locus. Alternatively, the TG₁₋₃ sequence may act to boost the spreading from the telomere farther into the chromosome. In this context, one-hybrid methodology can be used to address the mechanism by which an internal tract of telomeric sequence effectively boosts TPE. To analyze this phenomenon, a stretch of telomeric DNA adjacent to a promoter-defective *HIS3* gene was integrated 20 kb from a telomere. If a given protein were found to associate with this internal telomeric sequence in such a strain, one could then

systematically delete proteins involved in TPE to determine which telomeric proteins are involved in this spreading phenomenon.

END SPECIFICITY

A third way in which the telomere one-hybrid system might be employed is to test whether a given protein requires an actual end to bind. Such an end-specific complex has been identified in the ciliated protozoan *Oxytricha* (Gottschling and Zakian, 1986; Gray et al., 1991). This complex consists of a heterodimer of proteins that tenaciously bind the single-stranded extension at the telomere end. Recently, a candidate for such an end-specific protein in yeast has been identified. When the temperature-sensitive yeast *cdc13-1* is grown at semi-permissive temperature, it has large amounts of single-stranded DNA near the telomeres and an increase in recombination near telomeres as well (Carson and Hartwell, 1985; Garvik et al., 1995). The protein, Cdc13p, has been demonstrated *in vitro* to bind to single-stranded telomeric DNA of the G-rich strand (Lin and Zakian, 1996; Nugent et al., 1996). In the preceding chapter, the telomere one-hybrid system was used to show that the N-terminus of Cdc13p interacts with a telomere *in vivo*. Because single-stranded telomeric DNA is thought to only be present at the ends, Cdc13p is a good candidate for an end-specific yeast counterpart.

Another type of end-binding factor may exist. It has been demonstrated that the introduction of extra telomeres into a cell causes a decrease in TPE, presumably by titrating factors necessary for TPE away from the monitored telomere (Wiley and Zakian, 1995). The decrease in TPE is evident only when the introduced telomeric sequence is at an end and not when the telomeric sequence is introduced on a circular plasmid. This suggests that at least one protein involved in TPE has a higher affinity for telomeric ends than for internal sequences (Wiley and Zakian, 1995). Development of a

means to distinguish which proteins exhibit such end-specific binding is important for the complete understanding of the properties and functions of telomeres.

Most of the previously characterized telosomal proteins are thought to bind double-stranded telomeric DNA (Rap1p) (rev. in (Shore, 1994)), or to bind to the complex via protein-protein interactions via Rap1p (Rif1p, Rif2p, Sir3p, Sir4p) (Moretti et al., 1994; Wotton and Shore, 1997) (rev. in (Grunstein, 1997)). As such, all these proteins would be expected to bind internal tracts of telomeric DNA. In theory, the telomere one-hybrid system could distinguish between proteins that bind any double-stranded telomeric DNA, and those that require a *bona fide* telomere.

MATERIALS AND METHODS

YEAST MEDIA, STRAINS AND PLASMIDS

Yeast were grown in standard yeast media (Zakian and Scott, 1982). To eliminate the entire *SIR3* open reading frame, cells were transformed with PvuII digested pNO3. To construct pNO3, the plasmid pKL3 that contains the *SIR3* gene and ~700 bp of flanking DNA, was digested with EcoRI and partially digested with HpaI such that the entire open reading frame of *SIR3* was removed and the ~4.2 kb vector was left. This left ~600 bp of flanking DNA on the 5' end of *SIR3* and ~1 kb on the 3' end. Subsequently the 4.5 kb *LYS2* gene was excised from pTD27 (a kind gift from Tricia Davis) with EcoRI and PvuII, and ligated into the digested vector. The INTCAHIS strain was made by digesting the pYACAHIS plasmid with XmnI and transforming YM701. pYACAHIS was constructed in a 3-part ligation. The telomere tract for the ligation was obtained by cutting pCT300 with PvuII and NarI, then performing a partial digest with

EcoRI to liberate the ~550 bp fragment containing a ~270 bp telomere tract. Then a ligation was performed with the 550 bp telomere fragment, the 5.8 kb MscI-XbaI fragment of pYA4-2 and the 3.6 kb EcoRI-XbaI fragment of p601. All strain constructions were verified by Southern analysis.

WESTERN ANALYSIS

Samples were prepared for Western analysis essentially as (Lin and Zakian, 1994; Lin and Zakian, 1996). Protein samples were electrophoretically separated on 7.8% acrylamide gels. Gels were subsequently blotted to nitrocellulose (Lin and Zakian, 1994; Lin and Zakian, 1996). The primary antibody for detecting expression of fusion proteins was anti-HA antibody diluted 1:100 (Amersham Corp., Arlington Heights, IL). The secondary antibody was Horseradish peroxidase conjugated anti-mouse (Amersham Corp.). Enhanced Chemi-luminescence (Amersham Corp.) was used to detect bands. For the Sir3 western; the method was as (Palladino et al., 1993). The primary antibody was diluted 1:5000 (a gift from Jasper Rine). The secondary antibody was alkaline phosphatase conjugated anti-mouse diluted 1:3000. The development of the Sir3 blot was by nitro blue tetrazolium visualization (Promega, Madison, WI).

TPE ANALYSIS

Tested strains were grown overnight in YC medium and then spotted in 10-fold serial dilutions onto YC plates containing 1 mg/ml FOA (PCR, Inc.) or YC plates and grown for 7 days or 2 days respectively.

MATING ASSAY

Cells were smashed together onto YEPD plates, either as the strain alone, or mixed with tester strains PTa and PT α and allowed to incubate at 30°C for six hours. They were then replica plated to Ymin medium, which selects only diploids, and grown overnight at 30°C.

RESULTS

RAP1P, RIF1P, AND SIR4P DO NOT REQUIRE THE ENDOGENOUS SIR3 PROTEIN FOR ASSOCIATION WITH A TELOMERE

To determine whether or any proteins giving positive results in the one-hybrid system associated with the telomere via Sir3p, each of the fusion proteins was tested in a HISTEL strain lacking Sir3p. As described in Chapter 2, the telomere one-hybrid system was used to demonstrate that regions of the Rap1, Rif1, Sir4, Sir3, and Sir2 proteins each associate with the telomere. If this binding were dependent upon Sir3p, the *sir3* Δ HISTEL strain containing a particular fusion protein should grow no better than the same strain containing vector alone. To test for interactions in a *sir3* Δ strain, cells were plated on medium containing more 3-AT (50mM). This was necessary because, in contrast to cells with wild type *SIR3*, essentially all *sir3* Δ HISTEL cells with vector alone generated colonies on the usual telomere interaction plates (5mM 3-AT, data not shown). Presumably, this is because deletion of *SIR3* eliminates TPE thereby allowing some expression of the telomeric *HIS3* gene. 50mM 3-AT was determined to be the lowest concentration of 3-AT at which the *sir3* Δ HISTEL strain containing vector alone was unable to generate colonies. On test plates containing 50mM 3-AT, RapBActp, RifActp, and Sir4Actp expressing cells all generated colonies much more efficiently than cells carrying vector alone (Figure 3.1, left panel, compare rows 2-4 to

row 1). In contrast, the Sir3Actp and Sir2Actp expressing cells were unable to generate colonies on these plates (Figure 3.1, rows 5 and 6). From these data, I conclude that binding of Rap1p, Rif1p, and Sir4p to the telomere does not require Sir3p, whereas that of Sir2Actp and Sir3Actp (which encodes the C-terminal 541 amino acids of Sir3p) apparently do.

The Cdc13 protein was subjected to similar analysis. Consistent with the finding (Chapter 2) that full-length Cdc13p, or the *CDC13* middle region (13MActp), were unable to demonstrate telomere association, neither Cdc13p nor 13MActp bound to the telosome in the absence of endogenous Sir3p (Figure 3.2, compare rows 2 and 3 to row 1). In addition, whereas the N-terminal region of Cdc13p (13NActp) could associate with a telomere in HISTEL in a wild type *SIR3* background (Chapter 2), in the absence of *SIR3*, 13NActp was unable to demonstrate association (Figure 3.2, compare row 4 to row 1). This is consistent with 13NActp requiring the presence of the endogenous Sir3p to bind to the telomere.

DEVELOPMENT OF A MORE SENSITIVE TELOMERE ONE-HYBRID SYSTEM

Despite the fact that the reporter genes were placed such that their TATA regions were located in the previously defined hypersensitive region of a telomere, the *lacZ* and *HIS3* genes at a telomere were clearly subject to some degree of telomere position effect. This is evidenced by the fact that eliminating functional *SIR3* from the cells allowed some expression of the reporter genes' products (Chapter 2, and preceding section). Therefore, it might be possible to improve the sensitivity of the HISTEL system by eliminating TPE from the strain, hopefully allowing the detection of weaker, more distant, or more transient interactions. To accomplish this, HISTEL was manipulated such that the insertion derivative *sir3::LYS2*, which causes TPE to be eliminated, was its

only copy of *SIR3*. As was true for *sir3Δ* HISTEL, when the HISTEL strain carried the *sir3::LYS2* allele, it was able to generate colonies on Gal-his + 5mM 3-AT plates, much more frequently than the HISTEL strain containing wild type *SIR3* (data not shown). This indicated that the *HIS3* gene in HISTEL was somewhat repressed by telomere position effect, and that the *sir3::LYS2* allele reduced TPE in the HISTEL strain.

The *sir3::LYS2* HISTEL strain was used to detect potentially weaker interactions of the Cdc13 protein with a telomere. When Cdc13Actp was expressed in this strain, it grew no better than the strain bearing vector alone (Figure 3.3, compare row 2 to row 1). This result was consistent with the results in Chapter 2 that indicated that much of the effect of full-length Cdc13p on the transcription of *HIS3* was due at least in part to a decrease in TPE rather than to a transactivation effect. Since there was no TPE in this strain to affect, the full-length protein had no effect on the reporter gene. However, when regions of the Cdc13 protein were subjected to one-hybrid analysis in the *sir3::LYS2* strain, both the M and N regions, but not the C region, improved the colony forming ability of HISTEL approximately 100-fold compared to vector alone (Figure 3.3, compare bottom two rows to Vector row). These results indicated that in the absence of TPE, interactions of two Cdc13p regions, the middle and the N-terminus, with the telomere were detectable.

When a fusion protein made from the temperature sensitive *cdc13-1* allele was tested in the system, the M region showed decreased ability to activate the *HIS3* gene (Figure 3.4, compare row 3 to rows 2 and 1). These results suggest that Cdc13p can, indeed, be associated with a telomere *in vivo* and are consistent with the interpretation that the temperature-sensitive Cdc13-1p is defective in its interaction with a telomere.

DIFFERENCES IN THE TWO SIR3 KNOCKOUT STRAINS

Perplexingly, however, these results were not consistent with the results obtained for the strain in which *SIR3* was completely deleted (previous section). Specifically, the M and N fusion proteins of Cdc13p were able to activate *HIS3* in the *sir3::LYS2* HISTEL strain, whereas in the *sir3Δ* strain they were unable to do so (Figure 3.2). However, because the temperature sensitive version of the M region had reduced ability to activate, the interaction in the *sir3::LYS2* HISTEL seemed to reflect a functional difference evidenced *in vivo*. Since this difference between strains was unexpected, I tested the other fusion proteins for their ability to interact with the *HIS3* telomere in the *sir3::LYS2* strain. Consistent with the results in the *sir3Δ* background, RapActp, RifActp, and Sir4Actp activated the reporter (Figure 3.5, compare the last 3 rows with row 1), whereas Sir3Actp did not (data not shown). To confirm this result, the experiment was repeated in a second *sir3Δ*HISTEL clone; again no differences were detected (data not shown).

Because the *sir3::LYS2* HISTEL one-hybrid assay seemed to detect interactions that were not detected in *sir3Δ* HISTEL, I wondered if the difference between the two might be that the *sir3::LYS2* gene encoded a protein which was partially functional. To determine whether the *sir3::LYS2* strain might express a form of Sir3p that is partially functional, this strain was compared to the *sir3Δ* strain in several tests. Because strains with *SIR* deletions are sterile as a result of their inability to silence the silent mating type loci, if the strain could mate, it would indicate that the strain had partial function in silencing. I compared the abilities of the *sir3::LYS2* and the *sir3Δ* strains to mate. Wild type MAT a or α type, *sir3::LYS2*, or *sir3Δ* strains were mixed together and allowed to mate. After 6 hours, the plates were replica plated onto plates that allowed growth only of diploids resulting from the mating. In this assay, wild type

strains were able to generate diploids on the selective plates when mixed with the opposite mating type. However, as with the complete deletion, the *sir3::LYS2* allele did not mate with either mating type (Figure 3.6). This indicated that, for mating, the *sir3::LYS2* strain was functionally equivalent to the *sir3Δ* strain.

Next I wanted to ensure that the *sir3::LYS2* strain was indeed null for telomere position effect. Normally, when the *URA3* gene is placed near a telomere, it is transcriptionally repressed relative to the same gene located at an internal position on the chromosome (Gottschling et al., 1990). Because cells transcribing *URA3* are unable to live on medium containing 5-Fluoroorotic acid (FOA), the ability of the cells to grow on FOA plates is a convenient means to determine what percentage of cells are repressing the telomere adjacent *URA3* gene. In wild type strains, telomere position effect is evidenced in approximately 10-90% of cells (Gottschling et al., 1990). However, if any of the *SIR2-4* genes is defective, no cells are able to repress *URA3* and therefore, none of them will be able to grow on FOA plates. When 10-fold serial dilutions of the HISTEL strain were plated on FOA plates, cells were able to generate colonies out to the 4th or 5th dilution (Figure 3.7, top two rows). Comparison with the non-selective plate (which demonstrates plating efficiency), indicated that the frequency of cells repressing the telomeric *URA3* in the HISTEL strain was ~20%. In contrast, HISTEL strains containing the *sir3::LYS2* allele, or the complete *sir3* null were unable to form colonies on FOA plates (Figure 3.7, bottom four rows). These results indicated that the *sir3::LYS2* strain is indistinguishable from *sir3Δ* with respect to telomere position effect.

The knockout construct for the *sir3::LYS2* allele was made in such a way that the *LYS2* gene was inserted into *SIR3* such that it only disrupted the last 17 amino acids of the protein (Kimmerly and Rine, 1987). Therefore, I wondered if a truncated

protein was being expressed which might retain some of the functions of the full-length protein. To address this question, I analyzed strains containing the two alleles by Western analysis. A protein of approximately the right size was detected in the wild type strain and a strain overexpressing Sir3p (Figure 3.8, SIR3 lanes 3 and 5 respectively). As expected, no protein was detected in the *sir3Δ* (Figure 3.8, *sir3Δ* lanes). However, even though the blot was allowed to overdevelop, no band of the appropriate size was detected in the *sir3::LYS2* allele (Figure 3.8, *sir3::LYS2* lane). Furthermore, no additional bands of smaller or larger size were detected in this lane. However, because the strain behaves differently than the *sir3Δ* strain in the telomere one-hybrid assay, some form of Sir3p must be expressed in the *sir3::LYS2* strain. Perhaps it was not detected in these experiments, due to instability of the protein or because it was masked by another cross-hybridizing band. Alternatively, it may make a fusion protein with the integrated *LYS2* gene and therefore was too big to be detected in this Western Blot.

A TELOMERE ONE-HYBRID TO STUDY BOOSTING OF TELOMERE POSITION EFFECT SPREADING

Previously it has been demonstrated that internal tracts of telomeric sequence can cause TPE to be propagated further along the chromosome (Stavenhagen and Zakian, 1994). To study this phenomenon, a cassette consisting of a 270 bp tract of telomeric sequence immediately adjacent to the promoter-defective *HIS3* allele was integrated at the *ADH4* locus. This strain, INTCAHIS (for internal C₁₋₃A adjacent to HIS3), grew better than HISTEL on the original telomere interaction plates (5mM, data not shown). This was not surprising, however, because telomeric sequences have previously been shown to have an activation effect on nearby genes when they are integrated internally (Runge and Zakian, 1990). To allow the capability to distinguish

cells that are activating *HIS3*, more 3-AT (10mM) was added to the plates so that the strain carrying vector alone would not be able to generate colonies (Figure 3.9, row 1). When the fusion proteins RapBActp, RifActp, Sir4Actp, Sir3Actp, and Sir2Actp were expressed in INTCAHIS, they allowed cells to generate colonies approximately 100-1000 fold better than vector alone (Figure 3.9, compare the bottom 5 rows to the vector alone row). These results indicate that Rap1, Rif1, Sir4, Sir3, and Sir2 proteins are able to associate with telomeric sequence when it is located internally.

When the *SIR3* gene (and therefore TPE) was eliminated in INTCAHIS, even more 3-AT (50mM) was required to eliminate colony formation in the absence of a fusion protein (Figure 3.10). This indicated that, in spite of the aforementioned TG₁₋₃ enhancement of the *HIS3* gene, the *HIS3* reporter gene was still subject to telomere position effect. Therefore, whereas in a wild type strain, TPE only extends ~3-5 kb (Gottschling et al., 1990), in this strain a gene 20 kb from a telomere was affected. This suggests that the internal telomeric tract caused telomere position effect to be spread further along the chromosome than it would otherwise propagate.

When the fusion proteins were tested in *sir3Δ* INTCAHIS, only RapActp and RifActp expressing cells were able to generate colonies better than vector alone (Figure 3.10, compare rows 2 and 3 to row 1). These results suggest that the association of the Sir4 and Sir3, but not the Rap1 and Rif1 fusion proteins, with an internal telomeric sequence require the presence of the endogenous Sir3p.

THE TELOMERE ONE-HYBRID SIGNAL REFLECTS THE END-SPECIFICITY OF A PROTEIN

In theory, the telomere one-hybrid system could distinguish between proteins that bind any double-stranded telomeric DNA, and those that require a *bona fide* telomere. Therefore, I used the one-hybrid system to see if it could distinguish

between an end-binding factor and those components of the telosome that are thought to associate with a telomere by virtue of their interaction with Rap1p. As demonstrated in the preceding section, most of the fusion proteins, RapActp, RifActp, Sir4Actp, Sir3Actp, and Sir2Actp associated with the internal tract of telomeric sequence. However, the N-terminus of Cdc13p (13NActp) was unable to confer colony forming ability on the INTCAHIS strain (Figure 3.11, compare row 3 to row 1), even at this very low level of 3-AT (5mM) that allows many of the Vector alone cells to form colonies. These results are consistent with the idea that Cdc13p interacts with a telomere via an interaction with the single-stranded extension at the end of telomeres.

DISCUSSION

TELOSOME FORMATION

One-hybrid methodology was used to characterize various aspects of protein interaction with telomeric DNA. In Chapter 2 Rap1, Rif1, Sir4, Sir3, and Sir2 proteins were seen to associate with the *HIS3* adjacent telomere. In this chapter, the use of a HISTEL strain with a deletion of *SIR3*, allowed us to investigate whether formation of the telomeric complex required endogenous Sir3p. Results reported here indicated that the association with telomeres of Rap1p, Rif1p and Sir4p did not depend upon the presence of Sir3p (Figure 3.1). However, although the endogenous Sir3p was not required for these interactions, it may in fact stabilize the complex because the activation of *HIS3* by Sir4Actp appeared to be slightly weaker in the absence of Sir3p (data not shown). These results are consistent with the interpretation that recruiting Sir4p is not the primary purpose of Sir3p in the complex, nor does its presence impact strongly upon the binding of Rap1p and Rif1p.

The association of Sir2Actp did appear to be dependent on endogenous *SIR3* (Figure 3.1). However, because the Sir2Actp phenotype was difficult to detect in wild type cells (Chapter 2), this conclusion is less certain. Paradoxically, the binding of Sir3Actp, which includes the C-terminal 541 amino acids of the protein, was unable to associate with the telosome in the absence of endogenous Sir3p (Figure 3.1). One might have thought that in the absence of wild type Sir3p, Sir3Actp would generate a stronger signal because it would not have to compete with the endogenous protein. By two-hybrid analysis, however, we know that Sir3p can interact with itself (Moretti et al., 1994). Therefore, it is possible that the C-terminal *SIR3* construct does not contain the region required for binding to other parts of the complex (e.g. Rap1p and Sir4p), but that it does contain the region required for interaction with itself and is therefore positive in this assay via its homotypic interaction. Another possibility is that the proteins all have multiple interaction domains to associate with the other telosomal proteins in the complex. The C-terminal Sir3Actp may be missing these domains such that, in the absence of *SIR3*, the stability of the complex decreases enough to dissociate the truncated Sir3p. In support of this notion, Sir4Actp activated the telomeric *HIS3* more weakly than in the wild type *SIR3* background. Furthermore, several studies have found that the Sir2, 3 and 4 proteins have direct interactions both with other Sir proteins and with themselves (Hecht et al., 1996; Moazed and Johnson, 1996; Moretti et al., 1994; Strahl-Bolsinger et al., 1997). Therefore, each of the Sir proteins may contribute to the overall stability of the telosome complex.

BOOSTING OF TPE

Another application of the one-hybrid technique was as a means to investigate the molecular mechanism for the boosting of TPE by internal stretches of

telomeric sequence. Internal stretches of telomeric DNA have been shown to cause TPE to propagate farther along the chromosome than it does with no internal telomeric sequence (Stavenhagen and Zakian, 1994). In the work reported here, it was shown that Rap1p, Rif1p, Sir3p, Sir4p, and Sir2p associated with a 270 bp tract of telomeric sequence integrated at the *ADH4* locus, 20 kb from a telomere (Figure 3.10). Because the *HIS3* gene by itself integrated at this locus did not show association of these proteins (Chapter 2), these proteins must be recruited to the internal site via the telomeric sequence.

Interestingly, the presence of the telomeric sequence adjacent to the *HIS3* gene caused some activation in the absence of any fusion protein as evidenced by the fact that more 3-AT was required in this strain to eliminate the growth; 10mM 3-AT was required whereas no 3-AT was necessary when the *HIS3* gene was internal (Chapter 2). This is consistent with earlier work that demonstrated that TG_{1-3} sequences on a plasmid are capable of acting as enhancers to genes placed near them (Runge and Zakian, 1990), and that a 71 bp tract of TG_{1-3} enhances transcription from the *URA3* gene when it is integrated at the *ADH4* locus (Gottschling et al., 1990).

The system was also used to investigate the manner in which recruitment of proteins by the internal tract of telomeric sequence proceeded. To test this, the *sir3Δ* allele was introduced into the INTCAHIS strain. For this strain, even more 3-AT had to be added to the plates to reduce the background of His⁺ cells, indicating that some repression (TPE) affected expression of the *HIS3* gene in the *SIR3* strain. In wild type *SIR3* cells, TPE is only evident to approximately 4-5 kb from a telomere (Gottschling et al., 1990). Therefore, the fact that deletion of *SIR3* allowed an increase in expression

from the *HIS3* gene 20 kb from a telomere, indicated that TPE was being boosted by the presence of the 270 bp tract of telomeric DNA to a site that was normally not affected.

To investigate the molecular mechanism behind this phenomenon, fusion proteins of Rap1, Rif1, Sir4, and Sir3 were expressed in the *sir3ΔINTCAHIS* strain. Whereas Rap1 and Rif1 fusion proteins activated the *HIS3* gene in the absence of wild type Sir3p, the Sir4 and Sir3 fusion proteins did not (Figure 3.10). These results suggest that, in the absence of endogenous Sir3p, Rif1p and Rap1p interact with the internal telomeric sequence, but that Sir3p and Sir4p do not. Sir3p is known to be required for the phenomenon of spreading (Hecht et al., 1996; Renauld et al., 1993). Therefore, the fact that Sir3p is also required for Sir3 and Sir4 fusion proteins to interact with an internal tract is consistent with the idea that spreading is required for the boosting of TPE. However, we cannot rule out the model that the increase in transcription when telomeric sequence is integrated internally is due to the telomeric sequence nucleating a new seed of Sir3p and Sir4p and that this nucleation requires *SIR3*.

These results also suggest that Rap1p and Rif1p do not require the spreading in order to associate with internal tracts of $C_{1-3}A$. It seems that the telomeric sequence alone is not able to recruit Sir3p and Sir4p to an internal position, whereas it can recruit Rif1p and Rap1p. Finally, because Sir4Actp was able to associate with the telomere in the absence of Sir3p (Figure 3.1) it seems that, for this phenomenon, Sir4p can distinguish between telomeric sequence at an internal site, and that at a true telomere.

A PROTEIN'S SPECIFICITY FOR ENDS CAN BE ADDRESSED USING THE ONE-HYBRID SYSTEM

Cdc13p is a structural protein that appears to have regulatory functions. It has been postulated to regulate access to telomeres by two proteins involved in

telomere replication, telomerase, and a putative C-strand exonuclease (Nugent et al., 1996; Wellinger et al., 1996). Because it binds single-stranded telomeric oligonucleotides of the G-rich strand *in vitro*, Cdc13p has been postulated to accomplish its functions by binding the single-stranded overhang present at the ends of telomeres (Lin and Zakian, 1996; Nugent et al., 1996). Given this affinity for single-stranded DNA, Cdc13p is likely to have higher affinity for ends than it does for internal sequence. The Cdc13 N region has been demonstrated to bind telomeres *in vivo* (Chapter 2). If Cdc13p were truly an end-binding factor, it should not bind internal tracts of telomeric sequence. Here, we demonstrated that although Rap1, Rif1, Sir3, Sir4, and Sir2 proteins could associate with an internal tract of telomeric sequence, the region of Cdc13p found to bind telomeres, the N region (Chapter 2), was unable to do so (Figure 3.11). This is true even under very lax conditions, (i.e. 5mM 3-AT) that allow a high frequency of vector alone colonies to be generated (Figure 3.11). This is exactly the result that would be expected if the Cdc13 protein were functioning as postulated. Therefore, our data support models of telomere function that require Cdc13p to bind the single-stranded G overhang *in vivo*. Furthermore, these data demonstrate that the telomere one-hybrid system is able to discriminate between proteins that bind any tract of telomeric sequence, and those that bind specifically to the sequence located at an end.

A MORE SENSITIVE HISTEL ASSAY DETECTS THE INTERACTION OF THE M REGION OF CDC13 WITH TELOMERES

Because the HISTEL *HIS3* gene was subject to some degree of position effect (Chap 2, and this chapter), eliminating TPE might permit the detection of weaker interactions. Therefore, I eliminated *SIR3* function in HISTEL by introducing an insertion allele, *sir3::LYS2* into the strain. Full-length Cdc13p, as well as the regions (N, M and C)

of Cdc13 were expressed in *sir3::LYS2 HISTEL*. Full-length Cdc13Actp did not increase transcription of *HIS3*, consistent with the fact that its effects on the *HIS3* gene were primarily due to decreasing TPE rather than activation (Chapter 2). However, two of the regions, the N region, which was already demonstrated to associate with the telomere in this system (Chapter 2), as well as the M region, demonstrated an interaction (Figures 3.3 and 3.4). Moreover, these associations correlated with *in vivo* function, because the temperature-sensitive version of the M region was defective in the interaction (Figure 3.4).

Perhaps surprisingly, the full-length Cdc13p did not demonstrate an interaction in this, presumably more sensitive, strain. However, expression of the full-length protein, but not its regions individually, causes a decrease in plating efficiency, as well as speed of colony growth (Figures 3.2, 3.3, 3.12, CDC13ACT row), and therefore, any His⁺ signal might be more difficult to detect. Alternatively, dividing the protein into three regions may destroy some inhibitory function carried out by the full-length protein. Consistent with this idea, the fact that Cdc13p is involved in a cell cycle regulated activity (replication), makes it likely to be regulated such that it will dissociate from the single-stranded overhang at some point in the cell cycle.

Seemingly, *sir3::LYS2 HISTEL* was more sensitive and detected the Cdc13 M interaction because the insertion into *SIR3* eliminated TPE. However, a strain in which the *SIR3* gene had been completely eliminated, did not detect any Cdc13 M or N association (Figure 3.2). Why then were the M and N regions able to associate in the *sir3::LYS2* strain but not in a complete *sir3* null strain?

DIFFERENCES IN *SIR3*Δ VERSUS *SIR3::LYS2* HISTEL RESULTS

One way to explain these differences is that in the *sir3::LYS2* strain, a truncated Sir3 protein is likely to be expressed in the strain. The *LYS2* insertion is integrated into the XhoI site in *SIR3* which would only eliminate the last 17 amino acids (Kimmerly and Rine, 1987). Because Sir3p associates with the C-terminal region of Rap1p (Hardy et al., 1992), it may be an effect on Rap1p that is responsible. Telomeric sequences under some conditions have the effect of enhancing a nearby gene (Runge and Zakian, 1990). Because Rap1p has transcriptional activation activity (Shore, 1995), the situation in *sir3::LYS2*HISTEL may result from an effect on the Rap1 protein. Rap1p molecules bound to the telomere must have some mechanism for masking the activation activity, given that repression is the predominant activity at a telomere. For instance, given that activation and Sir3p binding have been mapped to approximately the same region of Rap1p (Moretti et al., 1994), the truncated Sir3p may mask the ability of Rap1p to be a transcriptional activator. If Rap1p were the cause of the enhancement, and this region were masked by a truncated Sir3p defective in TPE, it might result in a neutral situation — that is, there is no TPE, but neither is there any enhancement. In this scenario, very sensitive interactions might be detected in the *sir3::LYS2* strain that would be overlooked when Rap1p was not masked, because the weak signal would be lost in the background of an unmasked Rap1p enhancement. Consistent with this explanation, the *sir3::LYS2* strain requires less 3-AT than does the *sir3*Δ to eliminate the growth of the strain in the absence of any fusion protein. That is, *HIS3* transcription in *sir3*Δ HISTEL, perhaps by Rap1p, is more than *HIS3* expression in *sir3::LYS2*HISTEL. Arguing against this model, however, is the fact that the M interaction in *sir3::LYS2* does not appear to be any weaker than the N region (Figure 3.3).

One explanation for the differences between the *sir3::LYS2* strain and the *sir3Δ* strain, is that the *sir3::LYS2* strain may be expressing a truncated Sir3 protein, that is partially functional. Perhaps a truncated Sir3p can bind and assemble a normal telosomal complex. However, because it exhibits no TPE (possibly as a result of not being able to interact with histones) it might therefore be the perfect strain for testing interactions in—that is, the telosomal complex is intact, but *HIS3* is not repressed by TPE. In the Cdc13p case, for example, having a functional N-terminus of Sir3p present might allow the G overhang to be bent back such that a fusion protein binding to it might be close enough to reach the TATA. If this were true, study of this allele of *SIR3* would be interesting because it would be a case where the formation of a complex was uncoupled from the ability to perform TPE. Evidence in support of this model is the fact that most (all but Sir3Actp) other fusion protein interactions are present in the *sir3::LYS2* allele (Figure 3.5).

To determine whether the *sir3::LYS2* allele had any partial function, I tested the ability of this strain to mate and to repress a gene near a telomere. These analyses did not detect any partial function of Sir3p (Figures 3.6 and 3.7). Both explanations for the differences between the *SIR3* alleles rely on the fact that a changed Sir3 protein is being expressed in the strain. However, Western analysis did not detect any (Figure 3.8). Therefore, if a truncated Sir3p was being expressed, it must have been present at lower levels than that of wild type. It seems likely that some protein was expressed, given the differences between the strains. In fact, other groups have reported that this allele behaves differently than other alleles of Sir3p in genetic suppression with Sir proteins (Lorraine Pillus and Elisa Stone, personal communication). In addition, the fact that a Cdc13 M fusion protein made from the temperature sensitive allele *cdc13-1*

was defective in telomere interaction in the *sir3::LYS2* HISTEL assay (Figure 3.4) suggests that the *sir3::LYS2* HISTEL is detecting a biologically relevant interaction.

The second model can be tested in a straightforward way. If it were true, it would predict that the telosomal structure would be more like wild type than like a *sir3Δ* strain. The telomeric regions of strains defective for TPE (specifically *sir2*, and *sir4* strains) have increased accessibility to *dam* methylase than their wild type counterpart (Gottschling, 1992). Additionally, the wild type telosome has a Micrococcal nuclease hypersensitive site just proximal to the telomeric repeats (Wright et al., 1992). To test whether the telomere and sub-telomeric region of the *sir3::LYS2* strain has chromatin more like *sir3Δ* or wild type, the three strains could be probed for their telosome structure by *dam* methylase experiments, and/or Micrococcal nuclease digestion.

COMPARISON WITH OTHER RESULTS

Recent reports found that the presence of each of Sir proteins 2, 3, and 4 was required for the multi-protein complex associated with the DNA 0.77 kb away from the telomeric sequence (Hecht et al., 1996; Strahl-Bolsinger et al., 1997), suggesting that the presence of a multi-protein complex required, or was stabilized by, the presence of other members of the complex. My finding that Sir4p does not require endogenous Sir3p to associate with the telosome, might seem to contradict these findings. However, it is likely that the two assays are detecting different complexes. For instance, it is possible that the ability to associate with chromatin 0.77 kb from the telomere may result from the spreading phenomenon, whereas association with a telomere proper does not. Indeed, our assay suggested that the presence of Sir3p and Sir4p at a telomeric tract 20 kb internal required the presence of endogenous Sir3p. Perhaps the requirement for the other endogenous Sir proteins in other systems, reflects a requirement for these proteins

for spreading into adjacent chromatin, and this spreading is not required for binding the telosome.

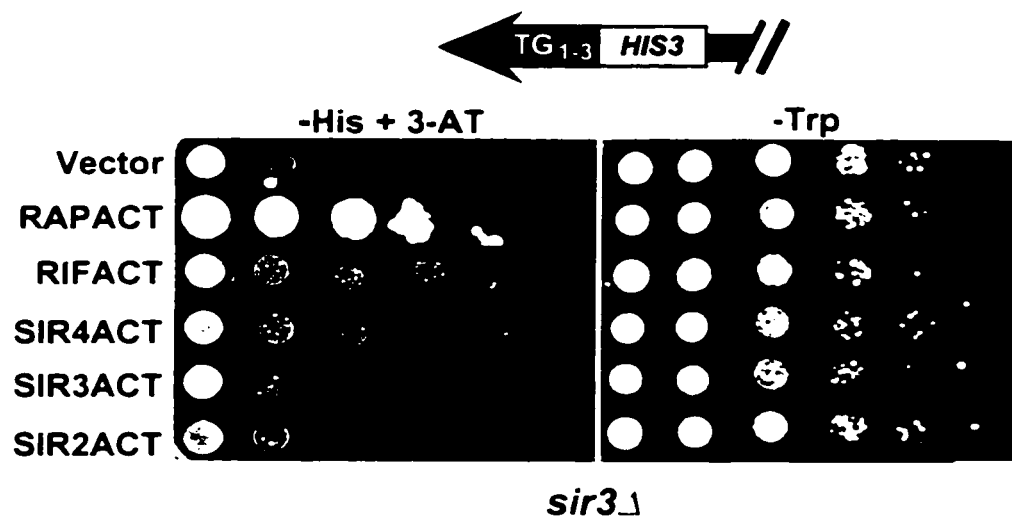


Figure 3.1 Rap1, Rif1, and Sir4 fusion proteins interact with a telomere in the absence of Sir3p. Strains containing the promoter-defective allele of *HIS3* integrated adjacent to a chromosomal telomere and expressing protein fusions were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for telomere interaction (Gal-His + 50 mM 3-AT, left panel) or onto plates selecting for plasmid-bearing cells (right panel) and allowed to grow for 13 and 4 days, respectively.

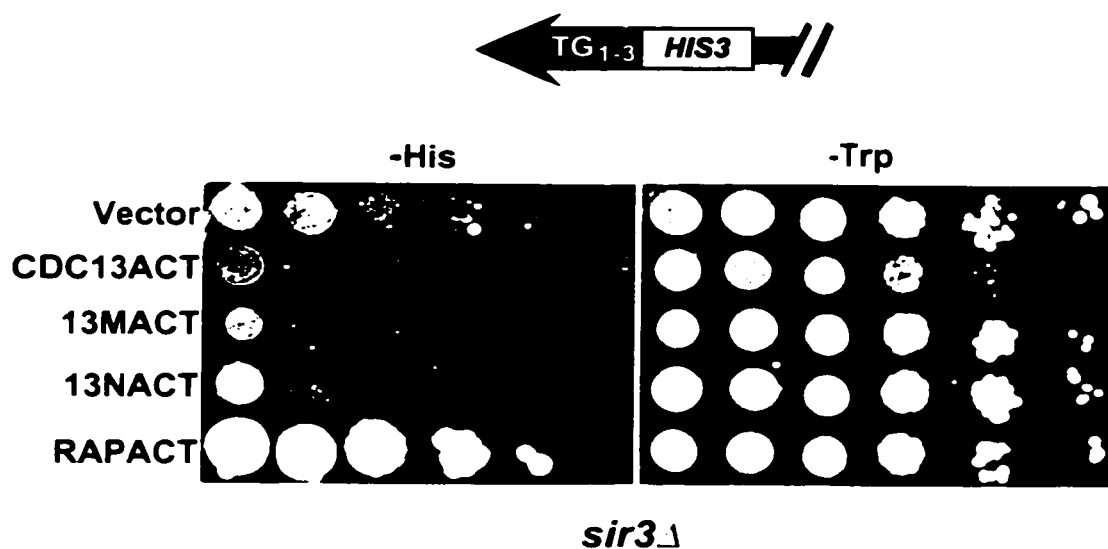


Figure 3.2 *CDC13* fusion proteins do not associate with a telomere in the absence of Sir3p. *sir3*Δ HISTEL cells expressing Cdc13 fusion proteins were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for the telomere interaction (Gal-His - 50mM 3-AT, left panel) or onto plates selecting for plasmid-bearing cells (right panel) and allowed to grow for 15 and 5 days, respectively.

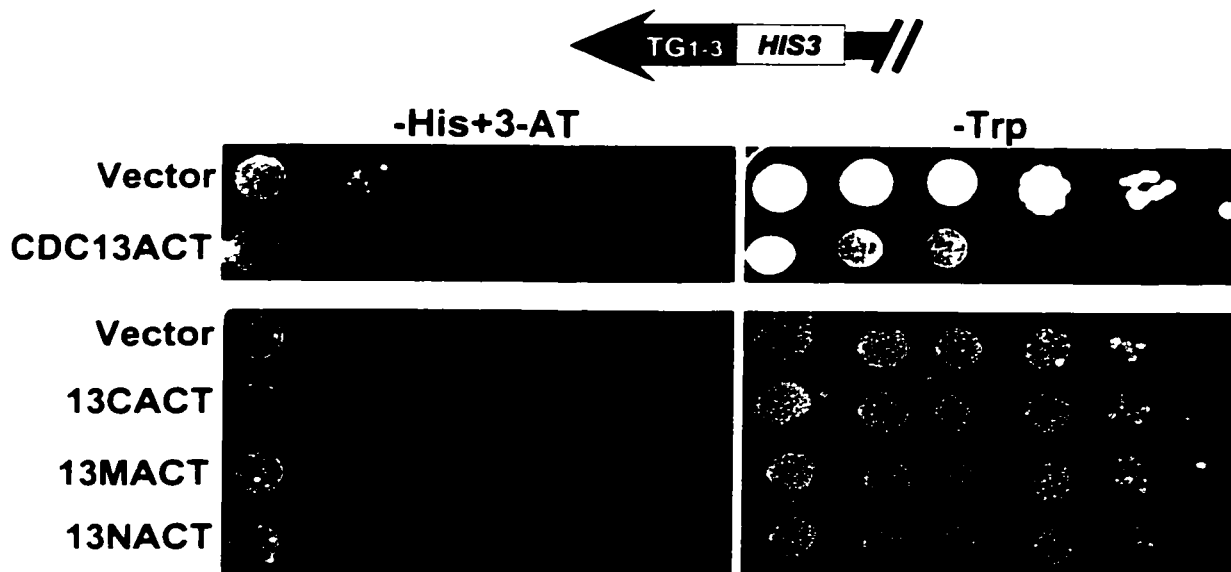


Figure 3.3 A more sensitive *HIS3* telomere one-hybrid system demonstrates the interaction of CDC13M and N regions with a telomere *in vivo*. Strains containing a promoter-defective allele of *HIS3* integrated adjacent to a chromosomal telomere and expressing Cdc13, Cdc13N, Cdc13M, and Cdc13C fusions protein were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for the telomere interaction (Gal-his - 50mM 3-AT, left panel) or plates selecting plasmid-bearing cells (right panel) and allowed to grow for 13 and 4 days, respectively.

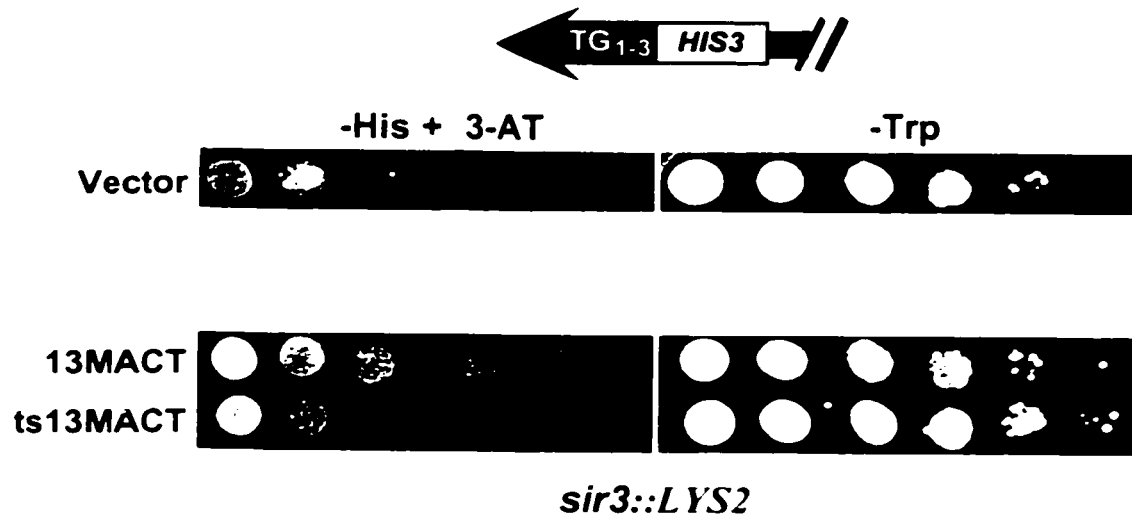


Figure 3.4 A fusion protein made from the temperature-sensitive allele of *CDC13* is defective for telomere association. Strains containing a promoter-defective allele of *HIS3* integrated adjacent to a chromosomal telomere and expressing protein fusions were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for the telomere interaction (Gal-his - 35mM 3-AT, left panel) or onto plates selecting for plasmid-bearing cells (right panel) and allowed to grow for 13 and 4 days, respectively.

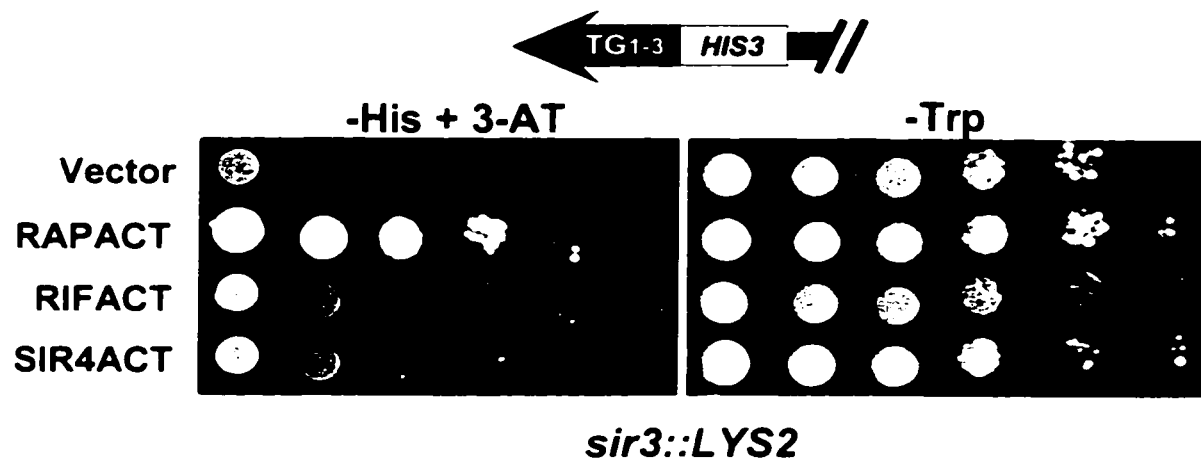


Figure 3.5 Interaction of Rap1, Rif1, and Sir4 fusion proteins with a telomere appear normal in the *sir3::LYS2* strain. Strains containing a promoter-defective allele of *HIS3* integrated adjacent to a chromosomal telomere and expressing protein fusions were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for the telomere interaction (Gal-his - 40mM 3-AT, left panel) or plates containing histidine (right panel) and allowed to grow for 8 and 5 days, respectively.

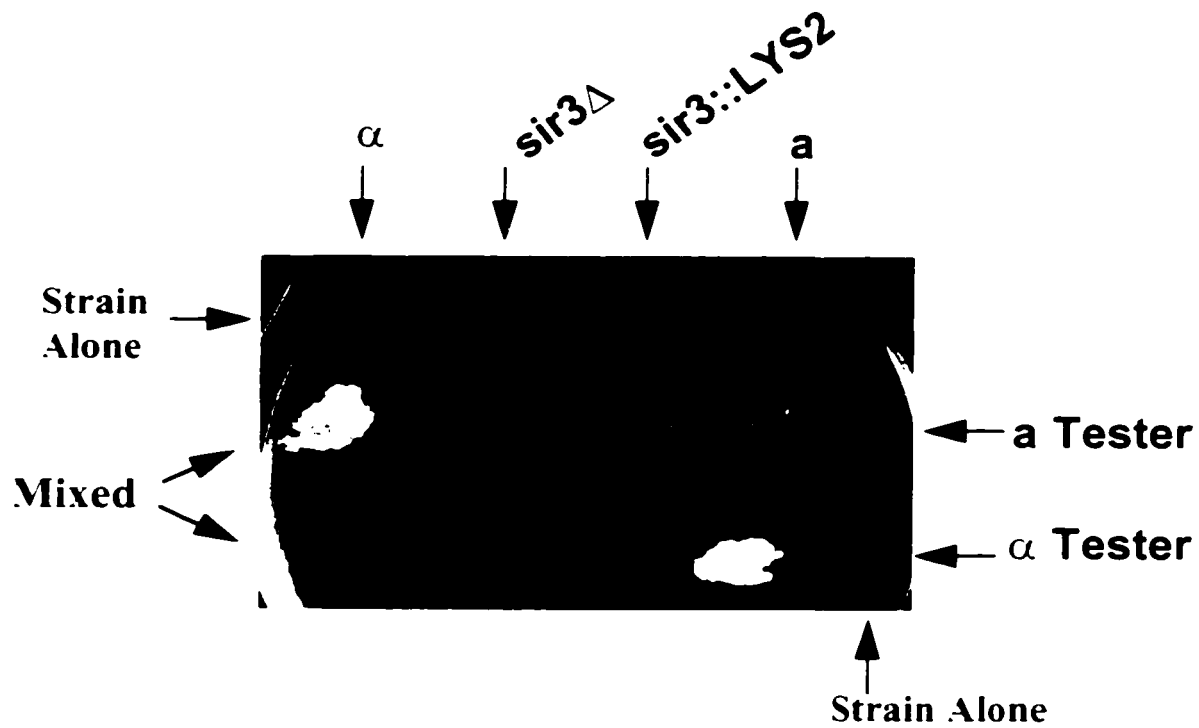


Figure 3.6 Both *sir3* Δ and *sir3::LYS2* HISTEL strains are sterile. The *sir3* Δ and *sir3::LYS2* strains, as well as wild type MAT **a** and MAT α control strains, were mixed on a YEPD plate with either MAT **a** PT cells or with MAT α PT cells. After incubation for 6 hours at 30 C, this plate was replica-plated to medium that only allows diploids resulting from the mating to grow. This plate was incubated for 1 day at 30 C.

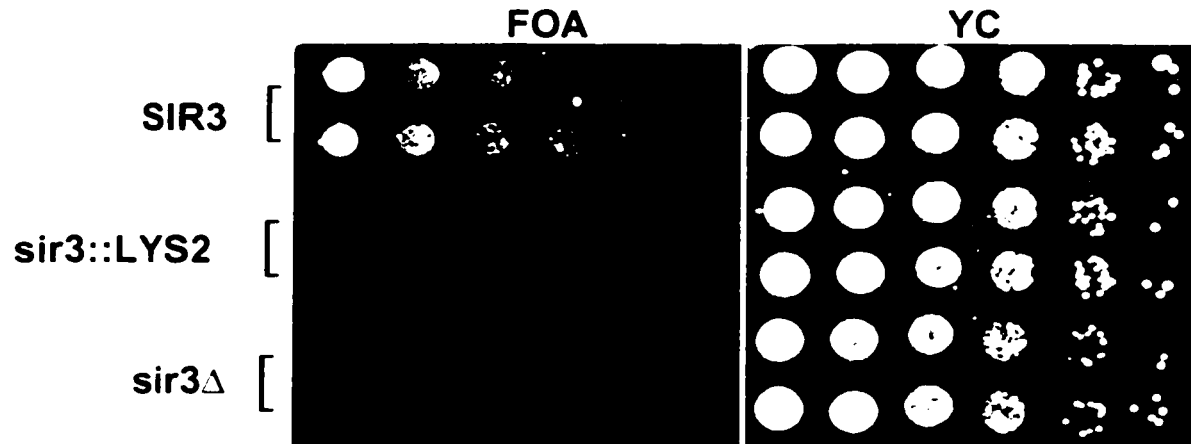


Figure 3.7 HISTEL *sir3*Δ and *sir3::LYS2* strains are defective in telomere position effect. Strains were grown in YEPD liquid overnight. Ten-fold serial dilutions were plated on FOA (left) or Yeast Complete (right) and incubated 6 or 2 days respectively.

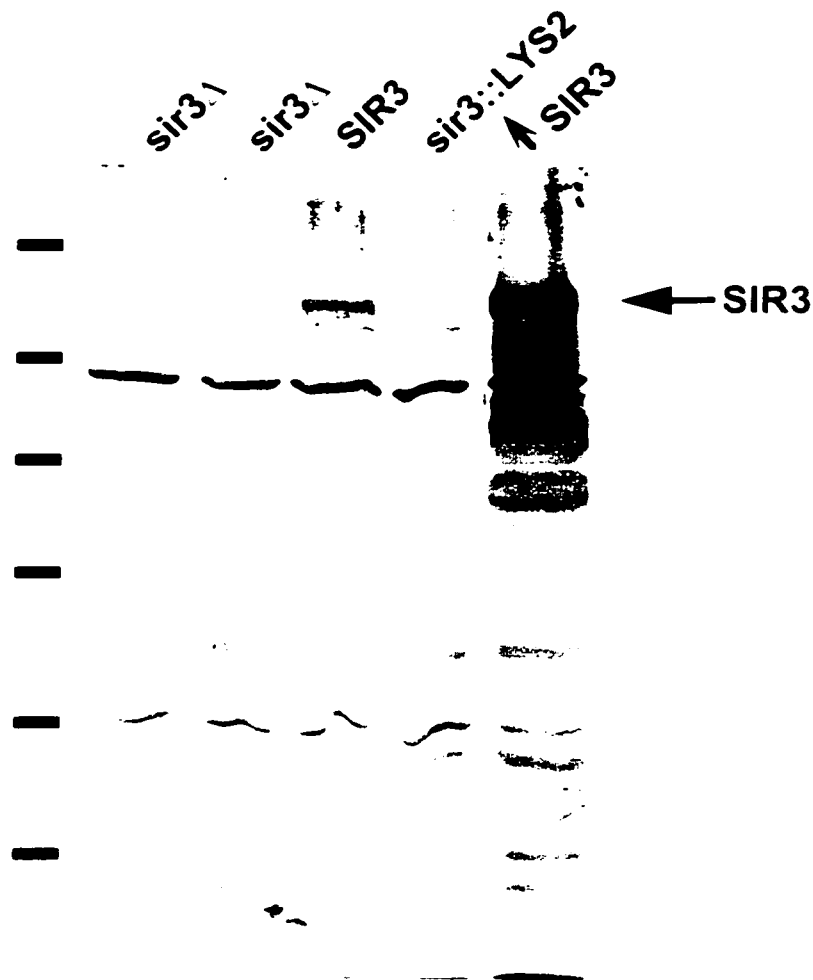


Figure 3.8 Western analysis does not detect Sir3p in *sir3Δ* or *sir3::LYS2*. HISTEL strains. Extracts made from HISTEL cells containing wild type *SIR3* (3rd lane), overexpressed *SIR3* (5th lane), *sir3Δ*, or *sir3::LYS2* (1st, 2nd and 4th lanes) were run on a 7.8% polyacrylamide gel and blotted to nitrocellulose. The blot was immunodetected for Sir3p and allowed to develop overnight.

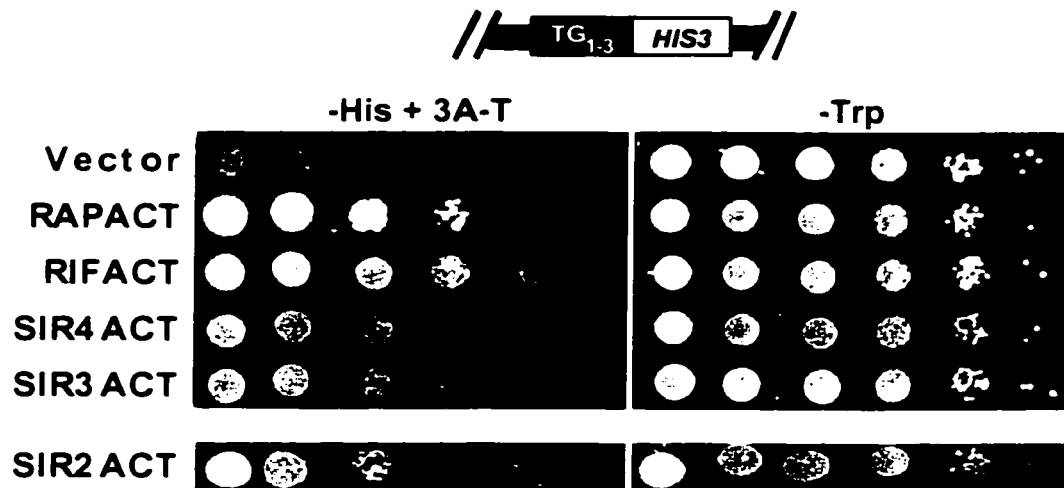


Figure 3.9 Rap1, Rif1, Sir4, Sir3, and Sir2 fusion proteins associate with an internal tract of telomeric sequence. INTCAHIS cells expressing Rap1, Rif1, Sir4, Sir3 and Sir2 fusion proteins were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for the interaction (Gal-his + 10mM 3-AT, left panel) or onto plates selecting for plasmid-bearing cells (right panel) and allowed to grow for 6 and 4 days, respectively.

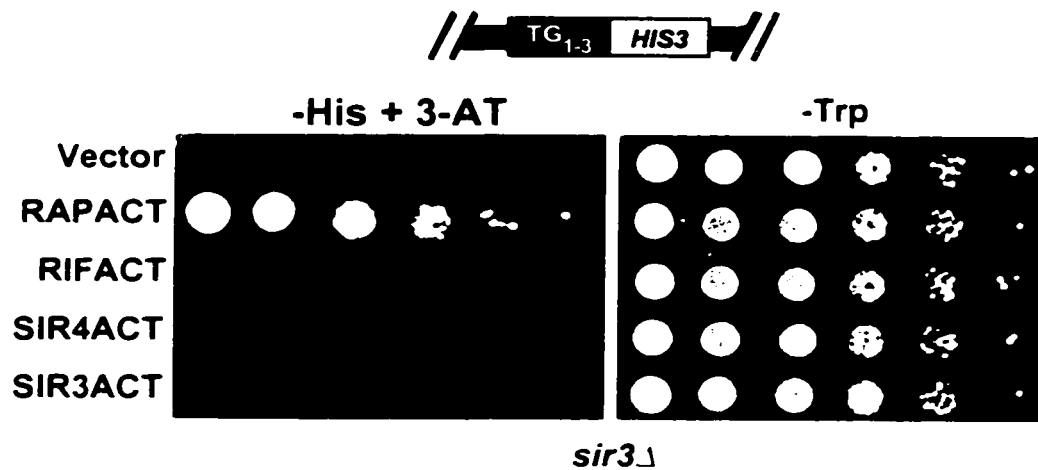


Figure 3.10 Sir4 and Sir3, but not Rap1 and Rif1 fusion proteins require Sir3p to associate with an internal tract of telomeric sequence. The *sir3*Δ strain bearing an internal tract of telomere sequence adjacent to the promoter-defective *HIS3* gene and expressing Rap1, Rif1, Sir4, or Sir3 fusion proteins were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for the interaction (Gal-his - 50mM 3-AT, left panel) or onto plates selecting for plasmid-bearing cells (right panel) and allowed to grow for 9 and 4 days, respectively.

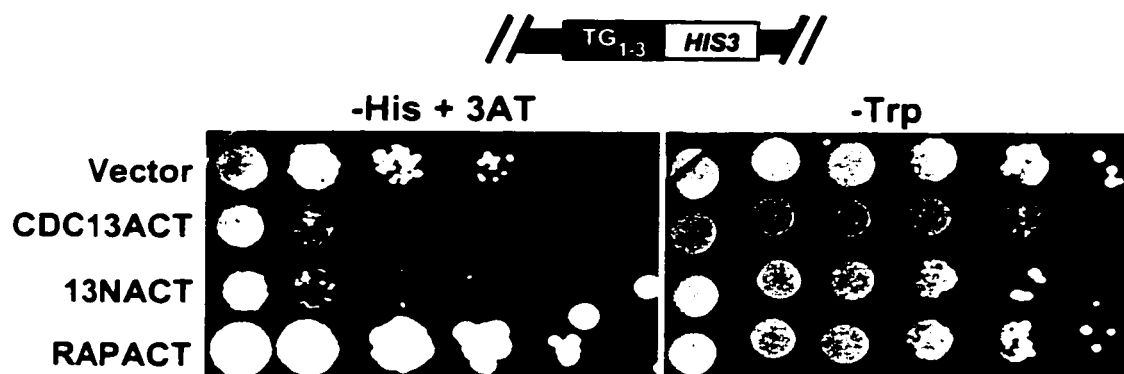


Figure 3.11 *CDC13* fusion proteins do not associate with an internal tract of telomeric sequence. Strains containing 270 bp of telomeric sequence and expressing the full-length Cdc13 (CDC13ACT), or the Cdc13 C-terminus (13CACT) fusion proteins were grown to stationary phase. Ten-fold serial dilutions were spotted onto galactose plates selecting for the interaction (Gal-his + 5mM 3-AT, left panel) or onto plates selecting for plasmid-bearing cells (right panel) and allowed to grow for 9 and 5 days, respectively.

CHAPTER 4: A TELOMERE ONE-HYBRID SCREEN FOR TELOMERE-ASSOCIATED PROTEINS

SUMMARY

In this work, I used the previously characterized telomere one-hybrid system (Chapter 2) to screen a yeast genomic fusion library for telomere binding proteins. Two previously characterized (Chapters 2 and 3) proteins, Rif1p and Sir4p were isolated, indicating that the system is indeed useful for identifying telomere binding proteins. Additionally, one other gene, *RSC6*, and three other hypothetical open reading frames were identified in the screen and are currently being investigated for possible telomere function.

INTRODUCTION

BACKGROUND

At the time this work was begun, several pieces of evidence suggested that telomeres contained additional structural proteins that had not yet been identified. For instance, biochemical analysis showed that a 57 kDa protein in addition to Rap1p copurifies with the telosome (J. Wright, Ph.D. thesis); this protein has, to my knowledge, never been identified. In addition, genetic evidence implicated the existence of a factor that has a higher affinity for telomere ends than for internal tracts of TG₁₋₃ (Wiley and Zakian, 1995). Finally, it was demonstrated that deletion of *RIF1* with concomitant overexpression of a non-DNA binding region of Rap1p, exacerbates the telomere lengthening phenotypes that each has independently. These results imply the existence of at least one other Rif1p-like factor (Wiley and Zakian, 1995). Recently, however, this

factor may have been identified in the protein Rif2p, which was identified by its ability to interact with Rap1p (Wotton and Shore, 1997).

One hybrid systems were initially developed as a method to screen for DNA binding proteins. The conceptual foundation for screening a library is much the same as it is for assaying designed fusion proteins (Chapter 2). The method involves placing a DNA binding site of interest immediately adjacent to a reporter gene, then transforming with a library expressing hybrids of candidate binding proteins fused to a transcriptional activation domain. If a fusion protein binds the DNA site, it brings the activation domain near the reporter gene and causes its transcription. The insert present in the plasmid harbored in the positive colony, encodes a potential sequence-specific DNA binding protein.

The first such screen was used to isolate the SAP-1 protein, a human protein that binds the c-fos serum response element. In this screen, yeast was employed as a host. The host yeast strain contained a plasmid with the human c-fos serum response element adjacent to a *lacZ* reporter gene. Subsequently, the yeast strain was transformed with a fusion library made from HeLa cDNA ligated in frame with a transcriptional activation domain. The isolation of a library plasmid conferring a *lacZ* positive phenotype allowed the identification of SAP-1 (Dalton and Treisman, 1992). One-hybrid methodology has also been used to screen for new yeast DNA binding proteins (Li and Herskowitz, 1993). In this screen, *ORC6*, which encodes a replication origin binding protein, was identified.

In the past, when one-hybrid systems were used to screen for DNA binding proteins, the binding site was taken out of its normal, chromosomal context (Dalton and Treisman, 1992; Li and Herskowitz, 1993; Wang and Reed, 1993). For example, in the yeast screen mentioned above, four tandem copies of the 11 bp ARS

consensus sequence (ACS) were used to screen for components of the yeast origin recognition complex (Li and Herskowitz, 1993). Whereas the ACS is only part of an *in vivo* origin of replication, the "bait" used for the one-hybrid system described here is a functioning chromosomal telomere. A novel aspect, therefore, to this telomere binding protein screen is that because the binding site is functioning in its normal capacity, all proteins normally associated with the telosome are likely to be present and any interactions detected by the system have a high probability of being functionally relevant.

Because the telomere one-hybrid system described herein uses a DNA binding site in its normal context, positives from the screen may generate information about binding domains in previously identified proteins that affect telomeres. Unlike most genetic approaches, using a one-hybrid system to screen for telomere binding proteins has the advantage that no specific telomere function is assumed. Furthermore, this approach may identify essential or redundant genes which would be overlooked in a mutagenesis-based screen.

MATERIALS AND METHODS

LIBRARY TRANSFORMATIONS

The strain containing the low-activity *HIS3* gene adjacent to the telomere at chromosome VII-L (HISTEL, Chapter 2) was transformed essentially as described (Gietz et al., 1995). For each screen, 10 µg of library DNA was transformed in 10 separate transformation mixes. After the 30 minute incubation at 30°C, DMSO was added to a final concentration of 10%. Cells were heat shocked at 42°C for 30 minutes.

After centrifugation, each of the reactions was resuspended in 1 ml of yeast complete medium and allowed to recover for one hour at 30°C.

INDIRECT SCREENING

For the indirect method, cells were first plated on yeast complete (YC) medium that only selected for uptake of the plasmid (YC-trp). Cells were plated at a density of $1\mu\text{g}/150\text{ mm}$ petri plate and allowed to grow at 30°C for two days. After the plates were placed at 4°C for 4 hours, cells were then scraped off plates with sterile microscope slides, and washed 2 times in 2 volumes of sterile water. They were then suspended in 65% glycerol, 0.1M MgSO_4 , 25mM Tris pH 7.5 and frozen at -80°C until needed.

For selecting transformants expressing fusion proteins that interacted with a telomere, cells were then thawed on ice and diluted 1:100 in galactose medium lacking tryptophan (Gal-trp), and incubated 4-24 hours to induce expression from the library plasmid. During this time the cells underwent less than one doubling. Fifty μl of cells were spread per plate onto galactose medium lacking histidine (Gal-his) + 5mM 3-Amino triazole (3-AT). This medium selects for transcription from the reporter gene. Transformation efficiency was approximately 5.5×10^6 transformants per μg .

DIRECT SCREENING

For the direct screen, cells were transformed as above except that after recovery in YEPD, cells were centrifuged and resuspended in Gal-trp medium. This medium both selected for the *TRP1* marked plasmid and induced expression of the fusion protein. Cells were allowed to grow for 4.5 hours, then centrifuged and resuspended in 1ml of sterile water. Fifty μl were spread per plate onto Gal-his + 5mM

3-AT. The transformation efficiency was approximately 1.16×10^5 transformants per μg . Therefore, a total of 1.16×10^6 colony forming units were plated onto 50 plates. These were incubated for 10 days at 30°C . At the end of this incubation, each plate was replica plated to YC-trp. ~350 big colonies grew per plate.

SPOT ASSAYS

Spot assays were performed as previously described (see Chapter 2).

YEAST COLONY PCR

The polymerase chain reaction was performed essentially as described (Maniatis et al., 1982), except that a modified procedure for amplification of DNA from whole yeast cells was used (Wang et al., 1996). The 5' primer was complementary to the pJG4-5 plasmid at the fusion junction and the 3' primer was just outside of the *SIR4* open reading frame: 5' GCCGCTCGAGCATTAAAATCGATTAAACACC 3'

β -GALACTOSIDASE ASSAY

The β -galactosidase plate assay was performed as described (Chapter 2).

SEQUENCING

Sequencing was performed as described (Sanger et al., 1977), or using the SequiTherm Cycle Sequencing Kit (Epicenter Technologies Corporation, Madison, Wisconsin), and the sequencing products separated on 6% acrylamide gels.

RESULTS

USE OF THE TELOMERE ONE-HYBRID TO SCREEN FOR TELOMERE BINDING PROTEINS

In an attempt to identify novel telomere associated proteins, I screened a library of yeast fusion proteins using the HISTEL strain. HISTEL contained the low-activity *HIS3* allele integrated adjacent to the telomere at chromosome VII-L (Chapter 2). The *HIS3* allele, which has no upstream activating sequence but retains its TATA region, typically confers a His⁻ phenotype when integrated into a chromosome (Alexandre et al., 1993), but is able to be activated and has been previously used successfully in a two-hybrid screen (Gyuris et al., 1993).

HISTEL was transformed with a yeast genomic fusion library constructed in pJG4-5 (Gyuris et al., 1993). This library expresses hybrid proteins of random yeast peptides fused to the transcriptional activation domain B42 (Ma and Ptashne, 1987).

For the initial screen I tried two different selection methods; referred to as direct and indirect. In the direct method, cells were transformed, immediately induced to express fusion protein, and then plated directly on plates selecting for interaction with a telomere. In the indirect method, cells were transformed with the library, then plated onto plates that selected only for plasmid uptake and allowed to grow before being harvested. Transformants were then harvested, induced and spread onto plates that selected for telomere interaction.

A DIRECT SCREENING METHOD

For the direct screen, transformed cells were grown in galactose medium lacking tryptophan (Gal-trp) to allow induction of the fusion protein. They were then spread onto plates that select for interaction of the fusion protein with a telomere: galactose medium lacking histidine plus 5mM 3 Amino-triazole (Gal-his + 5mM 3-AT;

hereafter referred to as test plates). In total, approximately 1.16×10^6 cells calculated to contain the plasmid were plated onto 50 plates (Figure 4.1, left column). These plates were then incubated at 30°C for 10 days, during which time approximately 17,500 cells grew to form colonies. When the colonies were of sufficient size, the plates were replica plated to medium that only allows plasmid bearing cells to grow (YC-trp). Approximately 7,500 colonies grew (Figure 4.1, left branch). Ten of the largest colonies from each plate, 500 total, were transferred onto a YC-trp master plate to characterize further. In this way, most of the large colonies were chosen, but many smaller ones were left on the plates.

AN INDIRECT SCREENING METHOD

For the indirect screen for telomere associating proteins, cells that had taken up the plasmid were selected for by plating a high concentration of transformants onto YC-trp. Cells were allowed to grow for 2 days into colonies ~2 mm in diameter, harvested, and frozen. After thawing, expression of the potential hybrid protein was induced by growing cells in galactose medium. Cells were then plated onto medium that selected for the telomere interaction (test plates). Approximately 2.23×10^5 total transformants were assayed (Figure 4.1, right column). Cells were allowed to grow for 10 days, during which time they generated heterogeneous size colonies. In this case, replica plating to YC-trp eliminated very few of them, presumably because they had been selected for their ability to manufacture tryptophan only a colony generation before.

Because there were so many colonies at this point, an attempt was made to eliminate uninteresting isolates. One potential avenue was to try to determine which transformants required expression of the fusion protein in order to grow on test plates. Because the library fusion proteins are under the control of *GAL1*, the cells should not

express fusion proteins when growing on glucose medium because the presence of glucose represses the *GAL1* promoter. Therefore the parent strain, HISTEL, was streaked onto YC-his plates containing increasing levels of 3-AT to determine at which concentration HISTEL would be unable to grow in the absence of an activating protein. Twenty mM 3-AT essentially eliminated growth of the parent strain on YC-his plates. Therefore, the original screen plates were replica plated to these glucose plates (hereafter referred to as repression plates), that should repress expression of the fusion protein. Those colonies that appeared to grow less well than their counterparts on the original telomere interaction selection plates were chosen for further analysis. This step proved difficult as most colonies appeared to be intermediate — that is, neither completely dead on repression plates, nor robustly growing. A total of 235 colonies were chosen that grew well on test plates, but poorly on repression plates (Figure 4.1, right column).

In an attempt to categorize transformants according to strength of phenotype, I first looked at the ability of previously constructed (Chapter 2) fusion proteins to confer growth of HISTEL cells on test plates. On Gal-his + 30 mM 3-AT, Rap1, Rif1, and Sir4 fusion protein containing cells conferred a clear His⁺ phenotype relative to cells containing vector alone (data not shown). When positive isolates from the indirect screen were compared on the same plates, none appeared to grow as well as the previously characterized telomere binding proteins (data not shown).

CONTINUATION OF THE SCREEN

From this point on, candidates from both the direct and indirect screens were processed the same way. An attempt was made to determine which colonies were able to grow only when the hybrid protein was induced. Master plates of both the directly and indirectly selected isolates were replica plated to repression plates and test

plates to determine which were transcribing *HIS3* by virtue of expression of the fusion protein. Patches were analyzed each day from 2-6 days to rate growth, and those that grew well on Gal-his, but not as well on glucose plates were selected.

Those that appeared promising, 53 from the direct, and 34 from the indirect (Figure 4.1), were streaked for single colonies on the same plates to confirm the protein dependent phenotype on a colony basis. Because so many cells are transferred during replica plating, a single colony analysis allows a much more accurate way to assess growth. Of the 87 isolates, 29 were selected for further analysis, based on their ability to grow well on test plates and as bad or worse than telomere binding proteins on repression plates. For each strain, the plasmid was recovered from yeast into *E. coli* and subjected to restriction digest analysis (data not shown). Of the 17 plasmids isolated from positives in the direct selection, 14 appeared to be unique, two apparently had no insert, and 3 appeared to be odd rearrangements of the plasmid. Two plasmids appeared to encode the same insert as the three redundant clones, but because I detected a slight variation in band size I chose to leave one of them in the screen. Of the 12 from the indirect selection, all 12 were unique. By this analysis 26 recovered plasmids were unique and the only obviously redundant two clones both came from the direct selection (data not shown).

The 26 potentially independent isolates were retransformed into HISTEL and tested by spot assay to determine His⁺ phenotype on test plates, and glucose repression plates. Isolates were rated according to frequency of colony formation as well as speed of colony formation (data not shown). Only a subset had a convincingly protein dependent phenotype, as many were able to grow well on repression plates. All isolates were saved that were still able to confer a His⁺ phenotype on test plates,

regardless of whether they were able to grow on glucose plates. Attributes of these eight isolates are summarized in Table 4.1.

Additionally, several of the promising clones were transformed into the strain with *lacZ* at a telomere and assayed for β -galactosidase activity. The ability to test positive isolates for activation of *lacZ* at a telomere is useful as a secondary screen to eliminate library plasmids that encode proteins that interact nonspecifically with the *HIS3* gene. However, because the HISTEL strain is more sensitive than the *lacZ* strain (Chapter 2), even if a candidate tested negative in the *lacZ* strain, it was not eliminated as it may be a weak interaction as with *SIR3* (Chapter 2). Because of this, even if a candidate tested negative in the *lacZ* assay, it was retained. Only clones D37, D34, and D45 were obviously positive for β -galactosidase activity, while D27 and D41 showed a very weak signal (Table 4.1).

SIR4 AND *RIF1* ARE IDENTIFIED IN THE TELOMERE ONE-HYBRID SCREEN

All 8 candidate clones that made it through the screen to this point (Table 4.1) were sequenced to determine which insert they bore at the fusion junction. Sequence analysis revealed that all the clones that tested positive for β -galactosidase activity encoded proteins that I had previously tested in the one-hybrid system (Chapter 2). Two clones of *RIF1* were isolated. They both encoded the same region of *RIF1* fused to the activation domain; the C-terminal 507 amino acids starting with the *AluI* site at amino acid 1409. They clearly arose from two separate transformants because the inserts were different sizes (data not shown). Additionally, the same region of *SIR4*, the C-terminal 115 amino acids, beginning at the *AluI* site at amino acid 1143, was identified 3 times. The 3 other clones (D7, D8 and D9) appeared to be the identical *SIR4* clone based upon the restriction digest analysis (data not shown).

OTHER CANDIDATES FROM THE TELOMERE ONE-HYBRID SCREEN

Additionally, two other plasmids identified in the screen were found to encode two other open reading frames. Isolates D18 and D26 contained inserts from *IMH1* (an open reading frame of unknown function), and an open reading frame associated with the Ty1 retrotransposon respectively. However, neither gene was in the correct reading frame to encode these open reading frame products (data not shown). As noted, because the *lacZ* reporter system is less sensitive, a clone that is positive in HISTEL, but negative in ZTEL may encode a telomere binding protein that happens to have a weaker interaction. Therefore, the two candidates, D18 and D26, whose insert came from a previously characterized open reading frame, were subjected to further analysis.

One type of false positive could arise that would test positive in HISTEL, but negative in ZTEL. This would be a protein that non-specifically interacts with the *HIS3* gene and has no interaction with telomeres. In order to rule out the possibility that D18 and D26 encode proteins that nonspecifically activate the *HIS3* gene, each plasmid was transformed into a strain with *HIS3* at an internal location (see Chapter 2), and analyzed by spot assay. Neither plasmid conferred upon HISTEL the ability to grow on test plates, even when no 3-AT was present (Figure 4.2). These two isolates met all the criterion for a true telomere associating protein, however, sequence analysis indicated that no fusion protein should be made.

MODIFICATION OF THE SCREEN

At this point I realized that the *SIR4* fusion protein containing cells were able to form colonies on repression plates better than vector alone (data not shown); seemingly some Sir4Actp was present in spite of the glucose repression of the *GAL1*

promoter. It is likely, therefore, that low level expression of the fusion protein containing the C-terminus of *SIR4* is able to activate *HIS3*. This should, perhaps, not be surprising considering that expression of Sir4Actp has dual effects on the telomere one-hybrid: activation and relief of TPE (Chapter 2). Because these strains grew on repression plates better than vector alone, and because few false positives had been isolated up to this point it is possible that the screen might have been too stringent. Therefore, more of the isolates that had been eliminated in the single colony assay for protein dependence (Figure 4.1, Step 5) were reevaluated.

At this stage, isolates had been eliminated for one of two reasons. First, they were required to have been able to grow nearly as well as HISTEL expressing known telomere binding fusions. Somewhat surprisingly, 5 of the 53 directly selected isolates, and 12 of the 34 indirectly selected isolates, grew poorly when streaked for single colonies on test plates (data not shown). A second reason isolates were eliminated was if they grew too well on the repression plates. Given the *SIR4* clones' ability to confer growth fairly well on plates that repressed expression of the fusion protein, it was possible that some true telomere associated proteins had been overlooked. Therefore, another 33 isolates (4 from indirect) that had been eliminated based upon the fact that they grew well on repression plates, were chosen to be analyzed further.

Because the previous screening scheme (Fig. 4.1) had eliminated a large proportion of the candidates upon retransformation and spot assays, these steps were performed first. Also since spot assay analysis gives a less ambiguous phenotype than replica plating, all transformants were subjected to spot assay analysis. Of these 33, 31 were successfully retransformed into the HISTEL strain (Fig 4.3). Seven of these candidates again tested positive in the spot assay. By this analysis, 5 grew well on test

plates, but also on repression plates (D31, 32, 50, 51, 44, Table 4.2). One clone generated colonies 10-fold less frequently than vector alone, and showed 10-fold reduced growth on glucose (D48). In addition, one isolate (D11) had very slow growing colonies on galactose selection plates, and grew well on glucose. Because so many positives of the previous screen had contained a *SIR4* fusion, I decided to use a Polymerase Chain Reaction (PCR) strategy to eliminate any *SIR4* clones. I performed PCR on whole yeast cells (Wang et al., 1996), and determined that 2 of the 7, D46 and D48 were indeed *SIR4*, and that D48 contained a longer insert than those previously isolated. Therefore I transformed the 5 that did not encode *SIR4*, and the one with the longer *SIR4* insert into *E. coli*, and proceeded to sequence them.

Sequence analysis revealed that, in addition to the two that were picked up by PCR analysis, one of the isolates, D32, contained *SIR4* (Table 4.2). This allele was indistinguishable from the previously identified 8 isolates by sequencing and restriction analysis (data not shown). In addition, sequencing revealed that the longer version of *SIR4* insert (D48) included the 840 C-terminal amino acids, from the HaeIII site at amino acid 518. D23 was determined to be an in frame fusion with a hypothetical open reading frame. However, further analysis determined that this insert was identical to the *SIR4* insert already isolated 9 times, but it was inserted in the opposite direction. Therefore, it is possible the Sir4 215 amino acid peptide was being expressed via a cryptic promoter and that the strain was His⁺ by virtue of the encoded peptide's ability to decrease TPE rather than a true trans-activation. If the termination region is acting as a cryptic TATA, there might be some expression of the C-terminus of *SIR4*, a region already known to decrease TPE (Singer and Gottschling, 1994).

Besides *SIR4*, the only other open reading frame identified in this second screen belonged to the chromatin remodeling factor *RSC6*. However, sequence analysis

indicated that this protein was not in the right frame to make a fusion protein between *RSC6* and the activation domain. Isolates D23 and D11 will be subjected to Western analysis to determine whether or not any fusion protein is expressed. In addition, D31 and D50 contained inserts that were not from any open reading frame. Two isolates, D44 and D51 remain to be sequenced.

DISCUSSION

The telomere one-hybrid system was used to screen for proteins that interact with chromosomal telomeres *in vivo*. The goal was to find novel interactions as well as to increase our knowledge about proteins already suspected to be involved with telomeres.

The HISTEL strain was used instead of ZTEL for two reasons. First, using a selection rather than a screen would allow for a larger scale screen, because selecting is intrinsically more efficient than screening. Second, the HISTEL system has been demonstrated to be more sensitive than the ZTEL system (Chapter 2). The increased sensitivity of this assay may allow detection of protein associations that are more transient than those of the structural proteins previously discussed.

The library made in pJG4-5 was chosen for several reasons. First, expression from pJG4-5 is galactose-inducible, which, in theory, should allow facile determination of whether the His⁺ phenotype is plasmid-dependent. Second, this library was made from genomic rather than cDNA. Use of a genomic library was thought to be advantageous because it avoids biases for abundant proteins such as Rap1p that might be over-represented in a cDNA library. The library was made from genomic DNA that had been partially digested with two different restriction enzymes, HaeIII and AluI. These enzymes are both 4-cutters that cut frequently in the yeast

genome. Consequently, the population of fragments obtained after partial digestion should have been fairly heterogeneous. Additionally, the partially digested DNA was size selected for fragments of DNA ~1000 kb in size. The small size of the inserts mean that there is a better chance of assaying any given protein in the genome. One further reason this library was chosen is that the activation domain that is used in the fusion is the *E. coli* sequence B42. This peptide causes strong transcriptional activation in yeast (Ma and Ptashne, 1987), but because it is not a yeast protein, it is not regulated by any yeast pathway.

DIRECT APPROACH VERSUS INDIRECT APPROACH

Two screening approaches were used: a direct approach and an indirect approach. At first thought, it might seem as though the indirect selection would be the more effective method. By allowing cells to first be selected simply on the basis of whether they took up the library plasmid, they were given a chance to recuperate before being required to integrate fusion proteins into their telosomes and activate the *HIS3* gene. However, the results reported here contradict such an assumption. In the indirect selection regime, only one clone made it to the sequencing step; and that clone, I22, did not appear to encode a gene. Conversely, the direct selection ultimately yielded two known telomere associated proteins, Sir4p and Rif1p, and it identified them several times.

Because the original test plates contained tryptophan in the direct screen, there was no selection for a plasmid other than the ability to make a fusion protein that could activate a telomeric gene. The reason the direct regime may have been more effective is that it allowed the advantage of replica plating to select only colonies that required the plasmid early in the regime (Figure 4.1). In contrast, in the indirect method,

cells were required to have the plasmid first, then selected on test plates. It is possible that non-plasmid dependent His⁺ cells would not have undergone enough divisions to have lost the plasmid by the time they were replica plated to YC-trp. Therefore, it was more difficult to eliminate the background of spurious His⁺ events. In the direct method cells that were His⁺ by virtue of an event not related to the plasmid, were screened out at this step. With the indirect selection, so many colonies appeared that I tried to enrich for true interactors by replica plating to glucose plates and then selecting those that grew poorly on glucose. However, this did not seem to be an effective step. In fact, it may have even complicated the situation because many of these isolates were unable to form colonies on test plates after they had been streaked to a master plate (Figure 4.1). Therefore, it appears that the most effective way to screen by one-hybrid for telomere interactors is by a direct selection in which the plasmid is not selected for on the screen plates.

SIR4

For the library screened, I calculated that ~330,000 transformants would allow a 99% probability of assaying each clone at least once. Empirically, however, in other hybrid screens, it seems that multiple positives have been isolated in a screen of approximately a million transformants. It is possible that the direct screen has been completed to saturation because *SIR4* was identified so many times. However, it might also be that cells expressing Sir4p might have a better chance of growing on test plates than any other protein because it has stronger activating characteristics, competes well with the endogenous protein, or has an additional advantage because it is likely to also be decreasing TPE (Chapter 2). Indeed, the fact that a *SIR4* clone in the opposite orientation (and therefore not making a fusion protein with the activation domain) was

isolated implies that the ability to decrease TPE is strong enough to give a positive phenotype in this assay. Additionally, it may be that this particular library has an overrepresentation of *SIR4* or, more likely, that *HaeIII*, and *AluI* restriction sites are located at positions that allow it to fold into a functional domain when fused to the activation domain. Therefore *SIR4* may simply have the best probability of being detected in this screen, and the screen could be continued if method of screening out *SIR4* earlier were employed.

IMH1

The isolate D18 was able to generate colonies on test plates at a very high frequency, as it generated colonies even more frequently than some of the *SIR4ACT* and *RIFACT* clones; although they grew quite slowly relative to known telomere binding proteins (Table 4.1). Additionally, it was unable to form colonies on repression plates (Table 4.1, Growth on glucose column). By these criteria, this appeared to be a promising candidate. However, by sequence analysis, the plasmid would be producing a very short peptide from the anti-sense strand of the *IMH1* open reading frame of unknown function. This makes it less likely to encode a real telomere binding protein.

TY1 ORF

The isolate D26 also had a promising phenotype in the retransformed HISTEL assay (Table 4.1). It generated colonies on test plates more frequently than vector alone, and did not grow at all on repression plates. It appeared to have an intermediate phenotype as it was able to generate colonies on test plates at least ten-fold better than vector alone and 1000-fold better than the other library plasmid containing cells. Additionally, when transformed into the strain with *HIS3* at an internal location, it was unable to generate colonies, even on plates selecting for a very

weak interaction (Gal-his). However, sequence analysis revealed that the fusion junction was from an open reading frame associated with the Ty1 retrotransposon. Given the phenotypes of both D18 and D26, I felt some further analysis was warranted. Therefore these isolates will be subjected to Western blot analysis to determine whether they encode a fusion protein.

RSC6

D11 was identified when I went back to reassess the isolates that had been eliminated based upon the fact that they grew better than known telomere binding fusion proteins on repression plates. This plasmid was found to encode the gene *RSC6* at the fusion junction, and it is the only open reading frame present on the insert (data not shown). However, by sequence analysis it did not appear to be in frame with the fusion. Oddly, when the other two frames were analyzed, they both encoded a stop codon within the next 20 amino acids (data not shown). This made it seem possible that despite the apparent open reading frame inconsistency, this plasmid was managing to express a region of Rsc6p. *RSC6* is an essential subunit in the 15-subunit complex RSC that has the capability to remodel the structure of chromatin (Cairns et al 1996). While its involvement with chromatin makes it tempting to speculate that it is likely to be a true telomere associated protein, it is also quite likely that it was having a non-specific effect on the expression of *HIS3*. Perhaps overexpression of this peptide was reducing TPE or, alternatively, it was affecting expression of the *HIS3* gene via a non-telomere specific association with chromatin. If this plasmid is seen to encode a fusion protein by Western analysis, it will be important to determine whether it is having a non-specific effect on the *HIS3* gene. It will have to be determined that the His⁺ phenotype

requires a fused activation domain, and that it requires the presence of a nearby telomere.

CONCERNING FREQUENCY

Even when cells start out with nearly all of them containing a plasmid capable of encoding a telomere binding fusion, usually only a fraction of the cells are able to generate colonies on test plates (Chapter 2). Expression of RifActp allowed colonies to be generated at higher frequency than the Sir4Actp, Sir3Actp, and RapActp (Chapter 2, and this Chapter), therefore, it might have been predicted to be one of the first telomere proteins isolated. However, because it generated slower growing colonies, some RifActp containing cells may have been overlooked at the stage where large colonies that replica plated to YC-trp (Figure 4.1) were selected. Indeed, there would be many more candidates, if I were to go back to the original YC-trp plates and characterize some of the smaller colonies.

MODIFICATION OF THE TELOMERE ONE-HYBRID SCREENING REGIME

The telomere one-hybrid screen could be modified in an attempt to isolate different proteins. For instance, choosing smaller colonies to characterize in the first step or increasing the 3-AT on the plates might lead to the identification of a different subset of telomere binding proteins. Another consideration is that, in the final analysis, this one-hybrid screen did not generate many false positives; of those that were sequenced, approximately two-thirds were genuine telomere binding proteins. Because of this, perhaps the screen was conducted too stringently. Decreasing the 3-AT on the test plates, or being more lenient about which candidates were further analyzed, might increase the likelihood of identifying different proteins.

It is interesting to note that the abundant telomere binding protein, Rap1p, was not detected in this screen. It may be that Rap1p is unable to activate sufficiently to be isolated in this screen. However, in a one-hybrid screen of a different library, Rap1p was identified 10 times and it was the only protein identified in this screen (A. Smith and B. Bourns unpublished observations). Therefore, it seems more likely that the reason it did not appear in this screen is that the gene does not have AluI or HaeIII sites in locations that allow it to function as a domain and compete with the endogenous protein. For this reason, different proteins are likely to be recovered from different libraries. Therefore it should be worthwhile to screen a different library for telomere binding proteins. In particular, a fusion library was recently reported that was made using five restriction enzymes for partial digestions, all with enzymes that were not used in the library I screened (James et al., 1996). It seems likely that a screening of this library will yield additional telomere-interacting proteins.

The fact that the library used in this work allowed expression of the candidate to be repressed on glucose plates did not appear to be as advantageous as one might have thought. Attempting to determine protein-dependence of the original screen colonies did not prove beneficial. Furthermore, going back to the single colony stage and allowing those that failed the repression step the first time to be characterized may not have been a useful exercise. The second round of screening seemingly generated more false positives, and it certainly identified disproportionately more *SIR4* clones (Tables 4.1 and 4.2), a result that one would like to avoid in any future screening. However, in a larger screen, the ability to test this phenotype at the single colony stage might be beneficial by allowing categorization of which isolates were most promising to pursue. Of the true telomere binding isolates identified, one *RIF1* clone and three *SIR4* clones appeared to grow less well on repression plates than some of the others (Tables

4.1 and 4.2). However, overall it is not clear that this attribute of the library was of any benefit. Because of this, it seems that the screen could be continued if a means to eliminate *SIR4* clones at an earlier step is employed.

CONCLUSION

To summarize, I have identified the same region of *SIR4* nine times, most likely all of the same region of the protein. I have also identified a larger region of Sir4p. In addition, I may have isolated the C-terminus of *SIR4* by virtue of its effects on TPE when overexpressed. I have identified the same region of *RIF1* two different times, on two different clones. Additionally, while no candidates appear very promising, 4 isolates are being subjected to further analysis. I have characterized the method of screening sufficiently that a future screen based on the modifications I have outlined stands a very good chance of being successful at identifying new telomere binding proteins.

Figure 4.1 Telomere one-hybrid fusion library screen scheme. The two different methods of screening are outlined. On the left is the direct method and on the right is the indirect method. After transformation, cells were spread onto Gal-his + 5mM 3-AT plates, which select for interaction of the fusion protein with a telomere. The number of isolates analyzed at each step is reported.

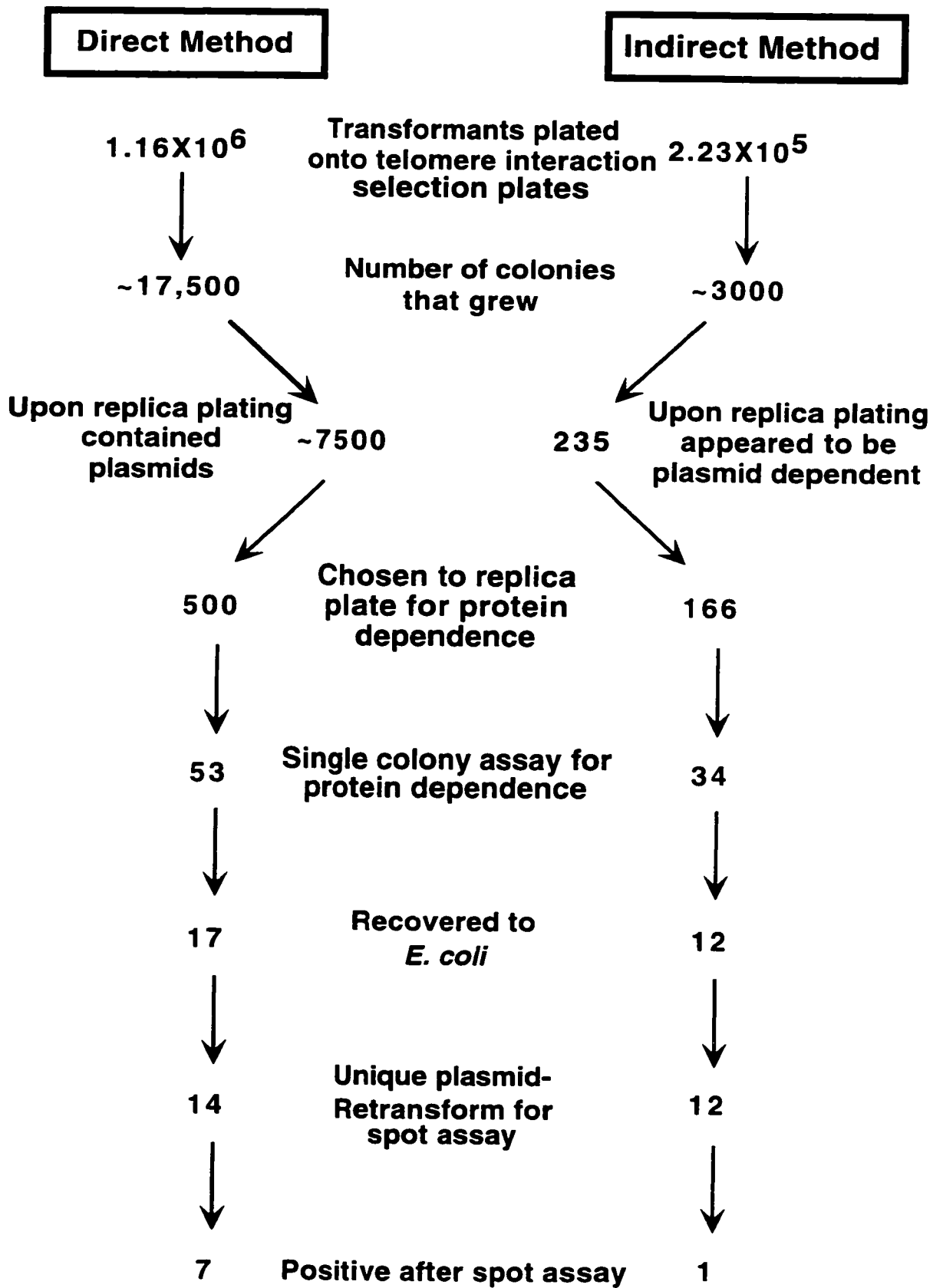


Table 4.1 Isolates that tested positive for containing a putative telomere associated protein were grown to stationary phase. They were then serially diluted in ten-fold increments and scored for growth on test plates (Growth on galactose) or repression plates (Growth on glucose). The last dilution at which they generated colonies is reported for test plates followed by the rate of colony formation. Relative growth compared to a *SIR4* positive control is reported for repression plates. The Activate *lacZ* column reports the ability of a particular plasmid to activate a telomeric *lacZ* gene. In the Gene column, results of sequencing for each isolate is reported. The Comments column contains any additional information about an isolate.

Clone	Growth on galactose	Growth on glucose	Activate <i>lacZ</i>	Gene	Comments
Vector	-2, mixed	+/-			
I22	-3, slow	-	-	No ORF	
D18	-5, slow	-	-	<i>IMH1</i>	Out of frame
D37	-3, moderate	-	+	<i>RIF1</i>	C-term 507 a.a.
D34	-5, fast	+/-	+	<i>SIR4</i>	C-term 215 a.a.*
D45	-4, fast	+	+	<i>RIF1</i>	C-term 507 a.a.
D41	-5, mod	+	~	<i>SIR4</i>	C-term 215 a.a.*
D27	-4, slow	++	~	<i>SIR4</i>	C-term 215 a.a.*
D26	-3, slow	-	ND	Ty1 ORF	Out of frame

*4 other isolates appeared to be identical by restriction analysis

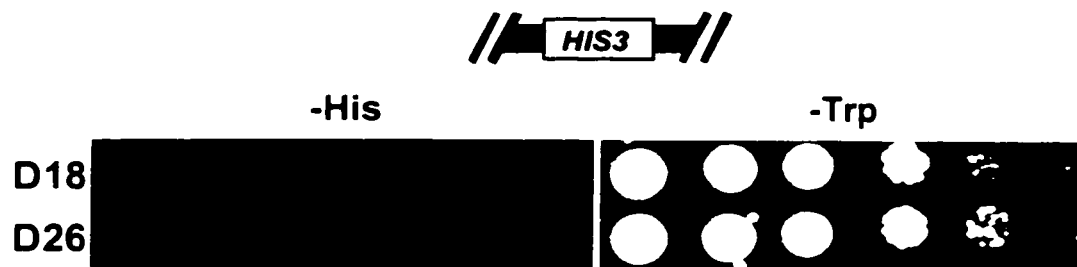


Figure 4.2 Screen positives do not nonspecifically activate *HIS3*. Strains containing a promoter-defective allele of *HIS3* integrated adjacent to a telomere contained library plasmids D18 and D26. Ten-fold serial dilutions were spotted onto plates selecting either for the activation of *HIS3* (left side), or for the presence of the plasmid (right side). Plates were incubated for 7 and 4 days, respectively.

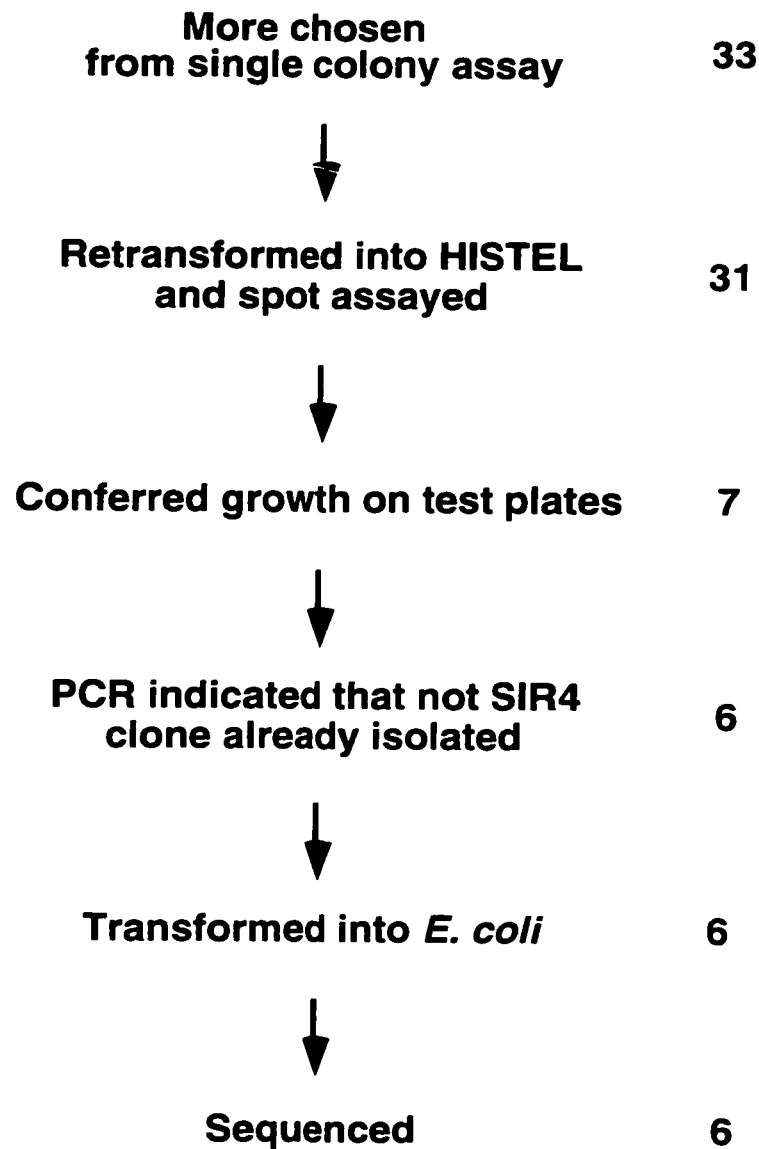


Figure 4.3 Screening of additional candidates. Isolates that had been eliminated in the first screening round (Figure 4.1) due to the fact that they grew well on glucose repression plates were subjected to further analysis. 33 isolates that grew well on plates selecting for telomere interaction (Gal-his + 5mM 3-AT), were taken through a slightly modified screening procedure.

Table 4.2 Isolates that tested positive for containing a putative telomere associated protein were grown to stationary phase and serially diluted in ten-fold increments and scored for growth on test plates (Growth on galactose) and growth on repression plates (Growth on glucose). The dilution at which they grew is reported for test plates and relative growth compared to the *SIR4* positive control is reported for repression plates. In the Gene column, results of sequencing for each isolate is reported. The Comments column contains any additional information about an isolate.

Clone	Growth on galactose	Growth on glucose	Gene	Comments
Vector	-2	-		Negative control
D11	-3	+	<i>RSC6</i>	Out of frame, tiny colonies
D23	ND	ND	<i>SIR4</i>	Opposite orientation
D31	-4	+	Mitochondrial genome	No ORF
D32	-4	+	<i>SIR4</i>	C-terminal 215 a.a.
D50	-4	+	Between ORFs	
D51	-4	+	Not sequenced	
D46			<i>SIR4</i>	
D44	-4	+	Not sequenced	
D48	-3	down 10X	<i>SIR4</i>	C-terminal 840 a.a.
SIR4	-4	+/-		

CHAPTER 5: CONCLUSIONS AND IMPLICATIONS

Telomeres are the DNA and associated proteins that constitute the physical ends of eukaryotic chromosomes. Telomeres allow the cell to distinguish natural ends from broken ends, facilitate the complete replication of linear DNA molecules, and are important for chromosome stability (rev. in (Zakian, 1995)). These functions as well as the proteins that mediate them appear to be conserved among organisms. For example, it has been shown that the process of telomere replication is conserved in a wide variety of organisms, as telomerase activity has been detected from ciliated protozoa to humans (Greider and Blackburn, 1985; Morin, 1989; Nakayama et al., 1997). In fact, organisms as diverse as yeast and humans have telomerase proteins that even share sequence homology (Nakamura et al., 1997). Even the structure of the telomere is conserved in diverse organisms. Not only are almost all organisms' telomeres composed of G/C rich repeats (rev. in (Zakian, 1995)), but the single-stranded G-rich extension is apparently conserved as well (Henderson and Blackburn, 1989; McElligott and Wellinger, 1997).

Interest in telomere processes has been increasing as it is appreciated more and more that telomere biology has important implications for two prominent concerns in human health, cancer and aging. Telomere replication in particular, impacts upon these processes, as the molecular clock model for aging and cancer is strengthened by accumulating evidence. As such, gaining an understanding of the processes involved in telomere replication is certain to shed light on the molecular mechanisms underlying cancer and aging.

In yeast telomere replication, the structural protein, Cdc13p, has important regulatory functions (Garvik et al., 1995; Lin and Zakian, 1996; Nugent et al., 1996). It seems likely that structural proteins are important for other functions of telomeres as well, especially for the stability of the chromosome. In support of this notion, functional homologues of another yeast structural protein that also has protective functions, Rap1p (Conrad et al., 1990; Lustig et al., 1990), have been found in a variety of organisms (Cooper et al., 1997; McEachern and Blackburn, 1994; van Steensel and de Lange, 1997).

Because the role of the telomere in chromosome stability and maintenance is so important, and because structural proteins are likely to be involved in this process, I developed an *in vivo* assay system that could determine whether a protein is structurally associated with a telomere. Given that the structure and replication of yeast telomeres are similar to that of other organisms, but yeast is genetically tractable, yeast provides an excellent model system in which to study telomeres. Such an assay promises to facilitate our understanding of the mode of action of telomere-associated proteins, not only in yeast, but across species.

Many models for telomere function depend upon the ability of a protein to bind the telomere *in vivo*. However, the *in vitro* assays used to identify and characterize telomere binding proteins have met with mixed success (Berman et al., 1986; Lin and Zakian, 1994; Nugent et al., 1996). Therefore, the development of an *in vivo* assay to facilitate understanding about specific protein interactions with a telomere was needed. This telomere one-hybrid system increases the repertoire with which to study telomere biology and function. In the preceding work, I described the use of a telomere one-hybrid system to assay protein association with a telomere *in vivo*. In this system a reporter gene was integrated adjacent to a telomere and then a fusion protein of a putative telomere binding protein to a transcriptional activation domain was

expressed. If the protein bound telomeres *in vivo*, it brought the activation domain within reach of the reporter gene and caused its activation. Activation of the reporter gene was therefore used as a signal to monitor whether an assayed protein bound a telomere.

Models to explain another aspect of telomere biology, the phenomenon of telomere position effect, are based on the association of the Sir proteins with a telomere *in vivo*. Especially in the case of *SIR2*, it was possible that the Sir proteins affected telomeres by an indirect mechanism. Given what was known of the *sir2* phenotypes, it was possible that it was functioning catalytically, modifying histones away from a telomere rather than associating with a telomere directly. Having developed a telomere one-hybrid system to analyze telomere-protein interactions, the second contribution of this work was to use it to shed light on various models by determining which proteins were associated with a telomere *in vivo*. It was demonstrated that, indeed, three Sir proteins, Sir2, Sir3, Sir4, and the Rif1 protein are directly associated with a telomere *in vivo*.

Because the telomere one-hybrid system can detect interactions of Rap1p, as well as the Sir proteins and Rif1p, it can identify protein-protein interactions in addition to protein-DNA interactions. Therefore, it may be able to detect all of the functionally important participants in the complex. Somewhat surprisingly, the telomere one-hybrid system has also been able to detect interactions that may take place farther away (i.e. at the G tail), or perhaps are more transient (such as Sir2 if it acts catalytically). In the future it will be of interest to determine whether the recently identified Rif2p (Wotton and Shore, 1997) is also a part of the complex.

In addition to identifying telomere-protein interactions, an interesting extrapolation of the methodology was to use it to characterize various aspects of

telomere-protein interactions. For example, this system provided a useful method to determine which proteins are required for the formation of the complete telomeric complex. The system might also be useful for determining whether proteins compete with one another for binding. For instance the Rif1 and Sir3 proteins are thought to compete with one another for binding the Rap1p C-terminus (Moretti et al., 1994). If this is true, eliminating one of them in the HISTEL strain may increase the signal of a fusion protein made from the other. Furthermore, this system may be helpful for determining specific information about protein-protein interactions. For example, there exist several alleles of *RAP1* that have been postulated to be defective in their ability to interact with specific proteins (rev. in (Shore, 1994)). Therefore, if the system were set up in a strain that had one of the various alleles of *RAP1*, it might be determined which interaction this allele of Rap1p is defective for by assaying several of the fusion proteins already constructed.

Another application of this methodology has been to demonstrate the end-specificity of the proteins assayed. For example, Cdc13 protein was demonstrated to specifically interact with telomeric sequence at the chromosome end, but not the sequence at an internal location (Chapter 3). In the future it would be interesting to test more thoroughly another protein thought to bind the single-stranded G-tails, Est1p.

Finally, because many telomere functions are not completely understood, it is likely that there remain other proteins associated with yeast telomeres that are thus far unidentified. This work describes the use of the telomere one-hybrid assay to search for novel interactions and to lay down the groundwork on which other screens might be undertaken or this one continued.

Thus far the telomere one-hybrid system has been used to screen two fusion libraries for novel interactions. The system was successful in allowing the

isolation of proteins that interact with a telomere, because *SIR4*, *RAP1* (Drew Smith, Princeton Molecular Biology Dept. undergraduate thesis), and two independent clones of *RIF2* were identified. The screen was also useful for identifying new regions of these proteins that are sufficient for the interaction. However, it is unlikely that the screen reported here was exhaustive because several proteins known to bind telomeres were not identified (i.e. Rap1, Sir2, Sir3, and Cdc13). Given these considerations, it seems likely that continuing this same screen or performing a new screen will yield more information about proteins that interact with a telomere. The characterization in Chapter 4 should allow refinements and modifications that will improve the chances of finding such a protein by this method.

If understanding telomere biology is of interest because of its impact on human health issues, this screen could also be modified to try to isolate human telomere binding proteins. A yeast strain could be constructed in which a stretch of human telomeric DNA was integrated immediately adjacent to the *HIS3* gene and a fusion library made from human cDNAs could be screened. This method would facilitate the identification of any human homologues of Rif and Sir proteins. In addition, because it has been shown that Cdc13p has some affinity for human sequence oligonucleotides (Nugent et al., 1996), it is possible that its human homologue also binds yeast telomeric G strand oligonucleotides. If this were true, screening human libraries using the yeast system described here, could identify such a protein.

In summary, the work described here has contributed a new assay to the arsenal of methods with which to study telomere structure and function. Furthermore, it has laid the groundwork for the use of this system to identify novel telomere-associated proteins.

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