Microbial Genome Methods

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Chapter 3

Methods in the Analysis of Telomere Function in *Saccharomyces cerevisiae*

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Contents

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I. Background

The yeast *Saccharomyces cerevisiae* has served as a unique model system for the characterization of the basic elements necessary for chromosomal stability. This chapter will focus on both the approaches and the methodologies designed to study the function of telomeres in yeast.

A. Telomere Structure in Yeast

The basic structure of telomeres in yeast and most higher eukaryotes is strikingly similar, composed of both GT-rich simple sequence DNA on the 3′ telomeric strand as well as larger species-specific subtelomeric elements[1] (Figure 3.1A). The most terminal sequences consist of a variable number of irregular G + T-rich repeats, following the consensus sequence poly($[TG]_{1\text{-}6}$ $TG_{2\text{-}3}$), commonly referred to as poly($G_{1\text{-}3}T$). While telomeres are maintained at a genetically defined average tract length (varying from 150 to 800 base pairs (bp) in different laboratory strains), the tract size of individual telomeres varies stochastically from the mean length by as much as 30%. This heterogeneity is the likely consequence of terminal degradation, RNA primer loss, and imprecise replication. Embedded throughout the tract are binding sites for the telomere-binding protein RAP1 (following the consensus sequence GGTGTGTGGGTGT), which binds efficiently to duplex telomeric DNA on average once every 20 bp.[2] The extreme terminus appears to contain a 3′ overhang of greater than 50 bp formed transiently during late S phase.[3]

Centromere proximal to the poly($G_{1\text{-}3}T$) repeats are a series of longer repeated elements that are organized in an end- and strain-specific manner. The highly conserved Y′ elements are present immediately distal to the telomeric tract at most telomeres, and are often reiterated as direct repeats, with smaller 50- to 150-bp poly($G_{1\text{-}3}T$) tracts occurring at Y′/Y′ junctions.[1] Centromere proximal to the Y′ elements (or to poly[$G_{1\text{-}3}T$] tracts at ends lacking Y′ elements) are a series of shorter and less conserved repeated elements[4] (Figure 3.1A). Four repeats designated A, B, C, and D (oriented from telomere to centromere) are present at some telomeres. Distal to these repeats, a core X element has been identified at the majority of telomeres. Although not all elements are present at each telomere, their relative order appears to be invariant. Both the core X and Y′ repeats contain autonomously replicating sequence (ARS) elements, which, at least in some instances, can serve as late-initiating origins of DNA replication near the telomere.[5] However, none of these subtelomeric repeats are essential for mitotic or meiotic chromosome stability.[1]

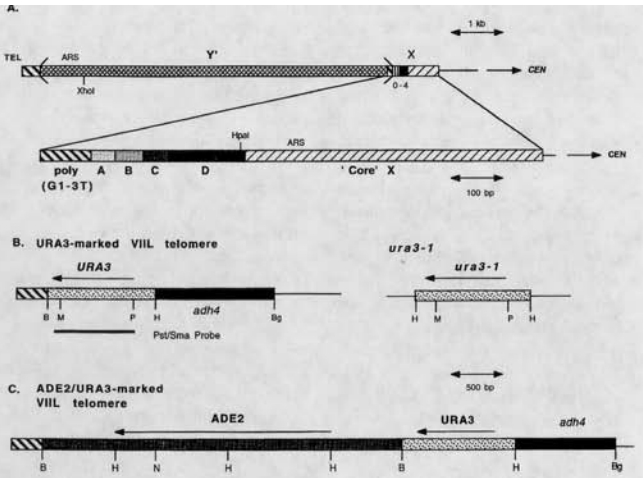

FIGURE 3.1
Structure of naturally occurring and marked telomeres in *Saccharomyces cerevisiae*. (A) General structure of the yeast telomere. The previously described X element,[1] displayed on top, is expanded on bottom, to demonstrate the substructure present within this region.[4] (B) Structure of the *URA3*-marked telomere, introduced at the *ADH4* locus on the left arm of chromosome VII (VIIL), and the internal *ura3-1* locus. The *PstI*/*SmaI* probe used for the *dam* methylase experiments is also shown. (C) Structure of the *ADE2*-marked VIIL telomere. Arrows refer to the direction of transcription. TEL, telomere; CEN, centromere; restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; M, *Mbo*I; H, *Hind*III; P, *Pst*I.

B. Genetic Approaches Toward Understanding Telomere Function

1. Telomere Replication

Telomere replication poses two fundamental problems. First, telomeres require a novel mode of synthesis as the consequence of lagging strand-associated RNA primer loss. In most organisms, the enzyme required to compensate for this loss is a ribonucleoprotein termed telomerase, which uses its RNA component as template for the synthesis of the G-rich repeats onto single-stranded telomeric substrates.[6] It appears that yeast utilizes a telomerase-based mechanism. The telomerase RNA, TLC1, has recently been identified in yeast,[6a] and several laboratories have identified telomerase activity in whole or partially fractionated extracts.[6b-d] The characteristics of telomere addition onto broken chromosomal ends are also most consistent with a telomerase mechanism of addition.[7] Hence, yeast is likely to serve as a powerful

model system for processes that occur in higher eukaryotes. Additional *RAD52*-dependent and -independent recombinational pathways for telomere addition and rearrangement have also been described that may serve to rescue the products of rare catastrophic events that result in loss of telomeric sequences.[8,9]

Second, telomeres are maintained in an equilibrium of sizes centered around a defined average length. Since telomerase is a highly processive activity in most organisms,[6] cellular mechanisms must be present to counterbalance this effect and regulate telomere length.

Four major approaches have been taken to identify mutations that affect telomere size control. First, early approaches focused on screening mutagenized cells for changes in poly($G_{1\text{-}3}$T) tract size. Using this approach, mutations in two genes, *TEL1* and *TEL2*, were identified that confer 80 and 50% reductions in tract length, respectively.[10] Carson and Hartwell[11] analyzed telomere tract sizes specifically in cell-division-cycle (cdc) mutants that display enhanced recombination rates near the telomere. Mutations in one gene, *CDC17*, which encodes DNA polymerase I (the DNA polymerase α homolog of yeast), confer ≈900 bp increases in telomere tract sizes. These elongated telomeres may be the consequence of an increase in nicked and gapped DNA at the telomere, which may serve as substrate for both recombination enzymes and telomerase. Increased tract size could also be mediated through enhanced frequencies of DNA polymerase slippage or a loss of coordination between leading and lagging strand synthesis.

Second, in an alternative approach to identify mutations defective in telomere addition, Lundblad and Szostak[12] took advantage of the ability of circular plasmids containing inverted repeats of telomeric tract sequences to resolve into linear plasmids terminated in poly($G_{1\text{-}3}$T) repeats. When the *URA3* gene was inserted between these inverted repeats, the circular plasmid frequently resolved into a linear form, resulting in a loss of the *URA3* gene and, consequently, in the ability of plasmid-containing cells to papillate on 5-fluoroorotic acid (5-FOA)-containing media. Following a screen to identify mutations that have lost the ability to form these FOA-resistant (FOA^r) colonies, a mutation in one gene, *EST1* (for "ever shorter telomeres"), was identified that conferred a slow loss of telomeric tract sequences and a concomitant loss in chromosome stability and cell viability. These phenotypes suggest an essential role of *EST1* in telomere addition. Using a similar screen, mutations in three additional genes, *EST2*, *EST3*, and *EST4* have been identified that confer identical telomere tract loss and senescence phenotypes.[13]

Third, Schulz and Zakian[14] screened for mutations that could stabilize spontaneously broken artificial chromosomes terminated in nontelomeric sequences. Although wild-type cells rarely stabilize broken chromosomes through the addition of poly($G_{1\text{-}3}$T) tracts, mutations in one gene, *PIF1*, conferred the ability to utilize very short G-rich sequences as substrate for telomere addition. Telomere tract sizes in *pif1* cells are also increased. These data suggest a reduced specificity for telomere addition in *pif1* cells. Interestingly, *PIF1* is a DNA helicase, raising the possibility that it may act by unwinding short hybrids between G-rich sequence and the telomerase template RNA.

Finally, genetic characterization of RAP1 (repressor/activator protein 1) has helped to define its function in telomere addition. In addition to being the predominant *in vitro* poly($G_{1\text{-}3}$T) binding protein, RAP1 also binds to sequences embedded within numerous UAS elements and within the silencer elements flanking the cryptic mating-type genes at *HML* and *HMR*.[14a] Both immunocytochemical studies and characterization of telomeric chromatin have confirmed the association of RAP1 with telomeres *in vivo*.[15,16] Two classes of *rap1* alleles have been identified which have marked effects on telomere tract size: (1) recessive *rap1* temperature-sensitive alleles that display a partial loss of the telomeric tract at semipermissive temperatures;[17,18] and (2) semidominant nonsense alleles that truncate the C terminal 144–165 amino acids of RAP1, conferring massive increases in telomere tract size and heterogeneity[19] (Figure 3.2). Overproduction of the C terminus of RAP1 also produces elongated telomeres, suggesting the titration of a factor necessary for telomere size control.[18] The phenotypes generated in these experiments suggest a critical role of RAP1 in telomere size control, possibly mediated through alterations in telomere chromatin structure. Also consistent with a role of RAP1 in telomere addition is the finding that mutations in synthetic telomeres that eliminate RAP1 binding *in vitro* are severely diminished in their ability to stabilize plasmids in the linear form *in vivo*.[17]

2. Telomere Position Effects

Several approaches have been used to probe the unusual properties of chromatin in subtelomeric regions. First, Gottschling et al.[20] have demonstrated that Pol II-transcribed genes placed adjacent to telomeric tracts undergo cycles of repression and derepression, with each state maintained epigenetically for multiple generations, a phenomenon termed telomeric silencing. The ease of marking telomeres with specific selectable genes (e.g., *URA3* or *ADE2*) in which both the repressed and derepressed states can be monitored has led to a rapid characterization of many of the factors that influence this process. These studies have uncovered a striking relationship between telomeric silencing and silencing of mating-type information at the *HML* and *HMR* loci.[21] Mutations in many of the genes that reduce or eliminate *HM* silencing abrogate telomeric silencing. These include mutations in *SIR2*, *SIR3*, and *SIR4*, encoding proteins of unknown function; mutations in the two subunits of N terminal acetyltransferase (NAT1 and ARD1); and mutations in the N termini of histones H3 and H4.[21,22] In contrast to telomeric silencing, however, *HML* and *HMR* are not dependent on telomeric position for silencing.

Another similarity between *HM* and telomeric silencers is the presence of binding sites for RAP1 at both set of loci. Recent studies have demonstrated that a 28-amino acid C terminal tail domain of RAP1 is essential for telomeric silencing.[23] Genetic, physical, and cytological studies suggest that this function may be mediated in part through interactions with SIR3 and SIR4.[23-25b] The RAP1 interacting factor, RIF1,[26] may play a different role in telomeric silencing. *rif1* mutations increase the efficiency of telomeric silencing, raising the possibility that RIF1 acts

as an antagonist of telomeric silencing, possibly by competing with SIR3 or SIR4 for association with RAP1.[23,24,27]

Genetic evidence has implicated an end-specific factor in telomeric silencing. While long 900-bp internal tracts of poly($G_{1-3}T$) can mimic the characteristics of telomeric silencing, internal poly($G_{1-3}T$) silencing is abolished by overproduction of poly($G_{1-3}T$) tracts on either circular or linear plasmids, probably due to the titration of RAP1.[28] In contrast, telomeric silencing is resistant to overproduction of poly($G_{1-3}T$) tracts on circular plasmids, but is abolished by the presence of poly($G_{1-3}T$) tracts present at the termini of high copy linear plasmids. These data suggest the presence of an end-specific factor that facilitates the association of RAP1 and associated factors to the telomere.[29]

A second assay for telomere position effects measures the relative accessibility of subtelomeric sequences to *Escherichia coli dam* methylase expressed *in vivo*. Subtelomeric *dam* methylase sites exhibit a marked resistance to modification, suggesting the presence of a more closed chromatin state near the telomere.[30] Interestingly, all mutations tested to date that abolish or reduce the efficiency of telomeric silencing also increase the accessibility of subtelomeric regions to the *dam* methylase, suggesting a mechanistic link between these two processes.

An independent assay for telomere position effects is the ability of fragments containing subtelomeric and telomeric repeats to increase the stability of ARS-containing plasmids, a phenomenon termed TEL plasmid stabilization.[31] This process is partially dependent on the SIR2, SIR3, SIR4, and RAP1 proteins, suggesting a mechanistic connection between TEL plasmid stabilization and telomeric silencing, possibly mediated through association of TEL plasmids with chromosomal telomeres.[31] In contrast, these telomeric fragments act to decrease the stability of centromeric plasmids, indicating an antagonism between the two mechanisms of stabilization.[32] Several suppressors capable of restoring the stability of these plasmids have been identified. These suppressors define a series of novel genes (termed the *RLF* genes), which when mutated result in a delocalization of RAP1 from its normal site at the nuclear periphery. Mutations in some of these genes (e.g., *RLF1, RLF2, RLF4*) also confer defects in telomeric silencing.[33]

Finally, Fangman's laboratory has used a combination of density shift and cell cycle synchronization methods to demonstrate that telomere-proximal origins of replication are initiated late in S phase.[5] Interestingly, when normally early-initiating origins are placed in a telomeric context, they invariably initiate late in S phase.[34] In contrast, a double-strand break induced adjacent to an origin of replication does not influence its time of initiation,[35] indicating that a bona fide telomere is required for late replication.

The factors that control telomere size and position effects are not mutually exclusive. This is most clearly demonstrated in the case of RAP1, which plays critical roles in both processes. Several additional mutations appear to affect both processes. *tel2* mutants, identified on the basis of decreased telomere tract size, display a severe reduction in telomeric silencing.[36] Conversely, *rif1* mutations, which enhance telomeric silencing, result in telomere tract elongation.[26] Similarly, mutations in *SIR4* and in the global transcriptional regulator *GAL11*, which severely reduce or abrogate telomeric silencing, also confer reductions in telomere tract size.[15,37]

II. Methods for the Investigation of Yeast Telomere Structure and Function

A. Telomere Identification

Classically, a telomere can be identified by several criteria including (1) restriction fragment size heterogeneity, reflecting the inexact replication of telomeric sequences; and (2) the presence of an apparent universal restriction site, corresponding to the chromosomal terminus. However, the most compelling evidence arguing for the presence of a telomere is the selective sensitivity of telomeric fragments to nuclease *Bal*31. *Bal*31 acts exonucleolytically at 3′ ends of duplex DNA and endonucleolytically at nicked and gapped DNA. Since DNA damage generally occurs at random, digestion of high molecular weight genomic DNA with *Bal*31 preferentially removes telomeric sequences. Due to variation among lots of *Bal*31, the rate of loss of a known chromosomal or plasmid telomere should first be determined. The optimal rate of degradation is ≈10 bp per minute. The degree of telomere tract degradation is monitored as a function of time relative to an internal control.

Procedure for Bal31 Digestion[38]

1. Isolate high-molecular weight DNA using the Cold Spring Harbor 40 ml miniprep method.[39] In all steps, care should be taken to minimize vortexing and centrifugation steps.
2. Phenol/chloroform extract DNA and precipitate DNA with EtOH. Following washes with 70 and 95% EtOH, dry the precipitate under vacuum and suspend gently in 0.5 to 1.0 ml of TE. Store samples at 4°C. Electrophorese undigested DNA on a 0.8% agarose gel to ensure that DNA migrates at limit mobility (>25 kb).
3. Prepare a reaction mix consisting of 30 µl of DNA (6 µg), 75 µl of 2 × *Bal*31 buffer (1.2 *M* NaCl; 40 m*M* Tris HCl, pH 8.0; 24 m*M* $MgCl_2$; 24 m*M* $CaCl_2$; 2 m*M* EDTA) and nuclease *Bal*31 (≈1 unit; New England Biolabs) in a final volume of 150 µl. Incubate at 30°C.
4. At various time intervals (usually at 10-min intervals from 0 to 60 min), aliquot 25 µl of the reaction mix into a tube containing 2.5 µl of 0.5 *M* EDTA, mix, and place on ice.
5. After the final time point, inactivate Bal31 by incubating samples at 75°C for 10 min.
6. Precipitate DNA with EtOH in the presence of 2.5 *M* NH_4OAc and 2 µg tRNA. Following centrifugation, wash consecutively with 70 and 95% EtOH, and dry under vacuum.
7. Resuspend Bal31-digested DNAs in 14 µl of TE.
8. Digest samples with a restriction enzyme that cleaves at a site greater than 700 bp upstream of the putative telomere.
9. Conduct Southern analysis of the digested products using a probe which recognizes the suspected telomeric fragment. An internal control is required for all Bal31 digestions to ensure the absence of random nucleases or overdigestion. Probes should be chosen that span the restriction site used for digestion of Bal31-treated samples, since Southern blots of these digests will detect both telomeric and subtelomeric fragments. This procedure eliminates the need to reprobe the Southern blot with an internal DNA sequence.

B. Assays for Telomere Replication in Yeast

1. Determination and Interpretation of Telomere Tract Size and Heterogeneity

Numerous physical and genetic assays have emerged to probe various aspects of telomere function in yeast. The most common assay used for detecting defects in telomere size control is a direct measurement of telomere size and heterogeneity. Telomeric tract size changes conferred by mutations normally occur slowly, removing only several base pairs per generation. Therefore, extensive subculturing is required to determine the extent of a defect in telomere size control. We normally subculture cells on solid media until most colonies attain a diameter of ≈1.0 mm, corresponding to ≈20 generations of growth. Individual colonies are then selected, and the process repeated for three additional rounds. DNA is isolated from cells obtained from each subculturing cycle after growth in liquid media. In most mutants, the full phenotype is observed within four cycles of subculturing (≈100 generations of growth). Several independent subculturings should be carried out to guarantee the reproducibility of the tract size changes. We note that plates containing cells subcultured for differing numbers of generations can be stored at 4°C without any subsequent change in telomere tract size. Subculturing by continuous growth in liquid media (with periodic dilutions) has been used as an alternative technique.[12] However, if mutant cells have a slow growth defect, care must be taken to avoid the accumulation of rapidly growing suppressors after extended growth in liquid media.

a. Determination of Y′ Class Telomere Tract Size

The majority of telomeric poly($G_{1\text{-}3}$T) tracts are associated with the conserved subtelomeric Y′ element which contains a conserved *Xho*I site 870 bp from the Y′/poly($G_{1\text{-}3}$T) tract junction. To measure the tract length of this class of telomeres, yeast genomic DNA, prepared by standard methods, is digested with *Xho*I; and the products are fractionated on a 22-cm 0.8% agarose gel in 1 × TBE at 70 V. Electrophoresis is terminated when the 830 bp marker of a λ *Eco*RI/*Hind*III digest has migrated ≈21 cm (≈16 h). Following transfer to a nylon membrane, the blot is probed with nick-translated poly(dGT)poly(dCA) (Pharmacia). This probe hybridizes to three classes of sequences: (1) telomeric poly($G_{1\text{-}3}$T) tracts, (2) poly($G_{1\text{-}3}$T) tracts at Y′/Y′ junctions, and (3) internal poly(GT) tracts.[40]

Conditions for Hybridization

1. Nick-translate poly(dGT)poly(dCA) by standard procedures using 1 μg of poly(dGT)poly(dCA) and 10 μCi of [^{32}P]-labeled dCTP (3000 Ci per millimole).[41] Assay incorporation of label into TCA-precipitable counts in an aliquot of the nick-translated sample. Typical specific activities are ≈10^7 cpm/μg.
2. Following standard prehybridization methods, denature ≈1 × 10^6 cpm of poly(dGT)poly(dCA) in 100 μl of TE for 4 min at 100°C. Calf thymus DNA should not be added during denaturation, since it contains multiple poly(GT) tracts which compete for hybridization with the labeled probe.
3. Add denatured probe immediately to the filter in 20 ml hybridization solution (6 × SSC, 0.5% SDS, 10% dextran sulfate).
4. Hybridize filters for 16 h at 55°C.
5. Wash filter briefly with 500 ml of 6 × SSC, 0.5% SDS to remove excess label.
6. Wash filter in 500 ml of 6 × SSC, 0.5% SDS at 55°C for 60 min.
7. Wash filter in 500 ml of 2 × SSC at 55°C for 30 min. Repeat wash three times.

Poly(dGT)poly(dCA) is difficult to remove from nylon membranes after hybridization. Therefore, when filters must be hybridized with both unique and poly(dGT)poly(dCA) probes, the unique probe should be utilized first. Following removal of the unique probe, the blot is rehybridized with poly(dGT)poly(dCA) in the presence of 10 × Denhardt's solution (0.1% Ficoll 70; 0.1% Ficoll 400; 0.2% polyvinylpyrrolidine; 0.2% bovine serum albumin [BSA]).

The typical blot contains several characteristic features (Figure 3.2). In wild-type strains containing a telomere trace size of ≈300 bp, the majority of telomeres are clustered at a fragment size of ≈1.2 kb. This signal is both intense and diffuse as a consequence of both the large number of overlapping telomeric fragments and the heterogeneity present at each individual telomere. In the 1.4- to 3.5-kb size range, several individual diffuse telomeric fragments are also detected, derived from termini that lack the conserved Y′ repeat. Three additional classes of fragments are present at sizes greater than 3.5 kb: (1) telomeric fragments with more distal *Xho*I sites, (2) 5.2- and 6.7-kb fragments containing poly($G_{1\text{-}3}$T) tracts between reiterated Y′ elements, and (3) fragments containing internal poly(GT) tracts.

An analogous procedure has been used to detect telomeres that lack the Y′ element, but contain the subtelomeric D element. Since many D elements contain a *Hpa*I site, 200 bp from the poly($G_{1\text{-}3}$T) tract,[4] Southern analysis of *Hpa*I-digested DNA, probed with poly(dGT)poly(dCA), yields a group of telomeric fragments that cluster at sizes of ≈500 bp in a strain containing poly($G_{1\text{-}3}$T) tracts of ≈300 bp.[38]

b. Analyzing Tract Sizes of Individual Telomeres

Several techniques have been used to detect individual telomeric fragments. First, two unique probes, near the left and right telomeres of chromosome III and chromosome I, respectively, have often been used to characterize individual telomeres of naturally occurring chromosomes.[18,19] Second, individual telomeres can be monitored by modification of naturally occurring chromosomes through the introduction of a unique gene (e.g., *ADE2* or *URA3*) adjacent to the telomeric tract[20] (Figure 3.1B and C, see below). DNA isolated from these strains is digested with *Nde*I or *Hind*III, to detect the *ADE2* and *URA3* genes, respectively, and is subjected to standard Southern analysis using *URA3* or *ADE2* probes. In wild-type strains containing a telomere tract size of ≈300 bp, both *ADE2*- and *URA3*-marked telomeric fragments migrate as diffuse species at ≈1.4 kb. Third, as noted above, several individual telomeres can also be detected on blots hybridized with poly(dGT)poly(dCA).

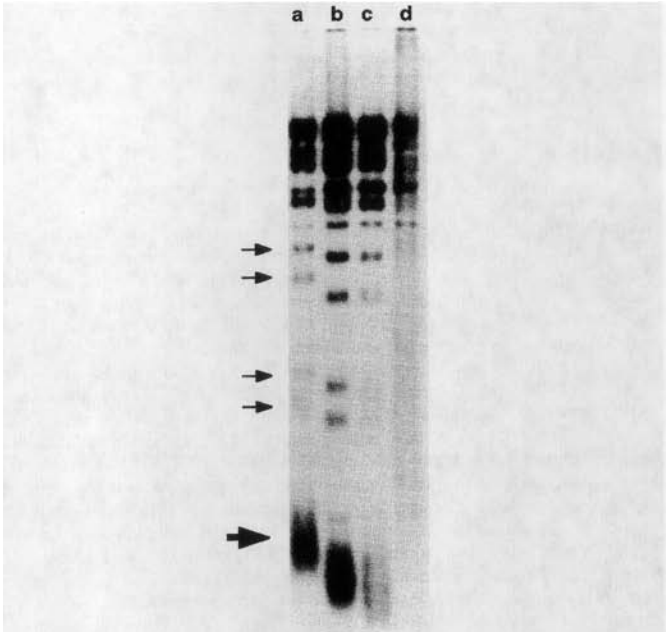

FIGURE 3.2
Analysis of Y′ class telomeres. DNA isolated from wild-type cells (b), cells containing the *rap1-5* temperature-sensitive allele grown at permissive (a) or semipermissive temperatures (c), and *rap1-18* cells (d), which contain a truncation of the C terminal 144 amino acids of RAP1, were digested with *Xho*I, and subjected to Southern analysis using poly(dGT)poly(dCA) as a probe. The large arrow refers to the Y′ class of telomeres, while the smaller arrows correspond to several individual telomeres lacking the Y′ element.

In some situations, the possibility that increases in fragment length are the consequence of subtelomeric rearrangement, rather than increases in tract size, must be ruled out. This can be accomplished by a modification of the Bal31 digestion procedure.[19,38] High molecular weight DNA is digested with Bal31 under conditions that remove the entire poly($G_{1\text{-}3}$T) tract, aliquoting digested DNA at appropriate time intervals. DNA is then digested with a restriction enzyme adjacent to a unique telomere. Southern blots of the digest are probed with the unique telomeric-specific probe, the probe is removed, and the blot is rehybridized with poly(dGT)poly(dCA). If the difference between the initial tract length and the point at which the hybridization to poly(dGT)poly(dCA) is eliminated (≈20 bp) is equivalent to the apparent tract size, then the possibility of telomeric rearrangements is effectively eliminated. Since poly(dGT)poly(dCA) hybridizes to all telomeric fragments, the success of this approach depends on the ability to detect the desired telomeric fragment. It may be necessary, therefore, to attempt this technique with several different telomeres.

c. Interpretation of Telomere Tract Size Data

Two distinct properties of telomeres can be assayed using these procedures. First, changes in the average tract length can be monitored. Mutations that influence telomere tract length normally affect all telomeres and are therefore best assayed by measuring the average size of Y′ class telomeric fragments. In contrast, changes in the size of individual telomeres may be obscured by overlapping variations in tract size among different clonal derivatives, a phenomenon also observed in wild-type cells.

Second, changes in the heterogeneity of telomeric fragments can be assayed. The heterogeneity of a telomeric fragment measures the variation in the length of the inherited tract generated over a specified number of generations, even in the absence of a change in average telomere tract size. Telomere tract size heterogeneity is therefore a reflection of the stability of the telomere tract, i.e., the ability of the telomere size machinery to maintain tract size within a discrete distribution. This information is best provided by measuring the distribution of individual telomeric fragment sizes. To this end, cells are grown on solid media for ≈20 generations, and individual colonies are patched onto solid media. A portion of this patch is then inoculated into 5 ml of liquid media, and the DNA is isolated. The degree of heterogeneity at individual telomeres is subsequently assayed by the techniques described above. In wild-type cells, individual telomeres do not differ by more than 100 bp from the mean length after 20 generations of growth.

Several factors can complicate the interpretation of telomere size data. First, telomere tracts exhibit subclonal variations (50- to 100-bp variations from the average tract length) even in wild-type strains. We therefore do not normally consider variations within this range as significant. Second, different wild-type strains vary from 150 to 800 bp in average telomere tract length. This variation is the consequence of polymorphisms in genes influencing telomere tract size control among the different strains.[38] It is therefore necessary to conduct experiments in an isogenic background. Third, since telomere tract size is often not rapidly reversible, wild-type and mutant strains can exhibit variations in the sizes of individual telomeres dependent on their genetic history. For example, a wild-type strain inheriting elongated telomeres from a *cdc17* or *rap1* mutant strain can maintain their increased tract length in a wild-type background even after extensive growth.[11,19] Since strains derived from a heterozygous diploid inherit both wild-type and elongated telomeres, these slow changes in telomere size can confuse the interpretation of experiments. We have overcome this complication by introducing a centromeric plasmid carrying the mutant allele into a strain in which the only wild-type allele is carried on a second centromeric plasmid. Subsequent elimination of the wild-type allele by a plasmid shuffle allows a direct determination of the mutant defect on telomeres of originally wild-type length.[19]

We note that changes in tract size reflect shifts in the equilibrium between processes that act to elongate and shorten telomeres. Changes in tract size may be caused by numerous factors including alterations in telomeric chromatin structure, the telomere addition machinery, the processes that act to limit telomere addition (e.g., endonucleases and recombination enzymes), and the coordination between leading and lagging strand synthesis. Ultimately, specific conclusions regarding the function of the gene product require additional *in vivo* and *in vitro* approaches.

2. Telomere-Associated Senescence

Several *est* mutations have been characterized that exhibit a slow loss of telomeric tract sequences and a concomitant loss in viability.[12] In recombinationally proficient cells, this population is ultimately overgrown by cells exhibiting *RAD52*-dependent recombination among subtelomeric elements that temporarily restores telomere function. In contrast, in *est1rad52* double mutants, the population as a whole senesces and ultimately is nonviable.[9] To determine whether a particular mutation displays a senescence phenotype, these assays should therefore be conducted in both a $Rad52^+$ and a $Rad52^-$ strain. To assay senescence, a diploid heterozygous for mutations both in the gene of interest and in *RAD52* is sporulated and the resulting spore products are subcultured. Mutations conferring a telomere-associated senescence phenotype should display a slow and continuous loss in both viability and telomere tract size.

3. Telomere Sequencing

Sequencing of telomeric tracts has been an important tool for determining both the organization of poly($G_{1\text{-}3}T$) tracts and the sequence specificity of telomere healing. However, as a consequence of both their terminal location and the unusual secondary structures formed by telomeric sequences, specialized methods are required to clone and sequence telomeric poly($G_{1\text{-}3}T$) tracts. Wang and Zakian[42] cloned the telomeres of linear plasmids (containing sequences allowing propagation and selection in both yeast and *E. coli*) by the following procedure: (1) removal of one of the two plasmid telomeres after digestion of yeast genomic DNA with an appropriate restriction enzyme, (2) generation of blunt ends with T4 DNA polymerase, (3) ligation of the products to generate intramolecular circular forms, and (4) transformation of the ligation mix into *E. coli*. Treatment with T4 DNA polymerase ensures that only 3′ overhang sequences (and not nicked or gapped DNA) are removed during cloning. Fragments generated from the cloned DNA were then end-labeled, strand-separated, and sequenced by the Maxam-Gilbert method. Chemical sequencing has been necessary as a consequence of the unusual secondary structures formed by telomeric DNA that has often precluded the use of other methods. However, shorter 41-bp poly($G_{1\text{-}3}T$) tracts have been sequenced by standard double-strand sequencing methods when 0.5 μg of *E. coli* single-stranded DNA binding protein (SSB; USB) is included in each sequencing reaction.[17]

Kramer and Haber[7] took two alternative approaches to clone and sequence the junctions of healed telomeres. In the first, after treating genomic DNA with T4 DNA polymerase, they ligated a specific oligonucleotide onto the blunt-ended molecules. They then amplified the healed telomere by a polymerase chain reaction (PCR)-based method, utilizing the ligated oligonucleotide and unique internal sequences as primers. In the second method, DNA was digested with a restriction endonuclease internal to the site of healing, ligated to form circular products, and subsequently amplified by PCR using unique sequences adjacent to the site of healing. After the products were cloned, junctional DNA was sequenced by double-strand sequencing methods.

We have sequenced up to 300 bp of cloned chromosomal poly($G_{1\text{-}3}T$) tracts using a modification of Sequenase procedures suggested by the manufacturer (USB). In the example described below, the telomeric tract was cloned into pUC18 in the absence of subtelomeric sequences, allowing the use of universal primers. This method is equally applicable, however, to larger cloned fragments using primers within subtelomeric sequences, adjacent to the tract.

Modified Sequenase Protocol

1. Isolate $CsCl_2$-gradient purified plasmid DNA from a 250-ml culture,[41] carefully avoiding the RNA pellet. After dialysis against TE buffer, extract the DNA with phenol/chloroform and precipitate with EtOH. Redissolve the DNA in 300 to 500 μl TE/gradient. The use of highly purified RNA-free DNA is critical for the success of this procedure.
2. Estimate the concentration of DNA following gel electrophoresis of varying amounts of restriction enzyme-digested plasmid DNA using *Eco*RI/*Hind*III digested λ DNA as a standard.
3. To denature DNA, dilute 6 μg of supercoiled DNA to a volume of 80 μl with H_2O, add 8 μl of freshly prepared 2 *M* NaOH and 2 m*M* EDTA, and vortex. Incubate at 37°C for 30 min. Three denatured samples should be prepared for the annealing and reaction conditions described below.
4. To neutralize, add 24 μl of 3 *M* NaOAc, pH 5.0 and 28 μl of H_2O, and vortex. The final pH, as determined by spotting 2 μl of the solution onto pH paper, should be 6.0 to 6.5.
5. To each tube, add 300 μl 100% EtOH and precipitate on dry ice for 5 min. Spin in a microfuge, and wash consecutively with 70 and 95% EtOH. Dry the precipitate under vacuum.

Sequencing the CA-Rich Strand:

6. Two of the denatured DNA samples are used for sequencing of the CA-rich strand to determine the sequence both proximal (steps 6 to 8) or distal (steps 6, 9, and 10) to the primer. To each dried pellet, add 2 μl of 5 × Sequenase buffer (USB), 2 μl (56 ng) of universal forward or reverse primer (depending on the orientation of the tract), and 6 μl of H_2O. Incubate at 65°C for 2 min. Cool slowly for 45 min.
7. To one tube, add 0.5 μl of *E. coli* SSB (USB; 1 mg/ml), 1 μl of 0.1 *M* DTT, 2 μl of dGTP labeling mix (USB; freshly diluted 1/20 with H_2O), 6 μCi of [^{35}S]-dATP (3000 Ci per millimole; 10 mCi per milliliter), and 2 μl of Sequenase 2.0 (USB; diluted 1/4 in enzyme dilution buffer). Incubate reaction at 19°C for 2 min.
8. Aliquot 3.5 μl of the above reaction to four 0.65 ml siliconized tubes containing 2.5 μl of either ddG, ddA, ddT, or ddC termination mixes (for dGTP sequencing; USB) prewarmed for 1 min. Incubate at 42°C for 3 min. Add 4 μl of formamide dye stop mix (USB) and place on ice.

9. Repeat the procedure for the second tube of annealed product including 1 µl of 5000µ*M* dCTP and 2 µl of [^{35}S]-dATP in the sequencing reaction.
10. Termination reactions are conducted as in step 8, except that 1 µl of the appropriate dideoxynucleotide and 1.5 µl of Sequenase extending mix (USB) are used for the ddA and ddC termination reactions. By both increasing the concentration of dATP and dCTP and decreasing the rate of termination, this procedure can often help to overcome the severe decrease in signal observed within the poly($C_{1\text{-}3}$A) tract under standard conditions. A combination of the two techniques allows the resolution of ≈150 to 200 bp of poly($C_{1\text{-}3}$A) sequence.

Sequencing the GT-Rich Strand:

11. To the third tube of denatured DNA, used for sequencing the GT-rich strand (steps 11 to 13), anneal the appropriate primer as in step 6.
12. Conduct sequencing reactions as in step 7 using 2 µl of dITP reaction mix (USB; freshly diluted 1/20 with H_2O) in place of the dGTP reaction mix.
13. Conduct termination reactions as in step 8, using dITP termination mixes (USB) in place of dGTP termination mixes. We find that dITP reactions are superior to dGTP reactions for sequencing of the GT-rich strand, since dGTP reactions tend to yield multiple strong stops. Although the dITP method still results in a background of weak stops, the relative intensities of signals in the dG and dT lanes can be used to unambiguously assign the base at these positions. Using this technique, we can resolve 100 to 150 bp of poly($G_{1\text{-}3}$T) sequence.

Following sequencing and termination reactions, digest SSB by adding 1 µl of freshly prepared 0.1 mg/ml proteinase K and incubate at 65°C for 20 min. Samples are then treated by standard procedures. We normally subject samples to electrophoresis on an 8% (19:1) –8 *M* urea polyacrylamide sequencing gel using an IBI wedge system. Using a combination of the CA-rich and GT-rich methods, the sequence of a cloned 300-bp tract (including overlapping regions) can be determined in a single set of reactions. Two independent clones should be sequenced to ensure the absence of mutations generated during the cloning process. In addition, if PCR amplification is used, the results should be confirmed in an independent PCR amplification and in independent clones from the amplified product.

4. Telomere End Structure

Wellinger et al.[3] have developed two assays for determining the presence of telomeric single-strand tails at specific stages of the cell cycle. The first assay consists of performing nondenaturing Southern analysis of genomic DNA isolated from synchronized cells using telomeric riboprobes complementary to the GT-rich strand. Telomeres containing either single-stranded terminal tails or gapped regions within the tract can be identified in this manner. However, prior digestion of DNA with *E. coli* exonuclease I, an enzyme that digests single-stranded, but not gapped, DNA, eliminates hybridization, indicating that hybridization is the consequence of single-stranded tails. The second assay relies on the ability of G-rich single-stranded tails to associate with one another via G-G base pairing. Linear plasmids present in cells synchronized in late S phase are present in a unique form which can be identified on two-dimensional neutral/neutral gel electrophoresis. The migration of this species is identical to the migration of circular forms of linear plasmids formed through intramolecular G-G base pairing between telomeric single-stranded tails.

5. Telomere Healing Assays

Plasmid telomere healing has been used as a model system for assaying the *cis*- and *trans*-acting requirements for telomere addition. In this assay, linear plasmids terminated by synthetic or natural telomeric repeats are transformed into yeast and the ability of the plasmid to be maintained in the linear form is tested. Stabilization in the linear form is dependent on the ability of the synthetic or natural telomere to "seed" the further addition of poly($G_{1\text{-}3}$T) sequences *in vivo*. Telomere healing is therefore a transient assay that measures the initial ability of the telomere addition machinery to add telomeric sequences onto telomeric substrates.

The replicating plasmids that we use contain synthetic telomeric repeats of 41 bp in inverted orientation, separated by spacer DNA (e.g., the *HIS3* gene, Figure 3.3) in order to maintain stability in *E. coli*.[17,43] Since this length of tract is close to the minimum size capable of efficient healing, these vectors provide a sensitive assay for differing efficiencies of telomere healing. Although the plasmid does not undergo frequent tract rearrangement in *E. coli* strain HB101, the telomeric tracts of these plasmids should be sequenced to ensure the absence of mutations, expansions, and contractions within the tract. Equally useful vectors have been constructed by several other laboratories.[8,12,43]

To introduce linear plasmids into yeast, the circular plasmid is linearized at a site adjacent to the telomeric repeats (e.g., *Bam*H1 in Figure 3.3), and the DNA is transformed into yeast using standard methods of spheroplast transformation.[39] Two points regarding this procedure must be noted. First, the addition of 10 µg of denatured calf thymus DNA improves the efficiency of transformation, probably by preventing the degradation of plasmid DNA. Second, it is important to transform equal amounts (≈0.3 µg) of digested plasmid DNA under each set of conditions or strain tested, since the number of plasmid molecules per transformed cell may increase at high DNA concentrations. Since linear plasmids have higher mitotic stabilities than their circular counterparts,[44] transformation with a high concentration of DNA may result in an overestimation of the efficiency of linear plasmid formation.

The efficiency of telomere healing is determined by physical analysis of the plasmid DNA isolated from transformants.[17] To prevent biasing the sample, all transformants within a random sector or plate should be patched onto minimal media under selection for the plasmid. Normally, 30 to 50 transformants are selected in order to observe statistically significant differences in healing efficiency. DNA is isolated from the transformants after growth in minimal media under selection for the plasmid. Both undigested DNA and DNA digested with an enzyme that cleaves the linear plasmid uniquely (e.g., *Pvu*II in Figure 3.3) are subjected to Southern analysis, probed with plasmid sequences, and reprobed with poly(dGT)poly(dCA).

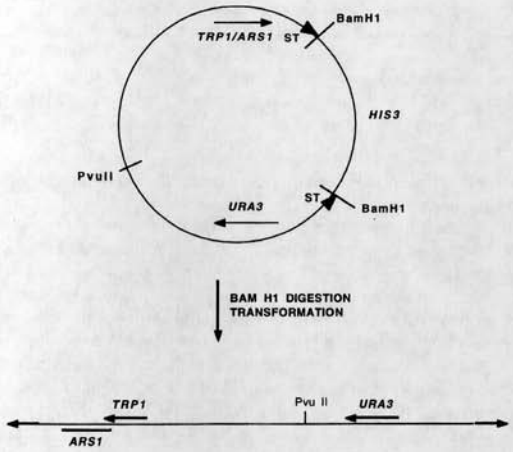

FIGURE 3.3
Telomere healing assays. The general structure of both the circular plasmids used for telomere healing studies and the healed linear plasmids identified in cells transformed with the *Bam*HI-linearized plasmid are shown. Drawings are not to scale. ST, synthetic telomeres.

Several plasmid forms have been observed in this assay:

1. Healed linear plasmids — These species are the consequence of the addition of poly($G_{1\text{-}3}T$) to the synthetic telomeres. The telomeres of this plasmid are maintained at tract lengths equivalent to chromosomal telomeres. Prior to cleavage, this form appears as a species slightly greater in size than the input plasmid (as a consequence of the poly[$G_{1\text{-}3}T$] addition onto both termini), and is capable of hybridization to both poly(dGT)poly(dCA) and pBR322 probes. After digestion with *Pvu*II, two diffuse fragments are produced that are capable of hybridization to pBR322. Each fragment is larger than the input plasmid by the size of the poly($G_{1\text{-}3}T$) tract.
2. Aberrant linear plasmids — This class is identical to the healed linear plasmids except that the tract length at one end is maintained at a size greater than found at chromosomal telomeres. These plasmids arise most frequently when synthetic telomeres that contain mutations within the tract (e.g., lacking RAP1 binding sites) are used, and are probably the consequence of promiscuous addition of poly($G_{1\text{-}3}T$) tracts during the initial steps following transformation.
3. Linear recombinants — The most common form of linear recombinant is the linear inverted dimer, the consequence of addition of poly($G_{1\text{-}3}T$) at one end, and recombination or ligation at the other. Analogous circular inverted dimer species have also been identified following transformation of DNA containing termini that lack homology to yeast sequences.[45] The formation of these species suggests a reduced efficiency of telomere addition, allowing a competition between telomere addition and recombination. In the absence of cleavage, the species are observed as dimer-sized products capable of hybridization with both poly(dGT)poly(dCA) and pBR322 probes. Southern analysis of *Pvu*II-digested plasmids using pBR322 as a probe reveals two fragments: an intense fragment corresponding to two copies of one of the two plasmid arms, and a second less intense fragment corresponding to an inverted dimer of the other plasmid arm. More complex linear forms have also been observed.
4. Circular forms — Two predominant circular species have been observed: (a) simple unit-size circles, the consequence of religation of the two linear plasmid ends; and (b) circular inverted dimers in which both ends of the linear plasmid are ligated in an inverted orientation to a second linear plasmid. The appearance of circular products is a strong indication of a defect in telomere addition. In undigested DNA, these plasmids appear in multiple forms that hybridize to the pBR322 probe. After digestion with *Pvu*II, these plasmids form either a linear monomer-length species, corresponding to a simple circular form; or two distinct dimer-length species, corresponding to a circular inverted dimer. More complex circular forms have also been observed.

The distribution of these classes of products define the efficiency of telomere addition. In the wild-type strain A364A, 92% of transformants contain healed linear forms and an additional 5% form linear recombinants.[17] These values, however, vary among strains. We note that conditions which lead to a lower frequency of healed linear plasmids often also confer statistically significant (1.5- to 3.0-fold) reductions in transformation frequencies. It is important to recognize that the plasmid healing assay may not reflect all of the events that occur at chromosomal ends. Nonetheless, since telomere healing is a highly sequence-specific process[46] and is influenced by mutations both in the RAP1 binding site[17] and in RAP1,[47] it is likely to share components with the cellular machinery responsible for telomere replication.

III. Assays for Higher Order Chromatin Structure at the Telomere

A. Telomere Silencing

Investigations of telomeric silencing have been restricted to the characterization of modified telomeric regions in which a Pol II-transcribed gene is placed directly adjacent to the poly($G_{1\text{-}3}T$) tract.[20] These marked telomeres are generated by a chromosome fragmentation method in which yeast cells are transformed with a linear fragment containing a marker gene, flanked on one side by unique chromosome-specific subtelomeric sequences, and on the other side by a short telomeric tract. Crossing over between the unique sequences (usually on the right arm of chromosome V or the left arm of chromosome VII) gives rise to a truncated chromosome containing a marked telomeric end (Figure 3.1B and C). Since the truncated sequences are not essential for viability, silencing can be characterized in haploid cells.

The most informative markers for telomeric silencing are the *URA3* and *ADE2* genes as they can be assayed in both the transcriptionally repressed and derepressed states (Figure 3.1B and C). The transcriptionally derepressed and repressed states of *URA3* are monitored phenotypically by the ability of cells to grow on uracil omission media and 5-fluoroorotic acid (5-FOA)-containing media, respectively.[20] 5-FOA allows the growth of Ura3$^-$, but not Ura3$^+$, cells.[48] In contrast, expression of the *ADE2* gene in an *ade2* strain results in white colonies or sectors, while repression of *ADE2* leads to red colonies or sectors, as the consequence of the accumulation of a red pigment in *ade2* cells.

Several important caveats to these assays must be noted. First, telomeric silencing is highly context-specific. Different chromosomal ends marked with *URA3* have significantly different FOA^r frequencies.[20,27] For example, telomeres marked at the left arm of chromosome VII normally repress transcription at frequencies ranging from 25 to 60%, while telomeres marked at the right arm of chromosome V have significantly lower median values (ranging from 0.05 to 5%). As a consequence, the latter telomere is very sensitive in detecting conditions or mutations that have subtle effects on silencing.

Second, telomere tract size can influence the efficiency of telomeric silencing: wild-type strains inheriting an elongated marked telomere from a *rap1* mutant strain are repressed at higher efficiencies.[27] Therefore, it is necessary to analyze the length of telomeres in the strains under investigation.

Third, telomeric silencing is highly strain dependent. This effect may be the consequence of differences in telomere tract size as well as in the large number of factors that are likely to be involved in this process.

Fourth, the efficiency of telomeric silencing is temperature dependent, necessitating growth of cells at identical temperatures. While we routinely grow cells at 25°C for the FOA assay, slightly higher FOA^r frequencies are observed at 30°C. The *ADE2* color assay appears to be highly dependent on temperature; colonies form red sectors more frequently at 30°C than at 25°C.

Finally, it is important to note that Northern and phenotypic analyses measure different parameters. Northern analysis measures the steady state level of mRNA. In contrast, the phenotypic assays detect the fraction of cells in a population that transcribe the *URA3* or *ADE2* gene at levels sufficiently low to produce an FOA^r or red colony phenotype. The latter assay is therefore highly sensitive to small changes in transcription within individual cells of a population. The procedures used for each of the assays are described below.

1. 5-FOA Assay

Protocol

1. Grow cells containing the *URA3*-marked telomere nonselectively and patch onto 5-FOA-containing media.[39] Incubate plates for 3 to 5 d at 30°C. This patch assay will yield a crude estimate of the FOA^r frequency, which can be used to estimate the dilutions required for the more quantitative analysis described below.

2. Grow cells nonselectively at 25°C. For strains yielding a median frequency greater than 10^{-4}, colonies should be grown to a diameter of 1 mm (≈500,000 cells). For strains yielding lower frequencies, larger colony sizes (1.5–2.0 mm) are required.
3. For each strain, pick 7 to 10 colonies of identical size and suspend into 300 µl of YPD. Vortex cells rigorously. Plate appropriate dilutions of cells onto both minimal complete media and FOA-containing minimal media, the latter prepared in 6-cm diameter Petri dishes.
4. Incubate control and 5-FOA-containing plates at 30°C for 3 to 5 d and count colonies.

Several factors should be considered in conducting and interpreting the results of this assay. First, the number of colonies required is dependent on the range of the frequencies obtained. Wild-type or mutant strains having low FOA^r frequencies also generate a broader range of frequencies. For this reason, we routinely use 7 to 10 colonies to obtain a statistically reliable sample size. Second, the *URA3* gene switches frequently between the repressed and derepressed state during growth on nonselective media and appears to reach an equilibrium before 20 generations of growth. Therefore, it is not possible to apply standard statistical methods to generate a mean rate of switching between transcriptional states. Due to the broad range of values that is often observed, standard deviations are also difficult to determine. Since the median value of the distribution is highly reproducible and more representative of the data, we conventionally present our data as the median value together with the ranges observed. Statistical significance between median values is determined by the Rank Sum method.[49] Third, FOA^r cells should be capable of switching to the Ura$^+$ phenotype, a characteristic feature of telomeric silencing. This is particularly important to confirm for strains displaying low FOA^r frequencies that may reflect spontaneous mutations in the *URA3* gene. To test this, FOA^r colonies are patched onto rich media, and then replica-plated onto uracil omission media.

2. *ADE2* Color Assay

Protocol

1. Grow cells containing the *ADE2*-marked telomere nonselectively at 30°C.
2. Suspend cells in YPD media and plate appropriate dilutions onto SC/low adenine media (SC media containing 7.5 mg adenine per liter).
3. Incubate cells for 3 to 5 d at 30°C.
4. Shift cells to 25°C.
5. Allow at least 5 to 8 d for full color development.

Cells containing an *ADE2*-marked telomere will form fully white (derepressed), fully red (repressed), and sectored colonies. Although the *ADE2* color assay is less quantitative than the FOA^r assay, it has the advantage of displaying both repressed and derepressed cells on a single plate. Distinct sectoring patterns are readily observed since the *ADE2* gene appears to switch between repressed and derepressed

states at significantly lower frequencies than the *URA3* gene, possibly as the consequence of the greater distance of the *ADE2* promoter from the telomere. In the wild-type strain W303, 2% of the colonies are red, 17% have white colonies with red sectors, and the remainder are fully white.[27] In this background, this assay has its greatest utility in detecting increases in the efficiency of telomeric silencing. We note that the color assay cannot be used to directly quantitate the rate of switching in a steady-state population, since the number of generations that a white- or red-centered colony has been in the repressed state is generally unknown.

B. The *dam* Methylase Assay

Several laboratories have developed an *in vivo* assay that uses *E. coli dam* methylase, which specifically methylates the adenine residue of GATC sequences, to probe the accessibility of different genomic regions.[30,50] This approach is possible since *S. cerevisiae* is fully unmethylated at these sites and methylation does not significantly affect viability. The *dam* methylase assay takes advantage of the ability of the restriction enzymes *Mbo*I and *Dpn*I to cleave only fully unmethylated and methylated GATC sites, respectively. In contrast, *Sau*3AI cleaves the GATC site regardless of methylated state. Resistance to *Dpn*I and sensitivity to *Mbo*I are an indication of the presence of unmethylated sites, suggesting resistance to the *dam* methylase, and therefore, a closed chromatin state.

Several plasmids have been constructed which can be used to introduce the *dam* methylase gene into a number of genetic backgrounds.[17,30,50] In order to prevent the *in vivo* repair of methylated sites, the *rad1* mutation, causing a defect in the excision/repair pathway, should be introduced into strains containing the *dam* methylase. Following integration of these plasmids, sufficient *dam* methylase should be produced from an advantageous promoter to differentiate between accessible and inaccessible regions.

The *dam* methylase assay has been most often used to examine the relative accessibility of a GATC site located within the coding sequence of *URA3* in internal and telomeric domains. The success of this assay depends on the ability to differentiate between telomeric and internal copies of *URA3*. In strains carrying the *ura3-52* Ty-insertion allele, the two alleles can be easily distinguished by restriction analysis.[30] In strains in which the *ura3* allele is not disrupted (e.g., *ura3-1* strains), the fraction of telomeric and internal restriction fragments resistant to methylation can nonetheless be assayed, taking advantage of a 70-bp C terminal truncation introduced in the telomeric *URA3* gene[27] (Figure 3.1B). The details of this latter assay are described below.

Protocol

1. Isolate yeast DNA from a 5-ml culture grown to stationary phase. Growth to stationary phase is required in order to eliminate high levels of hemimethylation found in logarithmically grown cells that can complicate the interpretation of the data.
2. Confirm the activity of the *dam* methylase gene by digesting genomic yeast DNA with *Dpn*I and *Mbo*I. Unmethylated yeast DNA is fully resistant to *Dpn*I, forming a species migrating at limit mobility, but is digested into a multiplicity of small fragments by *Mbo*I. In contrast, DNA isolated from strains carrying *dam* methylase is digested by *Dpn*I and (to a lesser extent) by *Mbo*I.
3. Carry out four parallel restriction enzyme digestions, each containing *Bam*HI and *Hind*III, to distinguish between the internal and telomeric copies of *URA3*. To each of three tubes add either *Mbo*I, *Dpn*I, or *Sau*3AI, and digest for 2 h at 37°C. Fractionate digests on a 0.8% agarose gel as described for the separation of telomeric fragments.
4. Probe Southern blots with a *Pst*I/*Sma*I fragment of *URA3*. This probe shares identical homology to both internal and telomeric copies (Figure 3.1B). Digestion with *Bam*HI and *Hind*III releases a 1.1- and 1.17-kb fragment from the telomeric and internal copies of *URA3*, respectively, that hybridize with this probe. The fraction of the 1.10-kb fragment that is resistant to digestion by *Dpn*I is equivalent to the fraction of cells in which the DNA is protected from methylation. The 1.17-kb fragment should be fully digested with *Dpn*I and serves as an internal control for the activity of the *dam* methylase.

It is important to note that while there is a correlation between defects in telomeric silencing and increased accessibility to *dam* methylase, there are some exceptions to this rule. For example, unlike mutations in the *SIR* genes which abolish resistance to methylation, *rap1* alleles that abrogate telomeric silencing confer only partial increases in accessibility to the *dam* methylase.[27] Partial *Dpn*I resistance may reflect either the fraction of cells in the closed conformation, or an intermediate state, which varies stochastically in its accessibility to *dam* methylase. Similarly, a wild-type strain containing a *URA3*-marked telomere on the right arm of chromosome V displays low levels of FOA^r cells, while exhibiting significantly higher levels of protection.[27] Both results imply that cells containing elements of the closed state can nonetheless be transcriptionally active. Thus, minor changes in accessibility of the *dam* methylase are correlated with profound effects on telomeric silencing, indicating that the chromatin structure defined by the *dam* methylase assay has more stringent requirements than transcriptional silencing.

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