INVESTIGATION OF THE RELATIONSHIP BETWEEN TELOMERE LENGTH AND DIRECT REPEAT RECOMBINATION RATES IN *SACCHAROMYCES CEVEVISIAE*

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ABSTRACT

INVESTIGATION OF THE RELATIONSHIP BETWEEN TELOMERE LENGTH AND DIRECT REPEAT RECOMBINATION RATES IN *SACCHAROMYCES CEREVISIAE*

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Telomeres function to protect the ends of chromosomes and to allow complete replication of linear DNA molecules. In yeast, the major replication pathway involves telomerase. A second pathway occurs independently of telomerase through recombination. Using a direct repeat recombination assay it was previously shown that C_{1-3}A-direct repeat recombination is specifically repressed near telomeres in wild-type cells (Stavenhagen and Zakian, 1998). This repression is independent of the ability of the telomere to repress transcription of nearby genes, a phenomenon known as telomere position effect. We hypothesize that the repression of telomere direct repeat recombination is under the control of telomere length. In order to further investigate the telomere direct repeat recombination pathway, *S. cerevisiae* cells with either short or long telomeres were created in strains harboring yeast telomeric direct repeats, recombination rates were measured, and recombination products were analyzed by genomic Southern blots. Short telomere cells were created by an *hdf1Δ* loss of function mutation. The rate of recombination specifically increased in *hdf1Δ* cells showing a preference for the *RAD1* recombination pathway. Recombination rates were similar for *hdf1Δ* cells grown at a permissive and a non-permissive temperature. Cells with long telomeres were created by harboring a high-copy plasmid containing 800 bps of yeast telomeric DNA, double mutations in *rif1* and *rif2* genes, or a single mutation in the *pif1-m2* gene. The long telomere cells had no significant change in the rate of recombination.

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INTRODUCTION

General Telomere Structure and Function

Telomeres are evolutionarily conserved structures located at the physical ends of linear chromosomes. Experiments performed in the 1930's in *Maize* and *Drosophila* first identified telomeres masking the end of the chromosome from cellular components that would otherwise recognize the ends as broken (Muller, 1938; McClintock, 1939; McClintock, 1941). As a protective cap, telomeres function to prevent degradation by endogenous nucleases and to stop the generation of di-centric chromosomes that could interfere in meiosis (McClintock, 1939). In most organisms, telomeres are composed of tandemly arranged simple sequence repeats. Telomere length varies greatly throughout nature ranging from ~35 bp in single celled ciliates to greater than 35 kb in some species of mice (Zakian, 1995). The length of these repetitive elements differs from cell to cell and chromosome to chromosome. In general, telomeres consist of a G-rich repeat that extends in the 5' to 3' direction towards the end of the chromosome and terminate with a single stranded 3' end. The exact composition of these repetitive elements varies from species to species. For example, *Tetrahymena* has a C_4A_2/T_2G_4 repeat, *Oxytrichia* has C_4A_4/T_4G_4 repeat, yeast has a $C_{1-3}A/TG_{1-3}$ variable repeat, and vertebrates have a T_2AG_3/C_3TA_4 repeat (Greider, 1995). The exception to the simple repetitive elements occurs in *Drosophila* where telomeres were first discovered. Instead, *Drosophila* have

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transposable elements which can be lost and added on to chromosome ends (Sheen and Levis, 1994).

In addition to their role in stabilizing chromosomes, telomeres allow for the complete replication of the 3' lagging strand during DNA replication (Watson, 1972). Most organisms are able to complete the replication of their chromosomes using a specialized DNA polymerase (Greider and Blackburn, 1985). To facilitate the complete replication of DNA, the 3' G-tail is extended by telomerase, an activity that adds telomeric DNA onto the end of the chromosome. One consequence of the method used by cells to maintain telomere length, is the generation of a 3' overhang that the cell could detect as a broken DNA end. To avoid detection as a broken DNA end, special telomere binding proteins form a non-nucleosomal structure that functions to protect the terminal end (Henderson, 1995). Also in some organisms the non-nucleosomal structure correlates with another telomere function, the ability to repress the transcription of nearby genes known as telomere position effect or TPE. TPE is a metastable event resulting in genes switching back and forth between transcriptionally active and inactive states (Gottschling *et al.,* 1990). Gene repression due to TPE is continuous from the chromosome end and occurs independently from chromosome to chromosome in the same cell (Shore, 1995).

Significance of Studying Telomeres

It is important to study telomeres because the regulation of their length has possible implications in aging therapy (Harley *et al.*, 1990). In human somatic cells there is little or no telomerase activity and consequently telomeres get shorter as cells replicate

(Kim *et al.*, 1994). Furthermore, somatic cells from older individuals have shorter telomeres than younger individuals (Harley *et al.*, 1990; Hastie *et al.*, 1990). This inability to maintain telomere length limits the proliferation of cells ultimately leading to senescence (Harley, 1991). Additional support for the loss of telomere length comes from experiments showing that somatic cells from human newborns usually divide in culture for many generations whereas cells from individuals later in life only divide 20 to 30 times (Hayflick, 1965). During somatic cell division telomeric sequences become shorter because most human somatic cells lack telomerase (Kim *et al.*, 1994). Furthermore in experiments where the telomerase genes were expressed ectopically in human cells, the cellular lifespan was dramatically increased (Hahn *et al.*, 1999). Taken together, these data strengthen the idea that telomeres are an essential cellular marker for aging.

The link between telomere length and aging, is supported by studies involving individuals with premature aging diseases. For example, fibroblasts from patients with an accelerated aging syndrome known as Hutchinson-Gilford progeria show decreased telomere length compared to normal fibroblasts of age-matched individuals (Allsopp *et al.*, 1992). Patients with trisomy 21 (Down's syndrome) also show some signs of premature aging. Interestingly, the rate of telomere loss is three times higher in the blood lymphocytes of trisomy 21 patients (Vaziri *et al.*, 1993). Another disease known as Ataxia Telangiectasia is characterized by immunodeficiency, neurodegeneration, genomic instability, and premature aging (Sedgwick and Boder, 1991). This premature aging is likely due to shortened telomeres that result in a large number of telomere fusions (Rotman and Shiloh, 1997). Finally, patients suffering from Werner's Syndrome,

another disease characterized by premature aging, have shorter telomeres that result from the fragmentation and accumulation of repetitive DNA sequences (Guarente *et al.*, 1998).

One possible therapy for individuals with progeria diseases focuses on lengthening telomeres in normal somatic cells as a means of avoiding cellular senescence. By either increasing telomerase activity or introducing additional telomere sequences, telomere length could be extended in cells from those patients. With longer telomeres, somatic cells could undergo more cell divisions before death, and slowing down cellular senescence could therefore slow down the premature aging process (Harley *et al.*, 1990). Currently, normal somatic cells have been designed to express ectopic copies of hTERT gene, the catalytic subunit of telomerase, *in vitro* leading to the bypass of senescence and the extension of viability in culture (Bodnar *et al.*, 1998).

Unlike normal somatic cells, most tumor cells do have detectable telomerase activity (Kim *et al.*, 1994). The presence of telomerase activity within tumor cells enables those cells to maintain telomere length, albeit at a length shorter than wild-type cells from the same tissue. The correlation of stable telomere length and tumorogenesis strongly suggests that telomeres are critical in maintaining the replicative potential of tumor cells. In a survey of different cancers, greater than 80% show stable telomere length due to telomerase activity. Thus if telomerase activity was eliminated from these tumor cells, a cancerous cell may no longer be able to maintain its telomere length. Furthermore, loss of telomere length may result in the end of cancer cell proliferation (de Lange, 1995).

Limiting telomere length could be a useful therapy for destroying tumor cells. Telomere lengths and rate of loss vary depending on the type of cancer (Mehle *et al.*,

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1994). In cancer cells, stable telomere length allows cancerous cells to proliferate indefinitely and are a critical step in tumor formation. In approximately 80% of human tumor cells, telomerase activity is present (Kim *et al.*, 1994). Specifically, reactivation of telomerase may increase cell life span promoting immortality during oncogenesis leading to the progression of tumor cells with stable telomeres (de Lange, 1995). In support of this theory, the majority of human tumor and transformed cell lines express telomerase (Kim *et al.*, 1994).

Even though telomere length is not equal in all forms of cancer, telomere shortening can act as a tumor supressor mechanism. This proposed tumor supressor mechanism is based on the observation that cells lacking protective telomere caps can activate DNA damage checkpoints. The activation of these checkpoints could stop cellular proliferation leading to cellular death (de Lange, 1995). Thus, one possible tumor therapy would be to severely limit the length of telomeres via inactivation of telomerase. By limiting telomere replication, telomere length would shorten. The short telomeres would reach a critically short length that would signal the cancerous cell to stop dividing and thereby shrink and eventually eliminate the tumor.

Yeast Cells as a Model for Telomere Research

Due to the significance and interest in both aging and cancer research, telomeres have become a chromosomal structure in need of further investigation. *Saccharomyces cerevisiae* is an important model organism for telomere research. *S. cerevisiae* is a preferred organism to work with in the laboratory because of a wealth of preexisting genetic knowledge, including the complete genomic sequence (*Saccharomyces cerevisiae*

genome database). In *S. cerevisiae*, telomerase is constitutively expressed and telomere length is maintained during cell division, which is similar to cancer cell characteristics (Kass-Eisler and Greider, 2000). However, when functional telomerase is inactivated in yeast cells, telomere loss occurs (Lundblad and Blackburn, 1993). Furthermore, yeast chromosomes that have had telomeres surgically removed with a site specific endonuclease are lost at a high rate (Sandell and Zakian, 1993). Chromosomes missing a single telomere are maintained for several generations before they are lost. Additionally, cells without functional telomerase can still divide for several generations and telomerase-independent survivor cells are produced (Lundblad and Blackburn, 1993).

Telomere Replication via Telomerase

One major role of telomeres is to facilitate the end replication problem diagramed in Figure 1. The end replication problem results from the primer requirement of conventional DNA replication (Watson, 1972). During replication of the lagging strand at the end of the newly synthesized 5' strand, the terminal RNA primer is removed to reveal a gap. This gap, which cannot be repaired by conventional DNA polymerase, results in the loss of DNA every time it is replicated (Zakian, 1997). Eventually, the inability to maintain telomere length can diminish cell viability by limiting proliferation (Harley, 1991)

The primary pathway for telomere replication requires telomerase, a ribonuclear protein that adds telomeric DNA onto the ends of chromosomes (Greider, 1995).
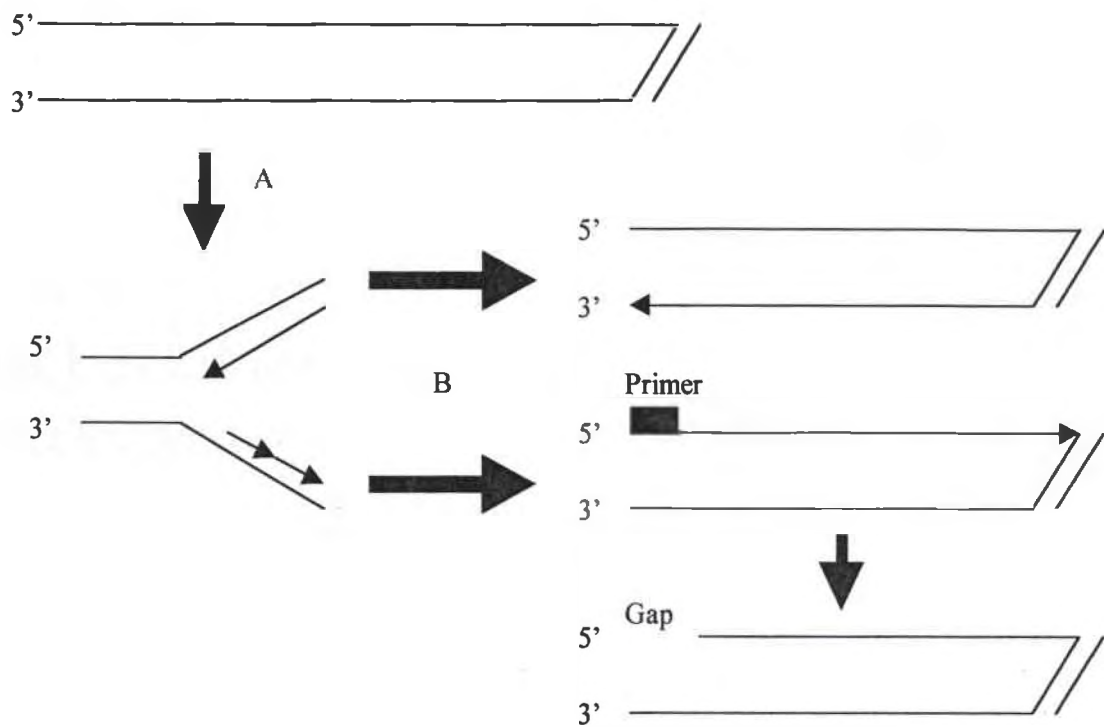
FIGURE 1. Telomere Replication. The replication of one end of the chromosome is shown above. DNA replication occurs after primase binds to the DNA, creating an RNA primer and then replicates the DNA on both the leading and lagging strands (A). Because DNA can only replicate in the 5' to 3' direction and a primer is needed, the lagging strand contains a gap after primer removal (B). This gap cannot be repaired by conventional DNA polymerase, and as a result DNA is lost after every round of replication.

First discovered in *Tetrahymena*, telomerase contains both an essential RNA subunit that provides a template for telomere synthesis and a protein subunit that catalyzes telomere additions (Greider and Blackburn, 1985). Since its discovery in *Tetrahymena*, telomerase has been found in many organisms indicating that it is conserved throughout nature. Telomerase prevents the loss of DNA by extending the 3' end of DNA through reverse transcriptase activity (Figure 2). Subsequent to telomerase activity the conventional DNA replication apparatus can complete synthesis of the 5' to 3' strand. Whether primase comes in and synthesizes a primer or a terminal hairpin forms that acts as a primer for DNA synthesis is currently being investigated. Either way, telomerase activity completes replication of the chromosome (Zakian, 1997).

Additional Telomere Replication Pathways

In addition to the major telomere replication mechanism, there are at least two other pathways for telomere replication. In most cells the major pathway involves the ribonuclear protein telomerase, which adds telomeric sequences to the ends of the telomeres without a DNA template (Greider, 1995). In *Drosophila*, telomere length is thought to be maintained by an RNA transposition intermediate that is converted into DNA by reverse transcriptase (Pardue, 1995). Recombination is another pathway that exists independent of telomerase based telomere replication (Lundblad and Blackburn, 1993). Studies on the mosquito *Anopheles gambiae*, where there is no evidence of telomerase activity, suggest that only a homologous recombination based mechanism is used to maintain telomere length (Roth *et al.*, 1997). Little is known about the
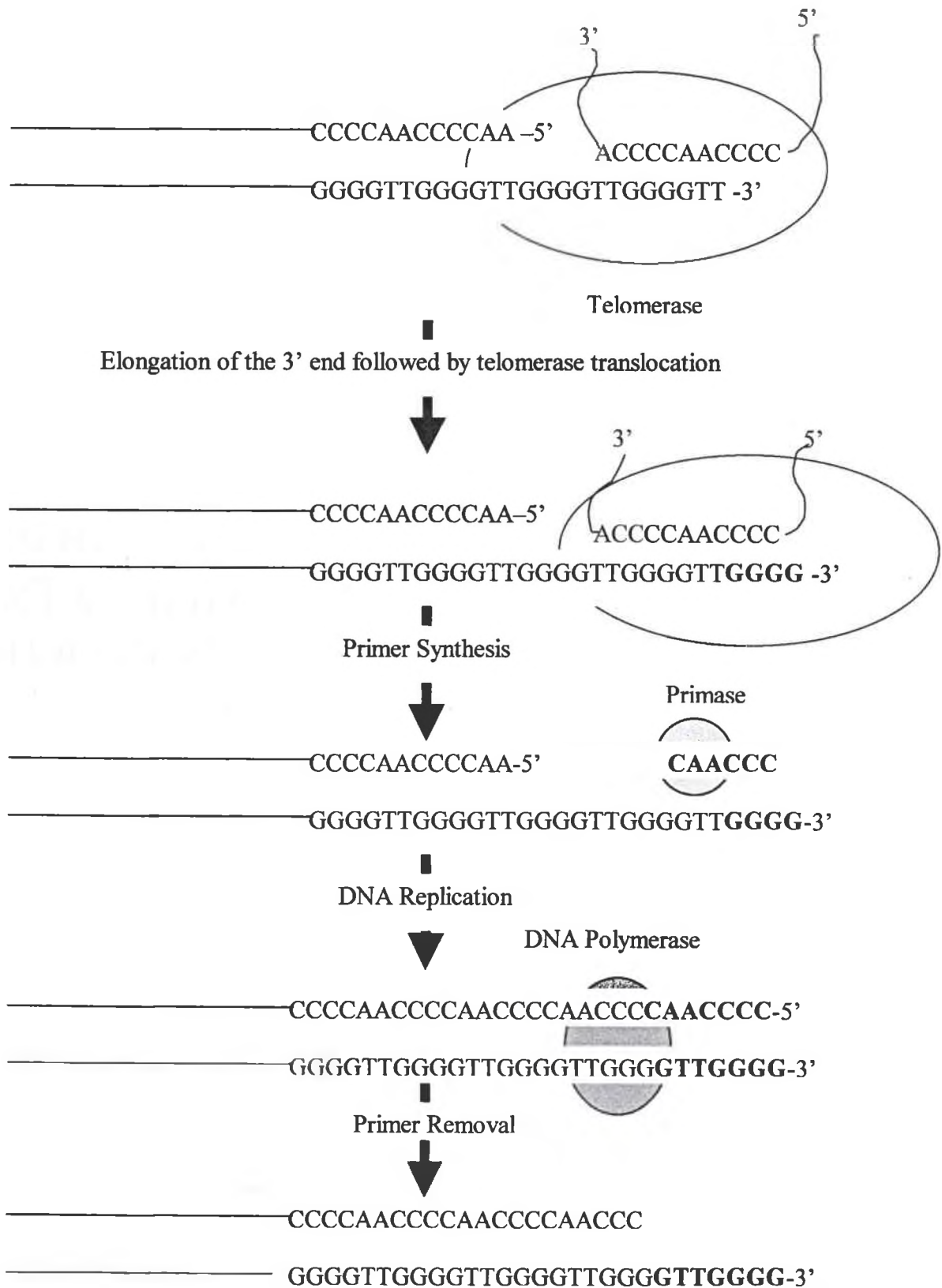
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FIGURE 2. Telomerase Functions. Telomerase binds to the gap left behind by the primer removal. Next, telomerase extends the 3' end by reverse transcriptase activity and translocates to extend the 3' end. Primase followed by DNA polymerase fills in the remaining gap. Finally, the primer is removed to reveal the 3' overhang.

mechanism of homologous recombination as a means of maintaining telomere length. Additional evidence suggests that recombination may occur in the presence of functional telomerase leading to further questions about the role of telomere recombination in wild-type cells (Kass-Eisler and Greider, 2000). By understanding the proteins that participate in the recombination pathway both in the presence and absence of telomerase, therapies that either enhance or inhibit recombination can be developed and ultimately applied to aging or cancer therapies. Also, studying the recombination pathway may determine if therapies that focus on telomerase can be overridden by a by-pass recombination mechanism for telomere replication.

Telomere Length Regulation

Telomere length is maintained through the complex interaction of different genetic pathways. In both human and yeast cells from different species a conserved set of proteins have been identified that are critical to maintain wild-type telomere length (Fang and Cech, 1995). There are several yeast proteins that regulate telomere length in the presence of active telomerase (Table 1). The key players involve several proteins including *RAP1, CDC13, HDF1, TEL1,* and a *RAD52* epistasis group including *RAD50, MRE11,* and *XRS2* (Shore and Nasmyth, 1987; Nugent *et al.*, 1996; Feldmann and Winnacker, 1993; Lustig and Petes, 1986; Johzuka and Ogawa, 1995). Rap1p is the first protein identified to bind telomeres *in vivo* and is known to interact with at least four other proteins at the telomere (Shore and Nasmyth, 1987). *RAP1* regulates telomere length by nucleating the formation of a non-nucleosomal altered chromatin structure

TABLE 1. Summary of *Saccharomyces cerevisiae* telomere binding proteins and their affect on the telomere.

Telomere Position Effect

Increase in Telomere Silencing		Decrease in Telomere Silencing	Regulation
ARD1	SAS2	MEC1	GAL1
H4	SDS3		
MPT5	SET1		
RAP1	SIR3		
RIF1	SIR4		
RIF2	TEL2		
SAS10			

Telomere Length

Mutation Results in Shorter Telomeres	Mutation Results in Longer Telomeres	Overexpression Results in Shorter Telomere	Regulates Telomere Length
HDF1	PIF1	RIF2	RAD50
MRE11	RIF1		RAP1
TEL1	RIF2		XRS2
TEL2			

Non-homologous end joining pathway

HDF1
HDF2
MRE11
RAD50
XRS2

Transcriptional Silencing

SIR1
SIR2
SIR3
SIR4

Telomerase Components

CDC13
EST1
EST2
EST3
TLC1

Protection of Telomere Ends

HDF1
HDF2

Recombination

HPR1
RAD1
RAD52
Y'-Help1

(Lustig *et al.*, 1990). Overexpression of *RAP1* leads to longer more heterogenous telomeres (Conrad *et al.*, 1990). Telomere length control by *RAP1* requires other proteins encoded by the *RIF* genes (Hardy *et al.*, 1992). Recent data suggests that these proteins are involved in the recognition of the telomere unique sequence junction (Roy and Runge, 2000). Support for this universal mechanism comes from homologs to *RAP1* that exist in a wide range of species including humans (Li *et al.*, 2000). The cell cycle gene *CDC13* functions as a mediator of telomere access for telomerase *in vivo* (Evans and Lundblad, 1999). Cells lacking *CDC13* have a senescence phenotype identical to cells lacking telomerase (Lendvay *et al.*, 1996). Specifically, *CDC13* is believed to regulate the maintenance of the single stranded tail at the end of the telomere (Wellinger *et al.*, 1996). Cells mutant for *CDC13* have a senescence phenotype due to the fact that *CDC13* has been invoked as important for providing telomerase access to the telomere (Evans and Lundblad, 1999). Null *cdc13* mutants have unusually long (50 to 90 nucleotides) 3' overhangs in the G-rich strand (Garvik *et al.*, 1995). Other mutations such as *hdf1* and *tel1* lead to the reduction of telomere length (Boulton and Jackson, 1996; Lustig and Petes, 1986). When an *hdf1* null mutation occurs, the telomeric tract is greatly reduced and is twice as heterogeneous suggesting increased sensitivity to nucleolytic degradation and to intrachromatid recombination (Polotnianka *et al.*, 1998). However after 30 to 45 generations the null mutant does not keep shortening telomeres, but rather establishes a stable shorter telomere (Fellerhoff *et al.*, 2000). Similar to *hdf1*, *tel1* mutants also result in a stable shorter telomere length. Specifically, the telomere tracts of *tel1* strains are approximately 50 base pairs long and result in about sevenfold shorter telomeres (Lustig and Petes, 1986). Null mutations in *tel1* have a higher rate of chromosome loss and

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meiotic recombination (Greenwell *et al.*, 1995). Finally, the *RAD52* epistasis group functions in genetic recombination and the recombinational repair of double stranded breaks (Sung, 1997; Ogawa *et al.*, 1995). Mutations in some genes in the *RAD52* epistasis group, including *RAD50*, *MRE11*, or *XRSII*, result in telomere shortening to the same extent as seen in *tel1* mutants. However, mutations in *RAD52* alone does not alter telomere length (Ritchie and Petes, 2000).

Telomere length is also regulated around a genetically defined equilibrium through telomere rapid deletion (TRD) events (Li and Lustig, 1996). In cells with artificially elongated telomeres, TRD is a recombination based mechanism used to shorten telomere length back to wild-type. The genetic requirements for TRD are different than those for recombination between $C_{1-3}A$ direct repeats suggesting that there are two-different recombination pathways (Li and Lustig, 1996). The relationship between $C_{1-3}A$ direct repeat recombination and TRD is unknown regarding both the initiation and the exact pathway of recombination (Stavenhagen and Zakian, 1998). TRD involves the *RAD52* epistasis group and is repressed by the *HPR1* gene product (Li and Lustig, 1996). TRD shortens telomeres to wild-type lengths at a faster rate than predicted for sequence loss due to replication (Kass-Eisler and Greider, 2000).

Regulation of Telomere Recombination in Cells Lacking Telomerase

The presence of non-telomerase based telomere replication in nature suggests that multiple pathways can function to replicate telomeres. Recently, data from human cells suggests that in some circumstances both tumor cells and immortalized cell lines can replicate telomeres in the absence of telomerase (Kass-Eisler and Greider, 2000). Some

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immortalized human cell lines do not have any detectable telomerase activity, but have varying telomere lengths ranging from very short to long (Bryan *et al.*, 1995). In the absence of telomerase, these immortalized cells use another pathway known as the Alternative Lengthening of Telomeres (ALT) (Bryan and Reddel, 1997). The ALT pathway was initially identified in human tumor cells that were able to maintain telomere length in the absence of telomerase (Bryan *et al.*, 1995). Immortalized cells replicating telomeres by ALT contain a microorganelle that interacts with an antibody to *RAD52* suggesting that ALT may be a recombination based pathway for telomere replication (Yeager *et al.*, 1999). So far, all immortalized cell lines either have telomerase activity or show evidence for the ALT pathway. The repression of ALT in telomerase negative cells leads to the loss of telomeric tracts. Alternatively, the reactivation of ALT leads to the rapid relengthening of telomeres (Perrem *et al.*, 1999). Thus in telomerase negative immortalized cells, ALT is the sole mechanism of telomere replication.

In yeast cells, removal of telomerase activity by mutation leads to loss of cell viability and eventual cell death. In cells lacking telomerase, a small fraction of cells are able to survive and return to wild-type growth (Lundblad and Blackburn, 1993; Singer and Gottschling, 1994; Lendvay *et al.*, 1996). In these survivors, a recombination dependent telomere replication pathway is required for the return to wild-type growth (Singer and Gottschling, 1994; Lendvay *et al.*, 1996). One possible explanation for a delay in cell death when telomerase is eliminated is the presence of a structure at the end terminus of the telomere. This structure may continue to be maintained without telomerase via proteins that assemble a protective telomere cap (Wellinger *et al.*, 1996). Within each successive cell division telomeres become shorter, eventually one telomere

or many telomeres reach a critically short telomere length resulting in cell death. Telomerase-independent survivors are viable and able to survive for many generations, because telomere synthesis via homologous recombination is a feasible option for cells. One possible reason recombination does not restore telomere length in all cells from a given culture is due to the ability of telomere loss to outpace telomere replication by recombination.

In *S. cerevisiae,* telomerase deficient survivors have been identified that recombine both the terminal telomeric elements and middle repetitive subtelomeric elements which are found more proximal to the centromere. These subtelomeric regions are known as X and Y' (Chan and Tye, 1983). Both X and Y' are found in yeast cells but are not necessary for life. The Y' elements are highly conserved and are present in both long and short forms, 6.7 or 5.2 kb, respectively (Louis and Haber, 1992). Of the two elements, X is less conserved, more heterogeneous, and ranges in length from 0.2 to 3 kb (Louis *et al.*, 1994).

Recombination between subtelomeric sequence elements leads to two different types of survivors in *S. cerevisiae*. The differences between the two survivors is indicated by genomic Southern blots hybridized to Y' DNA and growth rate differences. Type-I survivors are generated by gene conversion events between the Y' elements (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). Gene conversion events result in survivors with short terminal telomere repeats and slow growth. Alternatively, type-II survivors consist of long heterogeneous tracts of telomeric repeats. These heterogeneous tracts are likely due to gene conversion events between telomere repeat sequences (Kass-Eisler and Greider, 2000).

A recombination pathway in telomerase-negative survivors has been found in several types of yeast. In *S. cerevisiae, EST1* codes for a protein required to load telomerase onto the telomere (Evans and Lundblad, 1999). In the absence of *EST1*, cells lose telomere length, an event that correlates with cellular senescence and cell death. But the ability of some *est1* cells to survive reveals evidence for a recombination pathway that restores cell viability and telomere function (Lundblad and Blackburn, 1993). These *est1* survivors have very short telomeric tracts and long tandem arrays of middle repetitive elements that are due to a *RAD52*-dependent homologous recombination event (Lundblad and Blackburn, 1993). Survivors have been identified in yeast harboring mutations in four other genes required for telomerase activity, *in vivo* (Lundblad and Szostak, 1989; Lendvay *et al.*, 1996). In the budding yeast *Kluyveromyces lactis*, the *TER1* gene encodes for the RNA component of telomerase (McEachern and Blackburn, 1996). When *TER1* is eliminated, *K. lactis* cells have a decreased growth rate and increased senescence (McEachern and Blackburn, 1996). Investigation of the surviving sub-population reveals that they utilize a recombination based alternate pathway for telomere replication as a functional substitute for telomerase (McEachern and Blackburn, 1996). A back-up recombination pathway has also been shown in the fission yeast *Schizosaccharomyces pombe* (Nakamura *et al.*, 1998). When the telomerase catalytic subunit, *TRT1,* is eliminated in *S. pombe*, a small surviving sub-population reveals amplified telomere associated sequences (Nakamura *et al.*, 1998). These amplified sequences are similar to the amplified sequences found in budding yeast that are utilizing recombination-based survival (Nakamura *et al.*, 1998).

The majority of homologous recombination in yeast is facilitated through a group of genes known as the *RAD52* epistasis group. The *RAD52* epistasis group is responsible for the recombination based telomere replication in telomerase deficient survivors. Specifically, many yeast telomerase negative survivors are dependent on the *RAD52* epistasis group (Lundblad and Blackburn, 1993). The *RAD52* epistasis group repairs double-strand breaks in addition to being involved with homologous recombination (Freidberg, 1988). This epistasis group is composed of at least two pathways that affect recombination and generate survivors in the absence of telomerase activity (Le *et al.*, 1999). The first pathway includes *RAD51, RAD54,* and *RAD57,* which facilitates repair primarily through gene conversion events (Rattray and Symington, 1995). A second pathway composed of *RAD50, XRS2,* and *MRE11* works to facilitate telomere recombination. In addition to a role in homologous recombination, this complex is involved in the non-homologous end-joining pathway (NHEJ) (Haber, 1998).

Telomere Direct-Repeat Recombination

The research presented in this thesis specifically focuses on the influence of telomere length on internal direct-repeat recombination. Because it is difficult to measure recombination events between natural telomeres on the ends of different chromosomes, a recombination cassette has been developed for *S. cerevisiae* (Figure 3). This cassette is integrated 5 kb from the end of a chromosome VIIL and allows recombination events to be monitored through calculating the rate of *URA3* loss. Surrounding the *URA3* marker are non-tandem direct repeats that contain either $(C_{1-3}A)_n$ yeast experimental DNA or $(C_4A_2)_n$ *Tetrahymena* control DNA. Previously, this cassette

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system was integrated at different distances (~5kb, ~20 kb, and ~200 kb) to the telomere to determine if yeast telomeres exert a position effect on direct internal recombination. Telomere position effect on direct repeat recombination was observed between identical tracts of $C_{1-3}A$. The repression of direct repeat recombination is different than the gene silencing that occurs at telomeres as recombination between $C_{1-3}A$ tracts and is not dependent on the Sir protein complex, a complex required for TPE (Stavenhagen and Zakian, 1998; Aparicio *et al.*, 1991). The decrease in recombination is due to an absence of *RAD52*-dependent recombination and a reduced level of another independent recombination pathway known as *RAD1*. *RAD1* recombination occurs via single strand annealing (SSA). SSA is a non-conservative pathway for recombination between direct-repeats which eliminates DNA between repeats (Lin *et al.*, 1984; Fishman-Lobell *et al.*, 1992). TRD events are regulated negatively by *SIR3* and are *RAD1* independent (Li and Lustig, 1996). Furthermore, it was proposed that the $C_{1-3}A$ internal tracts form a Rap1p-mediated teleosome-like chromatin structure (Stavenhagen and Zakian, 1998). Support for this structure also comes from the silencing activity of internal tracts which increases as these tracts gets closer to the telomere. Overexpression of Rap1p in strains harboring direct repeats enhanced recombination between both internal $C_{1-3}A$ tracts and internal tracts at the natural telomere. Thus the Rap1p mediated structure does not repress direct repeat recombination (Stavenhagen and Zakian, 1994).

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Role of *HDF1* in Telomere Function

Through studies involving internal telomere repeat sequences, direct repeat recombination rates have been determined for wild-type yeast strains harboring either $C_{1-3}A$ or C_4A_2 internal sequences (Stavenhagen and Zakian, 1998). Because the wild-type recombination rates have been determined, the next direction would be to ask does altered telomere length lead to an increased rate of recombination, and if it does, what is the effect on telomere structure? To answer this research question, *HDF1* was eliminated in yeast cells harboring the internal repeat cassette. *HDF1* was chosen, because its elimination results in shorter telomeres (Porter *et al.*, 1996). The Hdf1p binds to the end of the chromosome and has been proposed to function as a protective cap preventing digestion by nucleolytic enzymes (Table 1) (Boulton and Jackson, 1996). *HDF1* is the yeast homolog to the mammalian *Ku70* gene that is part of the heterodimer complex that binds to DNA ends. Human *Ku70* was originally identified as an autoimmune antigen that has a high affinity for DNA ends (Dyan and Yoo, 1998). Mammalian cells without *Ku70* cannot repair double-stranded breaks or perform V(D)J recombination (Boulton and Jackson, 1996). *HDF1* serves several functions in a yeast cell including repairing double-stranded breaks, binding to the telomere, and localizing the telomeres to the nucleus.

In yeast *HDF1* functions in recombination by the non-homolgous end joining pathway (NHEJ) (Critchlow and Jackson, 1998). NHEJ is a minor recombination pathway in yeast, but it is evolutionary conserved and is a major recombination pathway in mammalian cells (Boulton and Jackson, 1996). When double strand breaks occur, *HDF1* binds the ends and functions as part of a complex to mediate recombination

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(Leiber *et al.*, 1997). At the site of the double stranded break, *HDF1* recruits the Sir protein complex which is required for non-homologous end joining in yeast (Table 1). It has been suggested that the *sir-hdf* complex forms a heterochromatin-like state, so the DNA can be repaired without inappropriate gene expression (Jackson, 1997). In addition to the *sir-hdf* complex, the *RAD52*, *XRS2*, and *MRE11* genes are necessary for NHEJ (Lewis *et al.*, 1999).

Additionally, telomere length maintenance also requires functional *HDF1*. At the telomere, *HDF1* protects the chromosomal termini from nucleolytic attack (Boulton and Jackson, 1996). When *HDF1* is eliminated from the yeast genome, telomeres are more susceptible to enzymatic damage and dramatically decrease in size (Porter *et al.*, 1996). Cells harboring a mutation in *HDF1* show reduced ability to repress transcription of genes located near the telomere indicating that *HDF1* is important for forming the heterochromatin-like state of the telomeric DNA (Jackson, 1997).

HDF1 is also involved in nuclear localization. Telomeres are thought to be located in the nuclear periphery at specific stages in the cell cycle. This localization organizes the chromosome domains into subcompartments in the nucleus (Zakian, 1996). *HDF1* was the first gene identified that acts directly in aspects of telomere organization and affects both the repression of telomere proximal genes and the subnuclear localization of telomeres (Laroche *et al.*, 1998). Through *HDF1,* the ends of telomeres interact with nuclear membrane pores (Galy *et al.*, 2000). *MLP1* and *MLP2* are proteins located at the interface between the nuclear envelope and the nuclear interior. Phenotypes resulting from mutations in *MLP1* and *MLP2* mimic that seen in *hdf1* strains. Both *MLP1* and *MLP2* are involved in the clustering of perinuclear telomeres.

Specifically *MLP2* physically tethers *HDF1* to the nuclear periphery linking chromatin to the nuclear envelope (Galy *et al.*, 2000).

Role of Telomere Length in Determining the Status of $C_{1-3}A$ Direct-Repeat Recombination

To determine if $C_{1-3}A$ direct-repeat recombination is under the direct influence of telomere length, mutations that lead to changes in telomere length were made in cells harboring cassettes of direct repeats of telomeric DNA flanking the *URA3* gene (Figure 3). In wild-type cells, telomeric $C_{1-3}A$ direct repeat recombination is repressed when compared to other direct repeat control substrates. This thesis proposes that the rate of $C_{1-3}A$ direct repeat recombination may be under the influence of telomere length. Direct-repeat recombination between $C_{1-3}A$ tracts and C_4A_2 tracts were examined in strains containing shorter telomeres and in strains harboring longer telomeres.

The results from the experiments in this thesis are summarized below. $C_{1-3}A$ direct recombination in *hdf1Δ* cells show an increase in when compared to wild-type. In contrast, recombination rates were similar between C_4A_2 direct repeats in wild-type and *hdf1Δ* cells. The mechanism of the internal direct-repeat recombination was further analyzed by additional mutations in recombination pathways in *hdf1Δ* strain backgrounds. Both *RAD1* and *RAD52* pathways were investigated in *hdf1Δ* cells. *RAD1* and *RAD52* are two alternative pathways that facilitate recombination in yeast. These two

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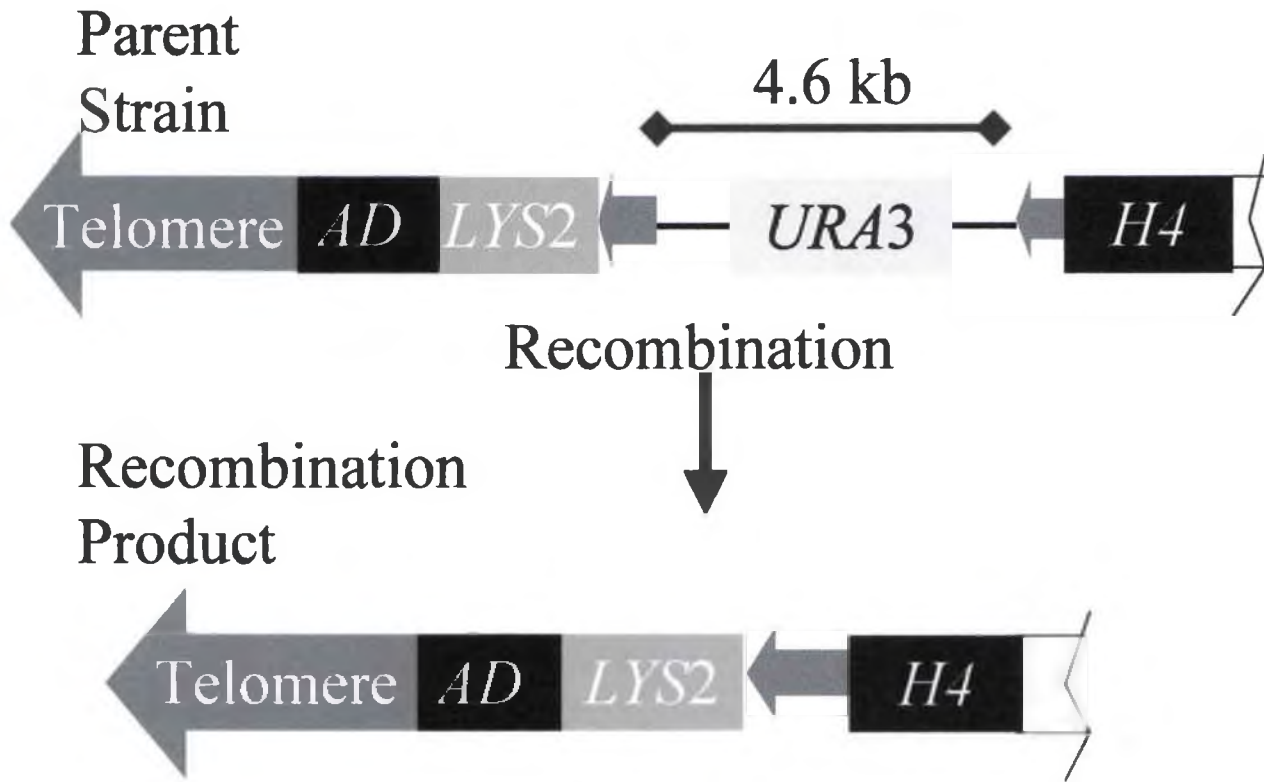
FIGURE 3. Recombination assay for measuring direct-repeat recombination. This assay monitors the loss of *URA3* in both C_4A_2 control and $C_{1-3}A$ experimental internal sequences. Both $C_{1-3}A$ and C_4A_2 direct repeats are integrated at 5kb from the end of the telomere on chromosome VIIL between the disrupted *ADH4* gene. The direct repeats are identical, approximately 300 base pairs in length, and are separated by a 4.6 kb segment containing the *URA3* gene surrounded by yeast plasmid DNA.

independent pathways are involved in telomere maintenance through recombination events between direct repeats (Rattray and Symington, 1995). Both recombination pathways share the same intermediate, so cells can shuttle between the two pathways. However, recombination is eventually resolved through two different mechanisms. Recombination through a *RAD52*-dependent pathway occurs through strand invasion after a pop-out deletion event (Petes *et al.*, 1991). Alternatively *RAD1* recombination occurs after single strand annealing (SSA), where endonucleases remove DNA until there are homologous sequences to facilitate recombination (Lin *et al.*, 1984; Fishman-Lobell *et al.*, 1992). Previous results have shown that recombination between $C_{1-3}A$ DNA in wild-type cells is greatly inhibited when the tracts are near the telomere. The reduction in recombination rate is due to a lack of *RAD52*-recombination and a reduction in the number of *RAD1*-dependent events (Stavenhagen and Zakian, 1998). In our strain background, *hdf1Δ* cells showed a preference for *RAD1*-dependent recombination. Finally, Southern blot analysis was used to visualize the influence of recombination on telomere structure in *hdf1Δ* cells.

As a means of comparison to *hdf1Δ* shorter telomeres strains, recombination rates were also measured in cells with long telomeres. To determine if long telomeres also have an increased rate of direct-repeat internal recombination, three different strains were constructed. These backgrounds include cells transformed with a plasmid containing 800 bp of excess $C_{1-3}A$ DNA (+100 bp of telomeric DNA), and two strains with either a *pif1-m2Δ* (+75 bp of telomeric DNA) mutation or *rif1Δrif2Δ* (+800 to 1000 bp of telomeric DNA) double mutations (Gottschling *et al.*, 1990; Schulz and Zakian, 1994; Hardy *et al.*, 1992; Wotton and Shore, 1997) . Excess $C_{1-3}A$ DNA was introduced into the $C_{1-3}A$

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direct repeat strain using a plasmid with three copies of a natural telomere (Stavenhagen and Zakian, 1994). The introduction of excess $C_{1-3}A$ DNA is thought to titrate telomere binding proteins from endogenous telomeres resulting in a long telomere phenotype (Gottschling *et al.*, 1990). The second long telomere strain resulted from a *pif1-m2Δ* mutation at the second methionine of the *PIF1* gene. This mutation has been shown to result in telomeres that are longer than wild-type because of changes in a telomere maintenance pathway (Schulz and Zakian, 1994). *RIF1* and *RIF2* code for two proteins that interact with Rap1p and are important for regulating telomere length (Sussel and Shore, 1991). Current models suggest that *RIF1* and *RIF2* compete with *SIR* proteins for access to Rap1p. Double mutants *rif1Δrif2Δ* have extremely long telomeres that can be as long as 4 kb (Hardy *et al.,* 1992; Wotton and Shore, 1997). The data from cells containing long telomeres showed that there is a modest change in recombination compared to wild-type length. This suggests that long telomeres have less of an influence than shorter telomeres on recombination between $C_{1-3}A$ repeats.

MATERIALS AND METHODS

Yeast Transformation

To transform a yeast strain with a fragment or plasmid, 5 ml tube of selective or complete media was inoculated with a yeast colony and grown overnight at 30^0 C. After centrifuging and washing with water, cells were resuspended in 10x TE and 1 M Lithium Acetate. The cells were centrifuged again and then resuspended in an additional milliliter of 10x TE and 1 M LiAc. The cells were placed in 30^0 C for an hour. At this point the cells were competent and were placed at 4^0 C overnight to enhance transformation efficiency. Ten microliters of plasmid or PCR amplified DNA, and 3 µl of carrier salmon sperm were added to 100 µl of the competent cells. The cells were vortexed briefly and placed at 30^0 C for 30 minutes. Seven hundred microliters of 50% PEG 4000, 1 M LiAc, and 10x TE were added. The cells were vortexed briefly and placed in 30^0 for 30 minutes. The cells were then heat shocked at 42^0 C for 15 minutes. An additional 0.5 ml water was added and the cells were left at room temperature for 30 minutes. The cells were centrifuged and the liquid was aspirated with a pipette. The cells were resuspended in 250 ml of water and plated on selective media. The plates were incubated at 30^0 C until there was cell growth.

Strain Construction

All strains used in this study are outlined in Table 2. Yeast cells containing an

$hdf1::HIS3$ null mutation were constructed using the one-step gene disruption method

(Lorenz et al., 1995). This gene disruption method is shown in Figure 4. The $HDF1$

disrupted $HIS3$ gene was amplified by PCR. Reactions were run for 2 cycles of 5

minutes at 95^0 C, 30 seconds at 94^0 C, 30 seconds at 45^0 C, and 2 minutes of 74^0C. Next,

the reactions were linked to another program that ran for 30 cycles of 30 seconds at 94^0

C, 30 seconds at 50^0 C, 2 minutes at 74^0 C, a dwell, and 5 minutes at 74^0 C. The PCR

amplified product was then transformed into either C_4A_2 or $C_{1-3}A$ yeast strains. Putative

mutants were then confirmed by PCR, temperature sensitivity, and Southern blot. To

make double and triple knockout strains, yeast cells that were either $rad1\Delta$, $rad52\Delta$, or

$rad1\Delta rad52\Delta$ were mated with $hdf1\Delta$ cells. These cells were then sporulated and

haploids were selected according to the method shown in Figure 5.

The $C_{1-3}A$ direct repeat yeast strain with FAT-RS303'b'-TT containing ~800 bp

of $C_{1-3}A$ DNA was made through transformation with the FAT plasmid. This plasmid

contains three copies of a natural telomere (Stavenhagen and Zakian, 1994). In addition,

the plasmid contains a defective promoter allel of the $LEU2$ gene, $leu2-d$, which results in

100-200 copies of the plasmid when the strain is grown on media lacking leucine. This

excess $C_{1-3}A$ DNA is believed to titrate telomere binding proteins from endogenous

telomeres resulting in a long telomere phenotype (Gottschling et al., 1990)

The $C_{1-3}A$ direct repeat yeast strain harboring a $pif1-m2\Delta$ mutation was

constructed as described previously (Schulz and Zakian, 1994). Mutations in the $PIF1$

TABLE 2. Summary of Strains

Strain	Genotype	Source
YJS(YPH499)	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δl*	Sikorski and Hieter (1989)
YPH500	MATα version of YPH499	Sikorski and Hieter (1989)
C$_4$A$_2$ (TPVN)	YJS; *adh4*::pTPVN (1.5 kb from telomere VII-L)	Stavenhagen and Zakian (1998)
C$_{1-3}$A (YPVN)	YJS; *adh4*::pYPVN (1.5 kb from telomere VII-L)	Stavenhagen and Zakian (1998)
C$_4$A$_2$*hdf1Δ*	YJS; *adh4*::pTPVN *hdf1::HIS3*	This study
C$_{1-3}$A*hdf1Δ*	YJS; *adh4*::pYPVN *hdf1::HIS3*	This study
C$_{1-3}$A*rad1Δ*	YJS; *adh4*::pYPVN *rad1Δ*	Stavenhagen and Zakian (1998)
C$_{1-3}$A*rad52Δ*	YJS; *adh4*::pYPVN *rad52::LEU2*	Stavenhagen and Zakian (1998)
C$_{1-3}$A*rad1Δrad52Δ*	YJS; *adh4*::pYPVN *rad1Δrad52::LEU2*	Stavenhagen and Zakian (1998)
C$_{1-3}$A*hdf1Δrad1Δ*	YJS; *adh4*::pYPVN *hdf1::HIS3 rad1Δ*	This study
C$_{1-3}$A*hdf1Δrad52Δ*	YJS; *adh4*::pYPVN *hdf1::HIS3 rad52::LEU2*	This study
C$_{1-3}$A*hdf1Δrad1Δ rad52Δ*	YJS; *adh4*::pYPVN *hdf1::HIS3 rad1Δ rad52::LEU2*	This study
Excess C$_{1-3}$A	YJS; *adh4*::pYPVN pYJS-TTL::*HIS3*	Stavenhagen and Zakian (1994)
C$_{1-3}$A*pif1-m2Δ*	YJS; *adh4*::pYPVN *pif1m2::URA3*	Schultz and Zakian (1994)
C$_{1-3}$A*rif1Δ*	YJS; *adh4*::pYPVN *rif1::TRP1*	Hardy *et al.* (1992)
C$_{1-3}$A*rif2Δ*	YJS; *adh4*::pYPVN *rif2::HIS3*	This study
C$_{1-3}$A*rif1Δ rif2Δ*	YJS; *adh4*::pYPVN *rif1::TRP1 rif2::HIS3*	This study

PCR amplification of the disrupted *HDF*1 sequence
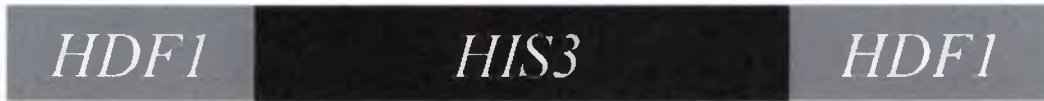
Creation of the *hdf*1 Δ strain by transformation
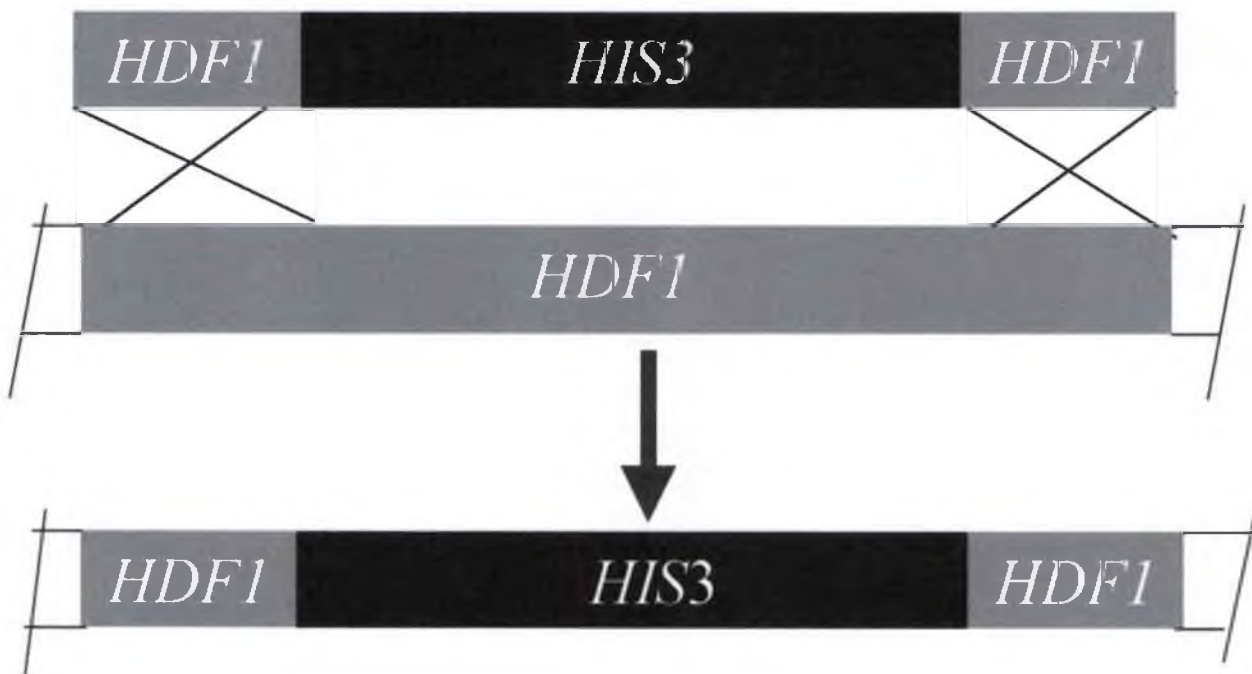
FIGURE 4. One-step gene disruption method. To create an *hdf1*::*HIS3* null mutant, an *HDF1* disrupted *HIS3* gene was amplified by PCR. The PCR amplified product was then transformed into either C_4A_2 or $C_{1-3}A$ yeast strains.

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Figure 5. Creation of double and triple knockout C_{1-3}A strains. To create double and triple mutants, *hdf1Δ* haploid cells were mated to *rad1Δ*, *rad52Δ*, or *rad1Δrad52Δ* cells, as indicated by the black arrow. The resulting diploid cells were then sporulated, as indicated by the gray arrow. Double or triple knockout haploid cells were selected according to appropriate markers.

gene leads to telomeres that are longer than wild-type due to changes in a telomere maintenance pathway (Schulz and Zakian, 1994).

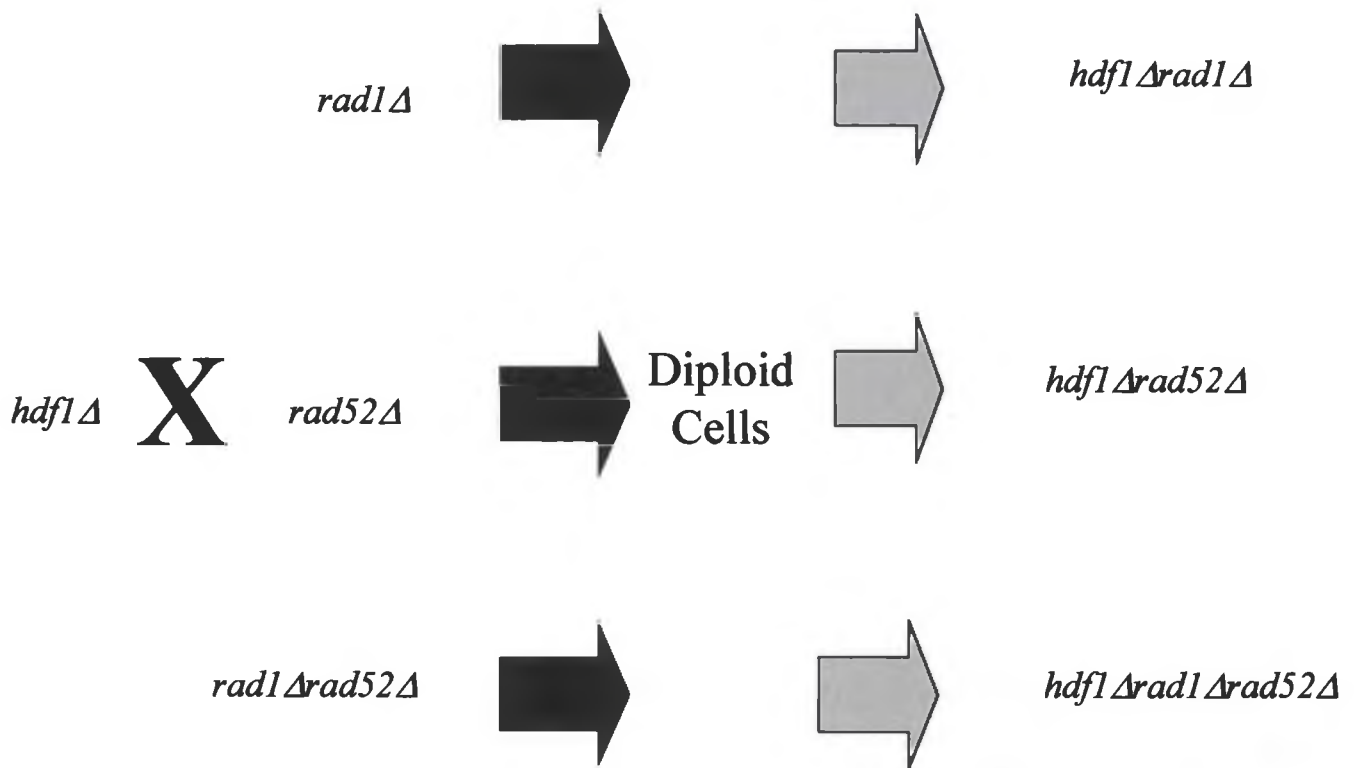Yeast cells containing the *rif1::TRP*1 mutation were constructed using the plasmid CH450 (Hardy *et al.*, 1992). Yeast cells containing the *rif2::HIS3* null mutation were constructed using the one-step protocol as previously described. The *rif1Δrif2Δ* double mutant was constructed by mating the *rif1Δ* and *rif2Δ* haploids, sporulating, and subsequently selecting a haploid containing both mutations. *RIF1* and *RIF2* code for two proteins that interact with Rap1p and are important for regulating telomere length (Sussel and Shore, 1991). Double mutants *rif1Δrif2Δ* have extremely long telomeres that can be as long as 4 kb (Hardy *et al.*, 1992; Wotton and Shore, 1997).

Recombination Assay

To determine the rate of direct repeat recombination in the previously described strains, a recombination assay was performed (Stavenhagen and Zakian, 1998). The recombination assay was performed on colonies from direct repeat recombination strains stored on YC-lys-ura plates to select for the presence of the direct repeat assay cassette. Colonies were then removed and struck for single colonies on YC plates lacking lysine. Figure 5 shows the recombination product that occurs when recombination excises *URA3* from the direct repeat cassette. Ten independent colonies from the YC-lys plates were picked, grown overnight in liquid YC-lys, and examined to determine the rate of $FOA^R Lys^+$ colonies. For the non-permissive temperature experiments, overnights in YC-lys liquid culture were grown for an additional four hours at $37^O C$. Aliquots from each of

the 10 colonies were spread on plates containing 5-fluoroorotic acid (FOA) and lacking lysine. FOA selects for ura⁻ cells. Cells from each colony were pooled and a viable cell count was performed to determine how many cells were present in each of the initial cultures. The FOA plates were then replica plated on YC plates missing uracil to make sure that *URA3* was in fact lost and growth on FOA plates was not due to *URA3* repression by telomere position effect (TPE). Also, in order to eliminate TPE in the recombination assay, cells were grown in the absence in lysine. When plates or liquid media does not contain lysine, the *LYS2* promotor is induced, transcription is driven towards the telomere, and TPE is no longer as significant. The rate for FOA^{R}Lys$^+$ colonies was calculated using the method of the median (Lea and Coulson, 1949). In each case the data reported as an average of two ten-colony fluctuations for two independently isolated mutants.

Yeast Genomic DNA Preparation

FOA^{R}Lys$^+$ cells were picked and genomic DNA prepared. Total genomic DNA was isolated by a glass bead DNA preparation procedure (Stavenhagen and Zakian, 1994). Yeast cells were grown in either selective or complete media overnight at 30^0 C. The cells were centrifuged at 2500 rpm for five minutes and the supernatant was discarded. The cells were washed in 5 ml of water and centrifuged for an additional 5 minutes. The supernatant was again discarded, the cells were washed in an additional milliliter of water, and transferred to a 1.5 ml eppendorf tube. The cells were centrifuged again and the supernatant was discarded. The cells were resuspended in 500 μl of yeast lysis buffer (0.1 M Tris-HCL pH8.0, 50 mM EDTA, 1% SDS). Sterile glass beads were

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added to each tube until they were approximately 1 mm from the meniscus of the cell-lysis suspension. The tubes were each vortexed at top speed for 30 seconds. Next, 50 µl of 5 M NaCl was added to each tube and vortexed for another 30 seconds. The liquid was removed from the tube using a P-1000 pipette tip, leaving the beads behind and redistributing the liquid into another eppendorf tube. Then 500 µl of a 1:1 phenol/chlorofom mixture was added to each tube. The tubes were vortexed for 30 seconds and microcentrifuged at 14,000 rpm for 5 minutes. The top aqueous layer was removed with a pipette and transferred into another eppendorf tube. A milliliter of isopropanol was added to each tube and then microcentrifuged at 14,000 rpm for 5 minutes. The supernatant was discarded, leaving behind a DNA pellet. To wash off excess NaCl, 500 µl of 70% ethanol was added to each tube and microcentrifuged for 5 minutes. The ethanol was discarded and this wash step was repeated two additional times. The remaining pellet was dried in a speed-vacuum until there was no more ethanol in the tube. The genomic DNA was then resuspended in 50 µl 1X TE.

Genomic DNA Digest

Genomic DNA from either parent strains or recombination events was digested with enzymes and bufferes from New England BioLabs. Twenty-five microliter digests were prepared by adding 5 µl of DNA, 2.5 µl of appropriate buffer, 1 µl of enzyme, and 11.5 µl of water. DNA was digested with XhoI for telomere blots of parent strains and BglII for FOA^{R}Lys$^+$ recombination events. To facilitate the enzymatic digest, tubes were incubated in a 37^0 C water bath overnight.

Southern Blot Gel and Transfer

One microliter of RNAse A and 3 μl of gel dye was added to each tube of digested DNA. The digested DNA was run on a 1% agarose gel. The gel was run overnight, stained with Ethidium Bromide, and photographed. The gel was washed with denaturation buffer (0.5 N NaOH, 1.5 M NaCl) for 30 minutes and then with neutralization buffer (0.5 M Tris pH 7.5, 1.5 M NaCl) twice for 15 minutes each. For the capillary transfer, a stack of thick absorbent paper soaked in 20X SSC was placed in Saran Wrap. One piece of thin paper was cut to the size of the gel and placed on top. The gel was positioned on top of the thin paper well side down. Nylon membrane (Amersham Hybond+) was cut to the size of the gel, soaked in 2X SSC, and placed on the gel. Two pieces of pre-cut thin absorbent paper were placed on top of the membrane followed by 3 additional thick sheets of paper. A weight was placed on top of the transfer to draw the 20X SSC upwards and facilitate the transfer of the DNA from the gel to the membrane.

Membrane Fixation and Hybridization

After transfer to a nylon membrane, the DNA was fixed by placing the membrane DNA side up on thick paper soaked in 0.4 M NaOH. After 20 minutes, the membrane was washed in 5X SSC. Next, the hybridization protocol was followed according to the directions in the Gene Images kit. The membrane was placed in a pre-hybridization solution (5X SSC, 0.1% SDS, 5% dextran sulfate, and a 20-fold dilution of liquid block) for 30 minutes at 60^0 C in a hybridization oven. After 30 minutes, a non-radioactive probe (Gene images Random Primer Labeling kit) was added to the membrane. The

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DNA probe fragment was diluted to a concentration of 2-25 ng/µl with sterile water. The diluted DNA sample was denatured by heating for 5 minutes on a 100^0 C heating block. The tube was then removed and placed on ice. In another eppendorf tube 10 µl of nucleotide mix, 5 µl of primer, 1 µl of Klenow enzyme, 50 ng of DNA, and water to a final volume of 50 µl was added. The mixture was incubated for 24 hours at 37^0 C and the luminescence of the probe was determined by comparing it to a reference strip. The probe for the $C_{1-3}A$ *hdf1Δ* telomere blot consisted of a $C_{1-3}A$ repeat telomeric DNA fragment. The probe for the $C_{1-3}A$ and C_4A_2 *hdf1Δ* recombination blots was derived from the 3' end of the interrupted *ADH4* gene (Figure 6). The C_4A_2 *hdf1Δ* recombination blot was then reprobed with a probe derived from the 5' end of the *LYS2* gene (Figure 6). The membranes were exposed to their respective probes overnight at 60^0 C.

Southern Blot Detection

Detection of the probed membrane was performed using the gene images CDP-STAR detection kit (Amersham). The hybridization buffer was poured out and the membrane was washed with two 15 minute stringency washes at 60^0 C. The first stringency wash was 1X SSC and 0.1% SDS and the second was 0.5X SSC and 0.05% SDS. After these washes, the membrane was removed from the hybridization oven. The buffer for the blocking, antibody, and Tween 20 wash solutions were all made with Buffer A (100 mM Tris-HCl, 300 mM NaCl, pH 9.5). First, a 10% blocking reagent in Buffer A was made with the reagent provided by Amersham. The blot was incubated in this blocking reagent (0.75-1.0 ml/cm^2) for one hour at room temperature. The antibody solution was made by preparing a 0.5% w/v of BSA fraction V was prepared in Buffer A
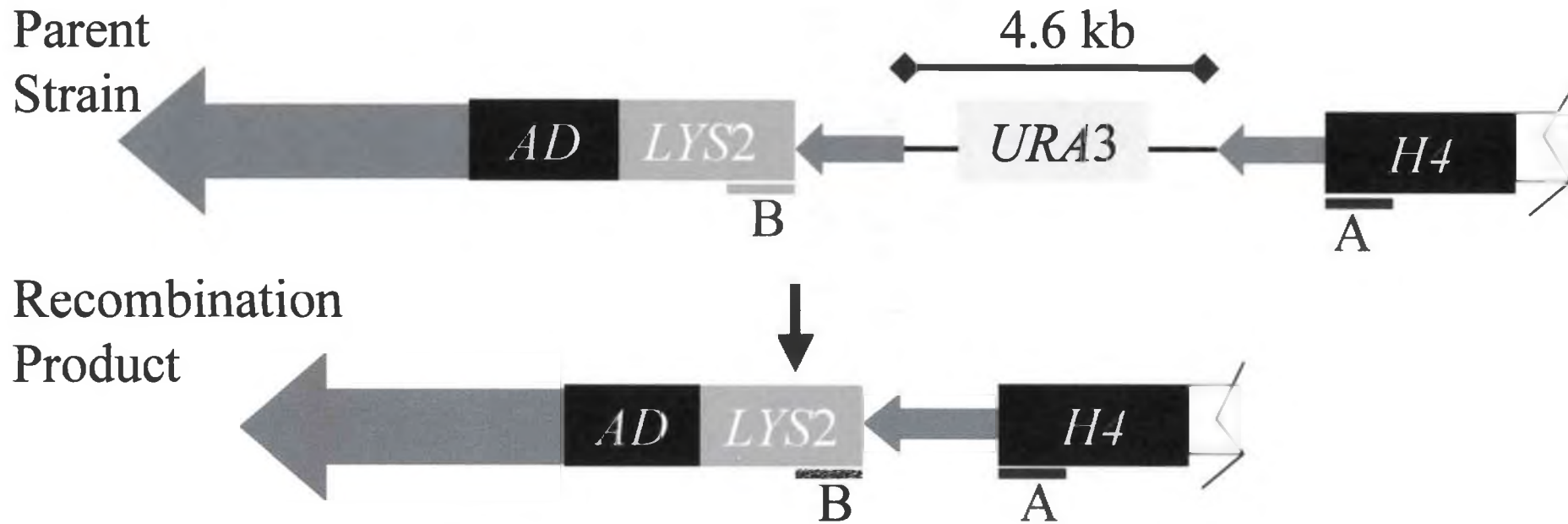
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FIGURE 6. Placement of the interrupted *ADH4* and the *LYS2* 5' probes. The recombination assay cassette consists of a telomere sequence (large gray arrow at the far left), and a disrupted *ADH4* gene. *ADH4* is disrupted by *LYS2* and either $C_{1-3}A$ (experimental) or C_4A_2 (control) internal sequence (smaller gray arrows). The direct repeats are identical, approximately 300 base pairs in length, and are separated by a 4.6 kb segment containing the *URA3* gene and yeast plasmid DNA. Both $C_{1-3}A$ and C_4A_2 direct repeats are integrated at 5kb from the end of the telomere on chromosome VIIL. The probe for the recombination Southern blots was derived from the 3' end of the interrupted *ADH4* gene as indicated above by a black bar (A). An additional probe derived from the 5' end of the *LYS2* gene was used to reprobe the C_4A_2 *hdf1Δ* recombination Southern blot. This *LYS2* 5' probe is indicated above with a gray bar (B).

and a 1:5000 dilution in a flourescein-AP conjugate. The membrane was incubated in the antibody solution for an hour at room temperature. Finally, the membrane was incubated in a 0.3% Tween 20 solution in Buffer A for 10 minutes. Incubation in the Tween 20 solution was repeated two additional times. Lastly, the CDP-Star detection reagent provided by the Amersham was pipetted over the blot and incubated for 2-5 minutes. The excess CDP-Star was shaken off and the blot was wrapped in Saran Wrap. The blot was placed in a cassette and exposed to a piece of X-ray film.

RESULTS

Analysis Using Direct Repeat Assay

Due to the repetitive nature of subtelomeric DNA at the terminal location and the lack of genetic markers, recombination events at the telomeres are difficult to detect. To overcome these difficulties in measuring telomere recombination, a direct repeat recombination cassette system was devised (Stavenhagen and Zakian, 1998). This cassette system is advantageous because it can be integrated at different chromosomal loci, is sequence specific, and allows both measurement of recombination rate and physical examination of the recombination products. In this experiment, the recombination cassette was integrated 5 kb from the *S. cerevisiae* VII-L chromosome and recombination was monitored through calculating the rate of *URA3* loss (Figure 3). The loss of *URA3* was determined on direct repeat cassettes harboring either C_4A_2 (*Tetrahymena* telomeric DNA) control or $C_{1-3}A$ (*Saccharomyces* telomeric DNA) experimental direct repeats. For all of these different strain backgrounds analyzed, recombination rates were measured using at least two ten-colony fluctuation assays from two independent strains.

Selective media was used to monitor recombination events in each strain. Selection for the loss of *URA3* occurs on 5-fluoro-orotic acid (FOA) plates lacking lysine. Specifically, FOA positively selects for cells lacking Ura3p because FOA is toxic to cells expressing *URA3* (Boeke *et al.*, 1987). In addition, cells are monitored on plates lacking

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lysine because expression of the *LYS2* gene blocks telomeric repression on transcription and prevents terminal deletions (Stavenhagen and Zakian, 1998). Using the cassette system, $FOA^R Lys^+$ cells can be generated by either interchromosomal recombination, intrachromsomal recombination, or sister chromatid exchange. Interchromosomal exchange occurs when the internal telomere tract is exchanged with the tract of another chromosome. In the cassette system presented in this study, interchromosomal exchange would result in the transfer of the terminal fragment containing *LYS2* to another chromosome. Both intrachromosomal and sister-chromatid exchange generate a recombinant chromosome that has a copy of the original tract at an internal site. Because both these events generate the same product and cannot be distinguished, they are collectively known as excision events. Additionally, $FOA^R Lys^+$ colonies can result from *URA3* silencing through $C_{1-3}A$ based silencing. To make sure that $FOA^R Lys^+$ cells did not result from silencing, cells were replicated onto media lacking uracil. The true number of recombinants were determined by subtracting the Ura^+ colonies from the $FOA^R Lys^+$ cell counts (Stavenhagen and Zakian, 1998). Method of the Median was then used to calculate the actual rate of recombination and the standard deviation (Lea and Coulson, 1949). Resistance to FOA can also take place through *URA3* point mutations, which occur at $\sim 10^{-7}$ events per cell division at this locus. Southern blot analysis was used to detect cells lacking uracil due to point mutations.

hdf1Δ Mutant Cells have Increased Rates of $C_{1-3}A$ Direct Repeat Recombination

To determine if there is an increased rate of recombination in cells with short telomeres, an *hdf1* loss of function mutation was made in cells harboring either the $C_{1-3}A$

or C_4A_2 direct repeat cassettes 5 kb from the telomere on chromosome VII-L. Yeast cells

containing an *hdf1::HIS3* null mutation were constructed using the one-step gene

disruption method shown in Figure 4 (Materials and Methods) (Lorenz *et al.*, 1995). In

summary, the *HIS3* disrupted *HDF1* gene was amplified by PCR and then transformed

into either $C_{1-3}A$ or C_4A_2 yeast strains. *HDF1* was chosen because it binds to the

telomere and the removal of this protein leads to a reduction in telomere length

(Polotnianka *et al.*, 1998). The *hdf1* loss of function mutation in both $C_{1-3}A$ and C_4A_2

strains was confirmed by temperature sensitivity and PCR to determine in the primers

bind to the *hdf1Δ* strain (Figure 7A). Also, Southern blot analysis with a $C_{1-3}A$ telomeric

probe was used to determine that the telomeres were in fact shorter (Figure 7B). This

Southern blot shows that the parent $C_{1-3}A$ cells have a telomere length of 1.24 kb and the

$C_{1-3}A$ *hdf1Δ* cells shown in lanes 1-4 have a telomere length of 1.14 kb, indicating a loss

of approximately 100 base pairs. The additional bands the hybridized to fragments that

ran at greater than 2 kb represent chromosomes that end in X subtelomeric regions,

uniques sequence, or $C_{1-3}A$ tracts at different internal locations.

Once *hdf1Δ* strains were constructed, the recombination assay was used to

monitor the rate of *URA3* loss. The recombination assay revealed that *hdf1Δ* cells result

in an increase of $C_{1-3}A$ direct repeat recombination when compared to wild-type cells

(Figure 8). Specifically, *hdf1Δ* cells have a 9.4-fold increase in $C_{1-3}A$ direct repeat

recombination rate compared to wild-type resulting in 2.72×10^{-6} (± 0.28) events per cell

division. Analysis of C_4A_2 direct repeat strains reveal that *hdf1Δ* cells have a

recombination rate of 5.35×10^{-5} (± 0.99) events per cell division, which is a 2.5-fold

increase in recombination when compared to wild-type cells. In *HDF1* cells, the C_4A_2
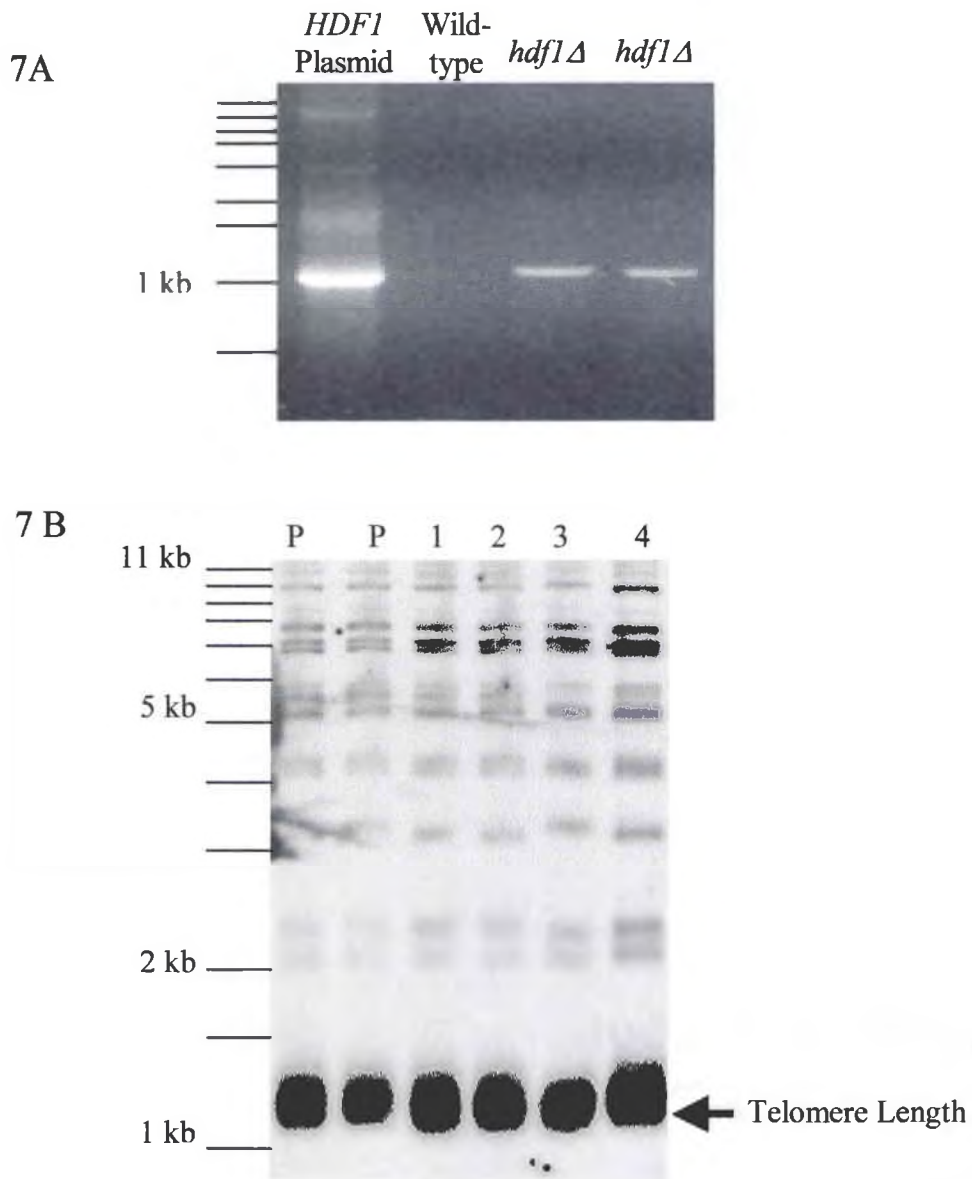
FIGURE 7. Confirmation of *hdf1Δ* cells through PCR and Southern blot analysis. (A) PCR was used to confirm that transformation of the one-step disrupted *HDF1* fragment. The band that corresponds to the *HDF1* plasmid was not present in wild-type cells, but was shown in *hdf1Δ* strains. (B) Southern analysis of telomere length from *hdf1Δ* $C_{1-3}A$ direct repeat strains was also used to confirm the mutation. DNA was isolated from both wild-type and $C_{1-3}A$ *hdf1Δ* cells, and digested with XhoI. The digested DNA was run on a 1% agarose gel, transferred to a nylon membrane, and hybridized to a non-radioactive $C_{1-3}A$ telomeric DNA probe. Lanes 1 through 4 contain DNA from $C_{1-3}A$ *hdf1Δ* cells resulting in a telomere length of 1.14 kb. The $C_{1-3}A$ parent strains have a telomere length of 1.24 kb. The arrow indicates where the telomere length was measured. Molecular weights are shown to the left of the gel.
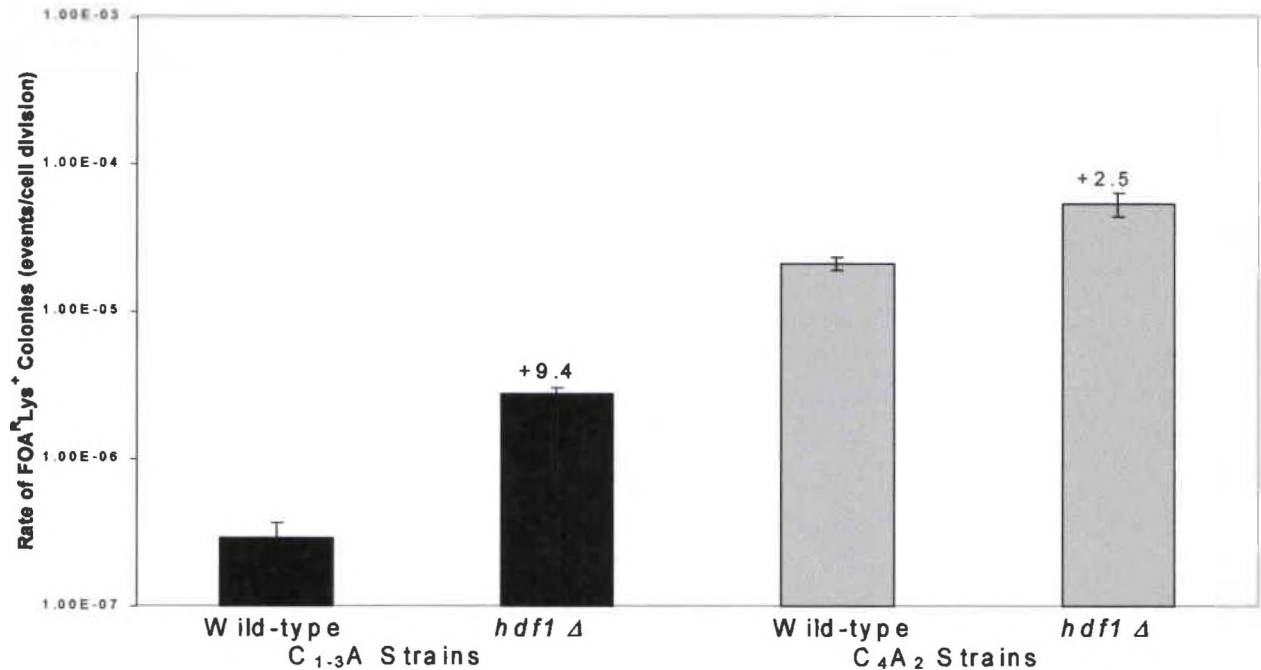
FIGURE 8. Comparison of direct repeat recombination rates for $C_{1-3}A$ direct repeat experimental (black) and the C_4A_2 direct repeat control (gray) cells. The rate of FOA^RLys^+ cells and the standard deviation were calculated by the Method of the Median (Lea and Coulson, 1949). Wild-type recombination rate is $2.9x10^{-7}$ (±0.2) events/cell division for $C_{1-3}A$ direct repeat cells (Stavenhagen and Zakain, 1998). In the presence of a *hdf1Δ* loss of function mutation in $C_{1-3}A$ direct repeat strains, the recombination rate is increased to $2.72x10^{-6}$ (±0.28) events/cell division. Alternatively, the wild-type rate for C_4A_2 direct-repeat cells is $2.10x10^{-5}$ (±0.2) events/cell division (Stavenhagen and Zakian, 1998). In the presence of a *hdf1Δ* loss of function mutation in C_4A_2 cells, the recombination rate is slightly increased to $5.35x10^{-5}$ (±0.99) events/cell division. The top of the bar represents an average of at least four independent assays. The vertical line through the top is the standard deviation. The number on top of the bar refers to the fold difference over wild-type.

direct repeat recombination rate is 69-fold higher than the $C_{1-3}A$ direct repeats compared

to a 19.7 difference in $hdf1\Delta$ cells (Stavenhagen and Zakian, 1998). Therefore, the

increase in recombination for $hdf1\Delta$ cells is specific for $C_{1-3}A$ direct repeats. However,

the increase does not completely alleviate the repression on $C_{1-3}A$ direct repeat

recombination as examined by comparison to the C_4A_2 rate.

$hdf1\Delta$ Recombination Rate is not Dependent on a Permissive Temperature

The recombination assay was also preformed on $C_{1-3}A$ and C_4A_2 $hdf1\Delta$ cells that

were grown at a non-permissive temperature. Previous studies on $hdf1\Delta$ mutants show

that in addition to the short telomere phenotype, growth is severely limited at $37^\circ C$

(Boulton and Jackson, 1996). Despite the severe growth defect, some $hdf1\Delta$ cells are

able to survive and form colonies at $37^\circ C$. These survivors show $RAD52$-dependent

amplification of the subtelomeric Y' region similar to the survivors seen in telomerase

deficient cells (Fellerhoff et al., 2000). To determine if non-permissive temperature also

alters the rate of internal direct-repeat recombination, $C_{1-3}A$ and C_4A_2 $hdf1\Delta$ cultures

were grown in YC-lys liquid culture media at $30^\circ C$ overnight and then for 4 hours at

$37^\circ C$ before being plated on FOA-lys to allow expression of the temperature sensitivity

phenotype. The recombination rate for the $C_{1-3}A$ $hdf1\Delta$ cells grown at $37^\circ C$ cells is

2.4×10^{-6} (± 0.21) events per cell division, which is an 8.2-fold increase over the wild-type

rate (Figure 9). The increase in recombination rate for $C_{1-3}A$ $hdf1\Delta$ cells grown at $37^\circ C$

is comparable to the 9.4 increase in rate observed for $C_{1-3}A$ $hdf1\Delta$ cells grown only at

$30^\circ C$. Similarly, C_4A_2 $hdf1\Delta$ recombination rates for cells grown at the non-permissive

temperature are close to C_4A_2 $hdf1\Delta$ rates for cells grown at $30^\circ C$. Specifically, C_4A_2
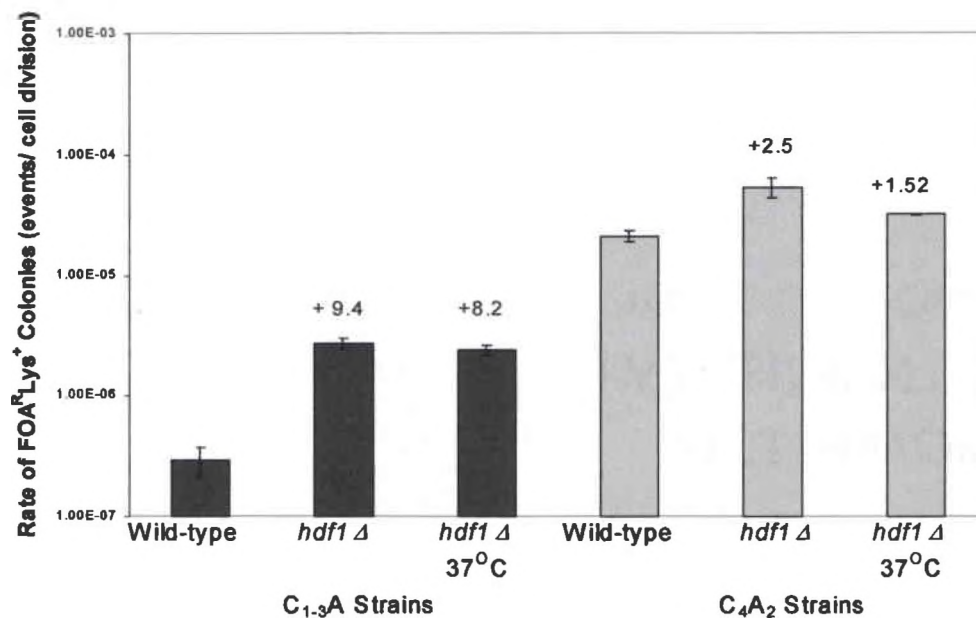
FIGURE 9. Rates of recombination for $C_{1-3}A$ experimental (black) and C_4A_2 control (gray) $hdf1\Delta$ cells grown at a non-permissive temperature of $37^\circ C$. The rate of $FOA^R Lys^+$ cells and the standard deviation were calculated by the Method of the Median (Lea and Coulson, 1949). The recombination rate for wild-type $C_{1-3}A$ cells is $2.9x10^{-7}$ (± 0.2) events/cell division (Stavenhagen and Zakain, 1998). Recombination rates increase to $2.72x10^{-6}$ (± 0.28) events/cell division in $C_{1-3}A$ $hdf1\Delta$ cells grown entirely at $30^\circ C$. Similarly, recombination rates increase to $2.4x10^{-6}$ (± 0.2) events/cell division when $C_{1-3}A$ cells are grown at $37^\circ C$ for an additional four hours. Alternatively, the recombination rate for wild-type C_4A_2 cells is $2.10x10^{-5}$ (± 0.2) events/ cell division (Stavenhagen and Zakian, 1998). Recombination rates slightly increase to $5.35x10^{-5}$ (± 0.99) events/cell division in C_4A_2 $hdf1\Delta$ cells grown entirely at $30^\circ C$. Similarly, the recombination rate for C_4A_2 $hdf1\Delta$ increases to $3.2x10^{-5}$ (± 0.04) events/cell division when cells are grown for an additional four hours at $37^\circ C$. The top of the bar represents an average of at least two independent assays. The vertical line through the top is the standard deviation. The number on top of the bar refers to the fold difference over wild-type.

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hdf1Δ cells grown for four hours at 37°C have a recombination rate of 3.2×10^{-5} (± 0.04)

events per cell division which is a 1.52-fold increase in recombination rate when

compared to wild-type. This modest increase in recombination rate is similar to the 2.5-

fold increase in recombination rate for C_4A_2 *hdf1Δ* cells grown at 30°C. Taken together,

the similarity between $C_{1-3}A$ and C_4A_2 *hdf1Δ* recombination rates at both permissive and

non-permissive temperatures indicates that in an *hdf1Δ* background, the increase in

temperature does not further enhance recombination. Thus the lethality of increased

temperature in *hdf1Δ* cells is not due to events that trigger $C_{1-3}A$ direct-repeat

recombination.

Direct Repeat Recombination Increase Occurs via a *RAD1*-Dependent Recombination Pathway

Additional mutations were made in cells harboring the $C_{1-3}A$ direct repeats to

determine if the increase in recombination occurs via the *RAD1* or *RAD52* recombination

pathway. *RAD1* and *RAD52* are important genes in two independent recombination

pathways that are responsible for intra-chromosomal excision type recombination events

between direct repeats (Rattray and Symington, 1995). Recombination through a

RAD52-dependent pathway occurs via a strand invasion event, facilitating recombination

through the formation of a Holiday junction (Petes *et al.*, 1991). Alternatively, single

strand annealing (SSA), a non-conservative pathway for recombination between direct-

repeats, is dependent on *RAD1*. Recombination occurs after endonucleases remove DNA

between repeats until homologous sequences are matched (Lin *et al.*, 1984; Fishman-

Lobell *et al.*, 1992). Previous results have shown that recombination between

$C_{1-3}A$ DNA in wild-type cells was greatly inhibited when the tracts are near the telomere. The reduction in recombination rate is due to a lack of *RAD52*-recombination and a reduction in the number of *RAD1*-dependent events (Stavenhagen and Zakian, 1998).

To determine if the increase in recombination in *hdf1Δ* cells is due to either a *RAD1* or *RAD52*-dependent recombination pathway, additional mutations were made in their respective genes. Double and triple knockout strains were made by mating either *rad1Δ*, *rad52Δ*, or *rad1Δrad52Δ* cells with *hdf1Δ* cells (Figure 5, Materials and Methods). After mating, these cells were then sporulated and haploids were selected. In the $C_{1-3}A$ direct repeat strains harboring a *hdf1Δrad1Δ* double mutation there is a 5.9-fold decrease in recombination rate compared to the *hdf1Δ* single mutant resulting in 4.61×10^{-7} (± 1.5) events per cell division (Figure 10). In addition, the decrease in recombination results in a rate that is similar to the wild-type rate suggesting that the *RAD1* recombination pathway plays a bigger role in *hdf1Δ* cells. This decrease also indicates that the recombination pathway is at least partially dependent on *RAD1* in *hdf1Δ* cells. In addition, $C_{1-3}A$ direct repeat recombination rates are similar in *hdf1Δ* and *hdf1Δrad52Δ* cells. Specifically, there is only a 1.7-fold decrease in *hdf1Δrad52Δ* cells when compared to *hdf1Δ* strains resulting in 1.43×10^{-6} (± 0.14) events per cell division. This modest decrease suggests that the *RAD52* pathway is not as critical as the *RAD1* pathway in facilitating recombination in *hdf1Δ* cells. Furthermore, this suggests that *RAD52*-dependent events are mostly absent in *hdf1Δ* cells. Finally, the rate of $C_{1-3}A$ direct repeat recombination is dramatically reduced, 120-fold, in the *hdf1Δrad1Δrad52Δ* triple mutants. The recombination rate was less than 2.04×10^{-8} events per cell division, but could not be quantitatively determined because there were "0" $FOA^R Lys^+$ colonies.
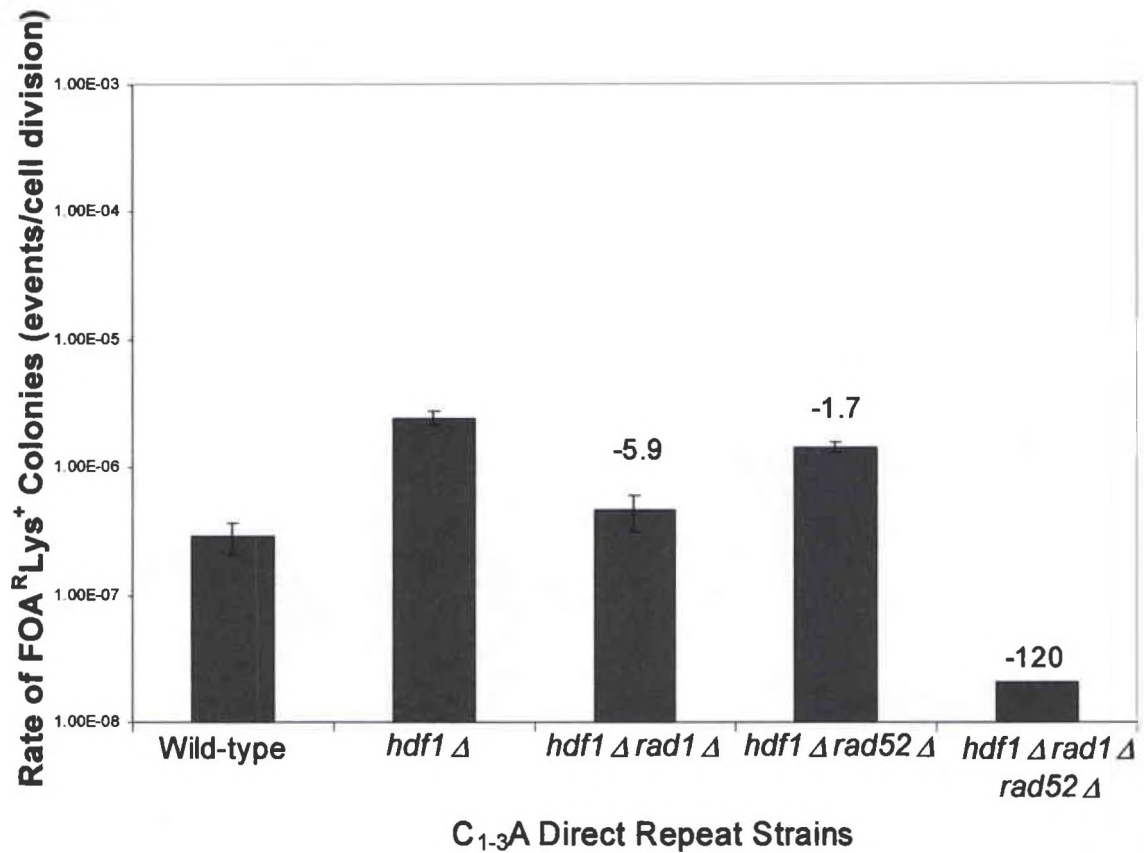
FIGURE 10. Rates of recombination for $C_{1-3}A$ direct repeat cells containing mutations in the *RAD1* or *RAD52* genes. The rate of FOA^RLys^+ cells and the standard deviation were calculated by the method of the median (Lea and Coulson, 1949). The recombination rate for wild-type $C_{1-3}A$ cells is 2.9×10^{-7} (± 0.2) events/cell division (Stavenhagen and Zakain, 1998). The recombination rate for *hdf1Δ* cells is 2.45×10^{-6} (± 0.28) events/cell division. In $C_{1-3}A$ cells harboring *hdf1Δrad1Δ* and *hdf1Δrad52Δ* double mutations, and an *hdf1Δrad1Δrad52Δ* triple mutation, the recombination rates are 4.61×10^{-7} (± 1.5), 1.43×10^{-6} (± 0.14), and less than 2.04×10^{-8} events/cell division, respectively. Because the recombination rates of the strains harboring *hdf1Δrad1Δrad52Δ* mutations resulted in 0" FOA^RLys^+ viable colonies, the standard deviation was not calculated. For *hdf1Δrad1Δ* and *hdf1Δrad52Δ* strains, the top of the bar represents an average of at least four independent assays. The vertical line through the top is the standard deviation. The number on top of the bar refers to the fold difference over *hdf1Δ* cells.

Also, there is no standard deviation calculation because of the absence of FOA^{R}Lys$^+$

viable colonies following the *hdf1Δrad1Δrad52Δ* mutation. The inability to recover

FOA^{R}Lys$^+$ colonies in *hdf1Δrad1Δrad52Δ* suggests that C$_{1-3}$A direct repeat

recombination requires at least one of these recombination pathways, which is similar to

what occurs in wild-type cells. For each mutant harboring a mutation in either *RAD1* or

RAD52, a recombination event can occur through the wild type recombination pathway

that is still available. The ability of some of the events to shuttle between recombination

pathways suggests that at some level they share intermediate products.

<u>In Cells Harboring Mutations that Increase Telomere Length there is a Modest Increase
in Direct Repeat Recombination</u>

Recombination rates were also measured in cells with longer telomeres, to

determine if an increase in recombination rate is a consequence of a non-wild-type

telomere length. The recombination rates of cells with longer telomeres were compared

to the rates of shorter telomere *hdf1Δ* cells. This comparison was done to determine if

increases in C$_{1-3}$A direct repeat recombination occurs due to either change in telomere

length or the shortening of the telomeres. Direct repeat recombination was examined in

three different genetic backgrounds that lead to longer telomeres. These backgrounds

include cells transformed with a plasmid containing 800 bp of excess C$_{1-3}$A DNA (+100

bp of telomeric DNA) (Stavenhagen and Zakian, 1998), and two strains with either a

pif1-m2Δ (+75 bp of telomeric DNA) mutation (Schulz and Zakian, 1994), or *rif1Δrif2Δ*

(+800 to 1000 bp of telomeric DNA) double mutations (Hardy *et al.*, 1992). Excess

$C_{1-3}A$ DNA was introduced into the $C_{1-3}A$ direct repeat strain using a plasmid with three

copies of a natural telomere (Stavenhagen and Zakian, 1994). In addition, the plasmid

contains a defective promoter allele of the *LEU2* gene, *leu2-d*, which results in 100-200

copies of the plasmid when the strain is grown on media lacking leucine. This excess

$C_{1-3}A$ DNA adds approximately 900 bp and is thought to titrate telomere binding proteins

from endogenous telomeres resulting in a long telomere phenotype (Runge *et al.*, 1991).

A mutation in the *PIF1* gene leads to telomeres that are longer than wild-type due to

changes in a telomere maintenance pathway (Schulz and Zakian, 1994). *RIF1* and *RIF2*

code for two proteins that interact with Rap1p and are important for regulating telomere

length. Double mutants *rif1Δrif2Δ* have extremely long telomeres that can be as long as

4 kb (Hardy *et al.*, 1992; Wotton and Shore, 1997).

Using these three strain backgrounds, $C_{1-3}A$ direct repeat recombiantion was

analyzed in cells harboring telomeres that range from ~375 bp to 4 kb. Using the direct-

repeat recombination assay, recombination rates for all three longer telomere strains were

determined (Figure 11). Cells harboring the plasmid with 800 bp of $C_{1-3}A$ DNA grown

under conditions that lead to 100 to 200 copies of the plasmid have a recombination rate

of 4.67×10^{-7} (± 0.3) events per cell division. This recombination rate is only a 1.7-fold

increase over wild-type rates of recombination. Similarly, *pif1-m2Δ* cells had a

recombination rate of 4.67×10^{-7} (± 0.02) events per cell division resulting in a 1.6-fold

increase. Finally, *rif1Δrif2Δ* cells have a recombination rate of 9.2×10^{-7} (± 3.2) events per

cell division resulting in a 3.2-fold increase in $C_{1-3}A$ direct repeat recombination when

compared to wild-type levels. Although excess $C_{1-3}A$, *pif1-m2Δ*, and *rif1Δrif2Δ* cells all

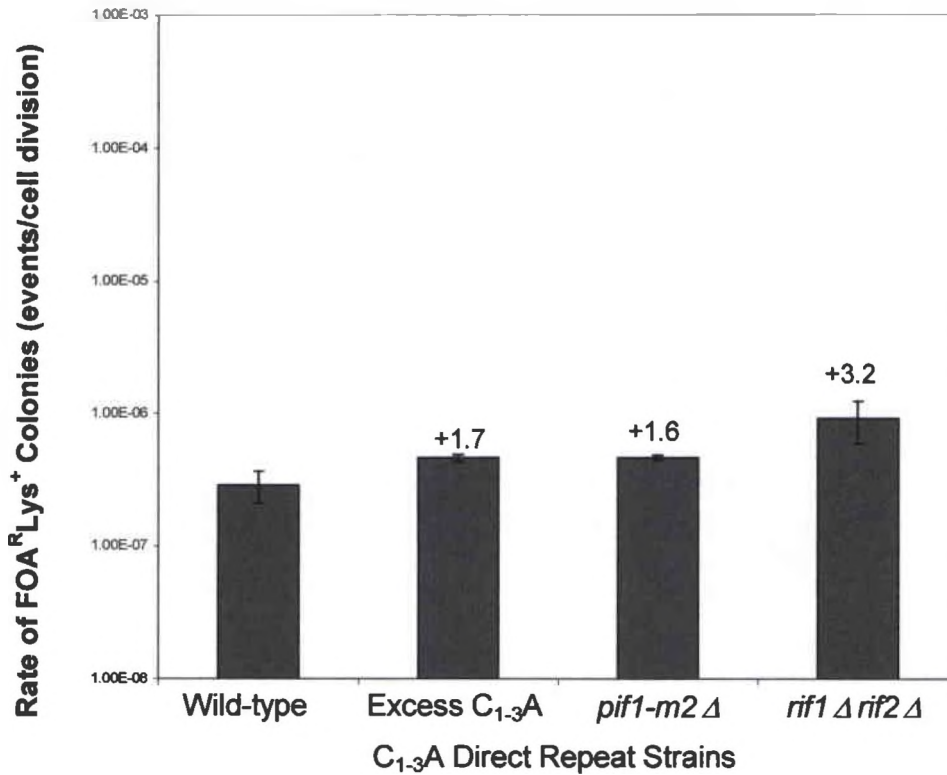FIGURE 11. $C_{1-3}A$ direct repeat recombination rates for cells with increased telomere length. The rate of $FOA^R Lys^+$ cells and the standard deviation were calculated by the method of the median (Lea and Coulson, 1949). The recombination rate for wild-type $C_{1-3}A$ cells is 2.9×10^{-7} (± 0.2) events/cell division (Stavenhagen and Zakain, 1998). The recombination rates in cells harboring excess telomeres, *pif1-m2Δ*, and *rif1Δrif2Δ* double mutations are 4.67×10^{-7} (± 0.3), 4.67×10^{-7} (± 0.2), and 9.2×10^{-7} (± 3.2) events per cell division, respectively. The top of the bar represents an average of at least four independent assays. The vertical line through the top is the standard deviation. The number on top of the bar refers to the fold difference over wild-type.

show an increase in recombination rate, the increase was modest in comparison to the short telomere effect. In addition, none of these genetic backgrounds had a significant effect on C_4A_2 direct repeat recombination (data not shown). The modest increase in recombination rate indicates that cells with long telomeres do not appear to have a significant influence on direct-repeat recombination. Genomic Southern blots showed expected recombination bands for all three long telomere strains (data not shown).

Recombination in *hdf1Δ* Cells Occurs via an Excision Type Pathway

Because there are a variety of recombination events that can lead to FOA^RLys^+ colonies, Southern blot analysis was used to determine that these colonies arise from recombination through excision events. Southern blots were also used to visualize the chromosome structure after specific recombination events. As shown previously, direct repeat recombination normally results in excision of the internal DNA and the presence of a single tract on the chromosome (Stavenhagen and Zakian, 1998). In $C_{1-3}A$ and C_4A_2 direct repeat strains this tract is variable in size because of the highly repetitive nature of the repeats. The repeated sequences are able to align in multiple registers to complete the recombination event. To observe recombination products, genomic DNA from both FOA^RLys^+ derived from both C_4A_2 and $C_{1-3}A$ direct repeat strains containing the *hdf1Δ* mutation was prepared, digested with BglII, and run on a 1% agarose gel (see materials and methods). Once the DNA was transferred to a nylon membrane it was exposed to a non-radioactive probe overnight. The probe for the *hdf1Δ* recombination products was

derived from the disrupted $ADH4$ gene (black bar, Figure 6). In addition, the C_4A_2 $hdf1\Delta$ Southern blot was reprobed with a probe derived from the 5' end of the $LYS2$ gene (gray bar, Figure 6).

Using a genomic Southern blot, recombination events were visualized for $C_{1-3}A$ $hdf1\Delta$ cells. For the parent $C_{1-3}A$ strain, there are three BglII cut sites that result in fragments approximately 7 kb and 13 kb including the $URA3$ gene and the direct repeat substrates (Figure 12). As expected, the parent strain hybridized to bands at 7 kb (Figure 13). The band hybridizing at 13 kb is likely due to partial digestion of the BglII cut site on the distal end of the $LYS2$ gene at chromosome VII-L (Figure 12). When recombination occurs through loss of $URA3$, 4.6 kb of the cassette including the $URA3$ gene and one tract is recombined out and FOA^RLys^+ cells result. After the loss of $URA3$, the $ADH4$ hybridizing band would be expected to range from 2.4 to 2.7 kb. In the case of partial digestion, an 8.7 to 9 kb band would be expected. The FOA^RLys^+ $C_{1-3}A$ $hdf1$ cells in this assay all show the product of recombination by a band at 2.36 kb (Figure 13, lanes 1-7). Also, lanes 1, 2, 4, and 5 have a partial digestion band at 8.4 kb. All seven of the experiemtental lanes contain faint bands at 7 kb and 13 kb, which correspond directly to the parent lane. These bands are likely due to mixed cell populations that sometimes arise because of the inclusion of small satellite colonies. These small satellite colonies form as a result of TPE and closely surround recombination cells on FOA-lys plates. Although the FOA-lys plates are replica plated on to YC-ura plates to prevent TPE, cells from the FOA-lys plates are chosen for genomic Southern blots. Furthermore, recombination rates are measured after accounting for TPE on YC-ura plates, so the overall rate should not be significantly altered by the satellite colonies.
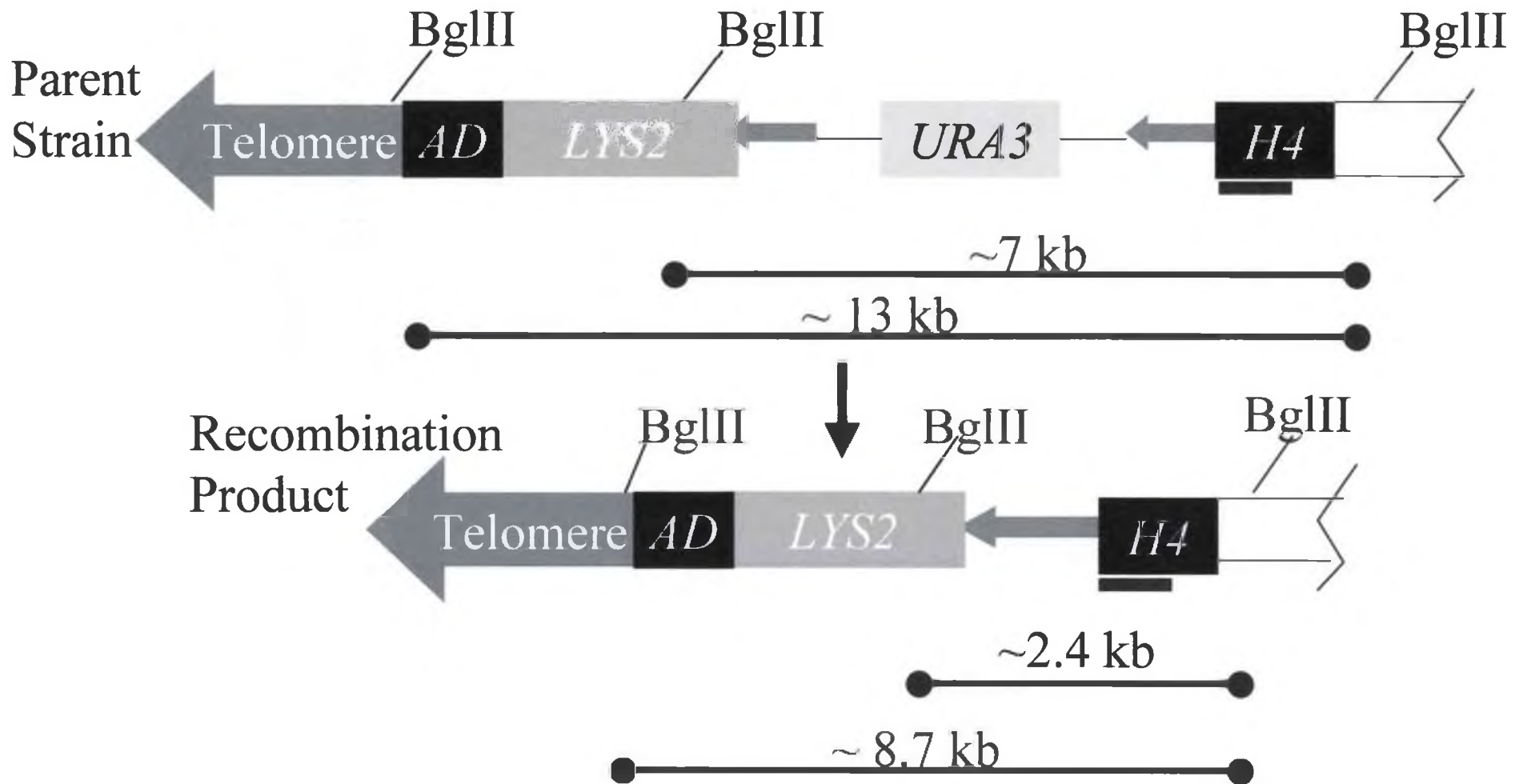
FIGURE 12. BglII cuts sites for $C_{1-3}A$ *hdf1Δ* cells. $C_{1-3}A$ cells have three BglII cut sites. Before recombination the cut sites detected by the disrupted *ADH4* probe are separated by approximately 7 kb, and if partial digestion occurs approximately 13 kb. The *URA3* cassette is 4.6 kb in length. After *URA3* is lost through recombination 2.4 kb remains between the two of the cut sites and approximately 8.7 kb remains between the partial digestion sites. The black bar indicates the placement of the disrupted *ADH4* probe.
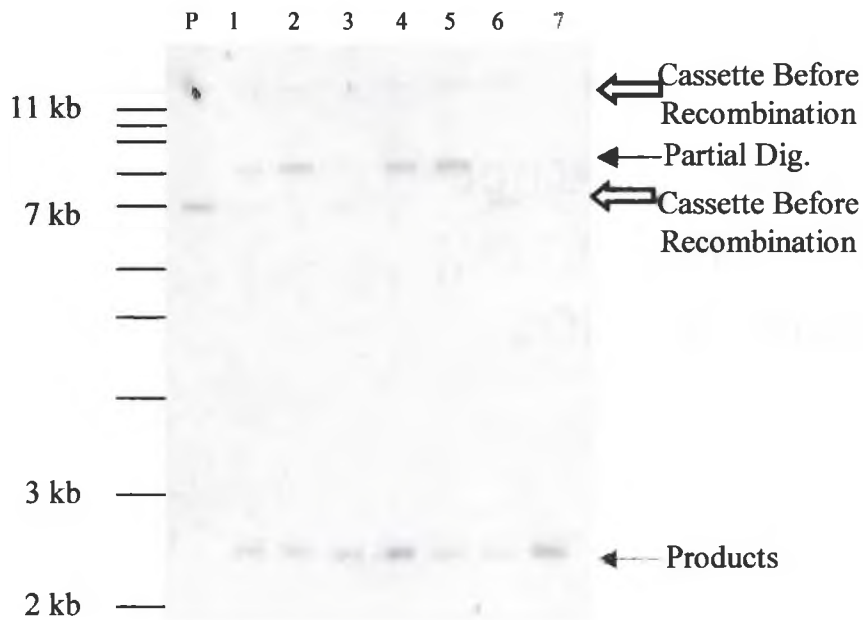
FIGURE 13. Southern analysis of recombinant chromosomes from *hdf1Δ* C$_{1-3}$A direct repeat strains. Genomic DNA from each FOA^{R}Lys$^+$ event was digested with BglII, run on a 1% Agarose gel, and transferred to a nylon membrane. The blot was hybridized to a non-radioactive probe derived from the 3' end of the disrupted *ADH4* gene. The parent strain shows bands at 13 and 7 kb (open arrow), indicating the position of the cassette before recombination. Lanes 1-7 contain genomic DNA from independent recombination events. The gray arrow indicates bands due to recombination events at 2.36 kb (lanes 1-7). The bands shown at 8.2 (black arrow) are likely partial digestion products (lanes 2, 4, and 5). In addition, the faint bands at 13 and 7 kb in the recombination products are probably attributed to mixed cell cultures.

A genomic Southern blot was also used to visualize recombination events for C₄A₂ *hdf1Δ* cells. For the C₄A₂ strain, there are four BglII cut sites producing only one fragment, at 1.7 kb, that should be detected by the interrupted *ADH4* probe (Figure 14). However, Southern blot analysis shows that for all lanes except lane 3 there are multiple bands (Figure 15A). These multiple bands are likely due to partial digestion. Specifically, partial digestion could produce fragments between BglII cut sites that are approximately 13 kb, 8 kb, and 1.7 kb apart from each other (Figure 14). The genomic Southern blot shows all three fragments for the parent strains as indicated by open arrows on Figure 15A. Specifically, the fragments in the parental lanes are 13kb, 7.9 kb, and 1.74 kb. The FOA^{R}Lys$^+$ colonies shown in lanes 1-13, are produced when 4.6 kb of the *URA3* marker is recombined out. When 4.6 kb of the *URA3* marker is subtracted from the 13 kb partial digest fragment, a fragment of approximately 8.4 kb results (Figure 14). The Southern blot shows a fragment at 8.7 kb, in lanes 1, 2, and 4-13, indicating that a recombination event has occurred (Figure 15A). Next, when the entire 4.6 kb of the *URA3* marker is subtracted from the 7.9 kb partial digest fragment, a fragment of approximately 3.3 kb results (Figure 14). The Southern blot shows that in lanes 1, 2, and 4-13, there are bands that range from 2.59 kb to 2.83 kb (Figure 15A). The difference in band length is likely due to the variability in telomeric tract alignment that can occur with repetitive sequences. Also, the band length does not center around 3.3 kb, because the recombination events may not align so the entire *URA3* fragment is lost. Finally, the 1.7 kb band on every lane of the Southern blot represents the distance between the last two BglII cut sites proximal to the centromere (Figure 15A). Because of the location of the interrupted *ADH4* probe, this band is the only one expected for both the parent and the
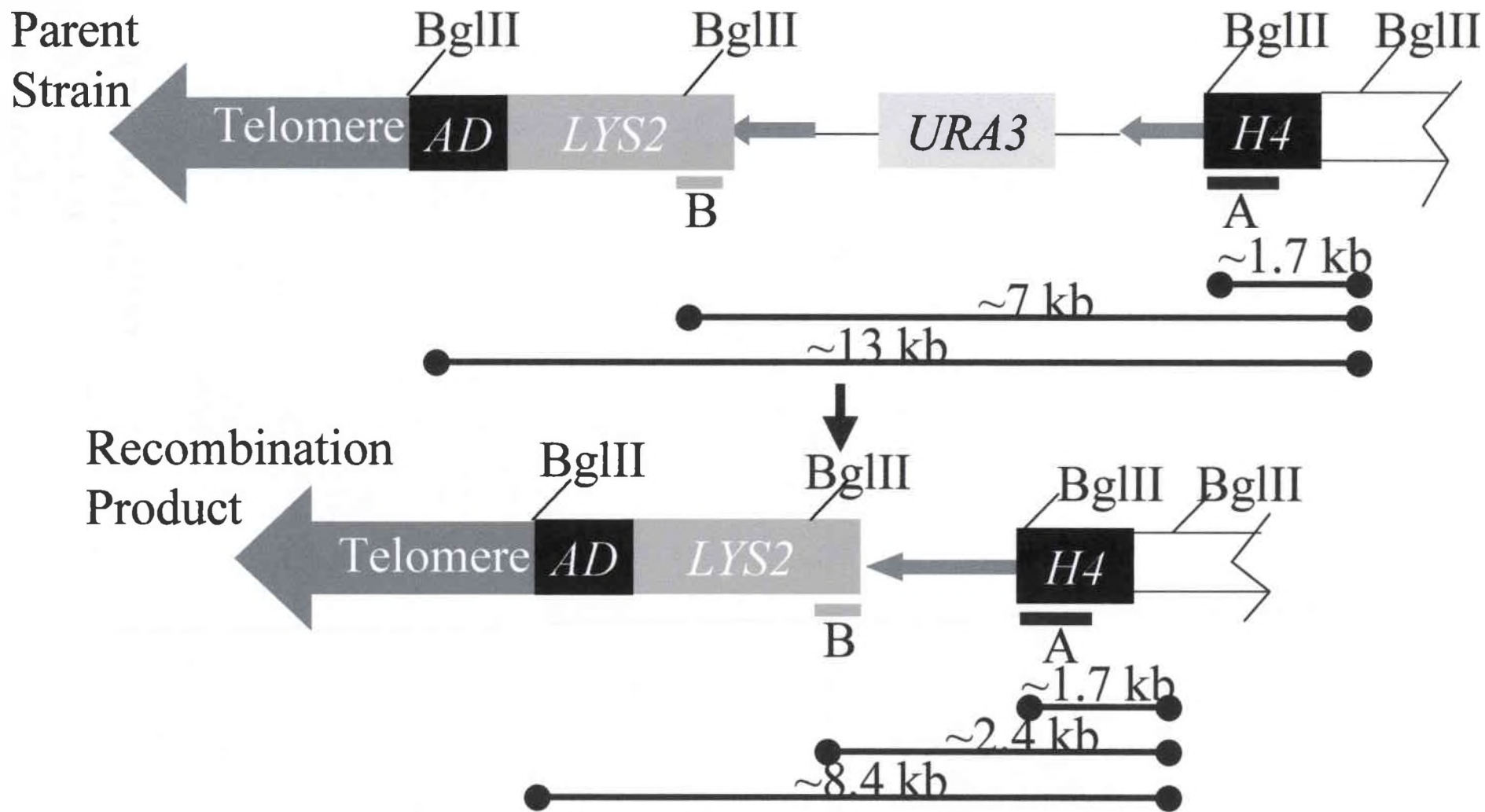
FIGURE 14. BglII cut sites for C_4A_2 *hdf1*Δ cells. C_4A_2 cells have four BglII cut sites. Before recombination, the partial digestion BglII cut sites detected by the disrupted *ADH4* probe (black bar labeled A) are separated by 7 kb and 13 kb. The *URA3* cassette between the C_4A_2 repeat sequences is 4.6 kb in length. After *URA3* is lost through recombination, 8.4 kb and 2.4 kb remain between the BglII cut sites. The last two BglII cut sites proximal to the centromere are separated by 1.7 kb. This length does change after recombination, because *URA3* is not separating the final two cut sites. When the blot is reprobed with *LYS2* 5' (gray bar labeled B), a fragment of 1 kb results from the length between the probe and the BglII cut site at the 5' end of the *LYS2* gene.
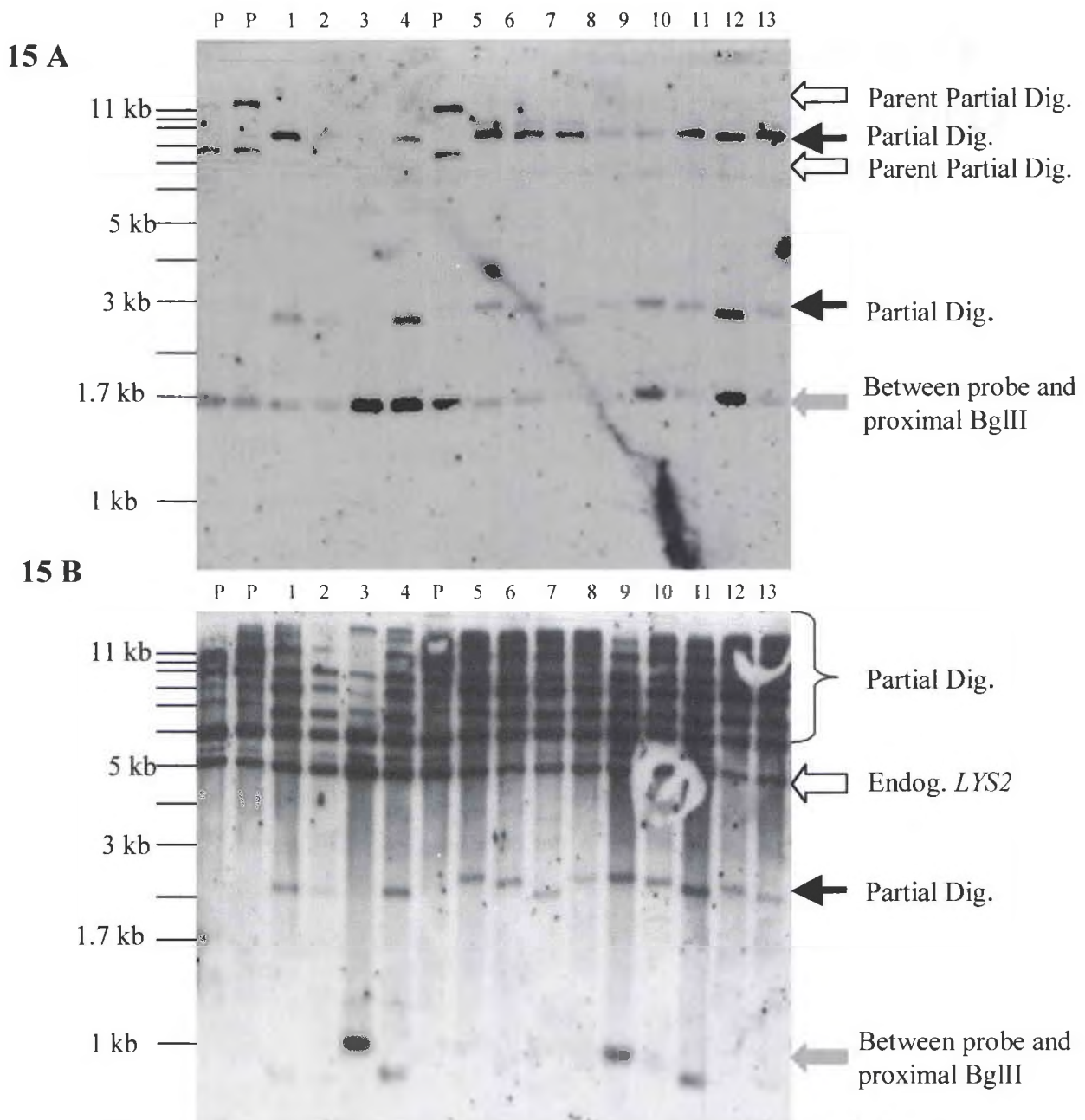
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FIGURE 15. Southern analysis of recombinant chromosomes from *hdf1Δ* C$_4$A$_2$ direct repeat strains. DNA from each FOA^{R}Lys$^+$ event was digested with BglII, run on a 1% agarose gel, and transferred to a nylon membrane. (A)The blot was hybridized to the 3' end of the disrupted *ADH4* gene. All lanes show the expected 1.7 kb which represents the distance between the probe and the BglII cut site proximal to the telomere (gray arrow). The parent strains show two additional partial digest bands at 13 kb and 7.9 kb, as indicated by the open arrows. Lanes 1 through 13 contain DNA from independent recombination events, as shown by the black arrows. Recombination events resulted in partial digest bands at 8.7 kb, and between 2.59 kb and 2.83 kb (lanes 1, 2, and 4-12). Lane 3 shows only the expected band at 1.7 kb. (B) The blot was hybridized to the 5' end of the *LYS2* gene. All lanes show partial digestion products greater than 5 kb (indicated by a bracket) and an endogenous *LYS2* band at 5kb (as shown by an open arrow). Partial digest recombination products are shown between 2.59 kb and 2.83 kb in lanes 1, 2, and 4-13, as indicated by a black arrow. The distance between the *LYS2* 5` probe and the BglII cut site at 5' end of the *LYS2* gene is shown at 1 kb in lanes 3, 4, 9, and 11 (gray arrow). Lane 3 shows only the expected band at 1.1 kb. Molecular weight standards are shown to the left of the gel.

FOA^{R}Lys$^+$ strains. The distance between the final two BglII cut sites does not change during recombination, because the two cut sites are not separated by the *URA3* tract. One exception to the FOA^{R}Lys$^+$ cell recombination banding pattern occurred in Lane 3. Lane 3 shows only the expected 1.7 kb band, suggesting that digestion went to completion.

Because several partial digestion bands were seen in the C$_4$A$_2$ *hdf1Δ* Southern blot with the interrupted *ADH4* probe, the blot was reprobed with a *LYS2* 5' probe (Figure 6). This probe was chosen because it binds more distal to the centromere and gives additional information about the partial digestion. Specifically *LYS2* should hybridize to the same fragment as the *ADH4* probe confirming the identification of the recombination product. This additional information was needed to make sure that the aberrant bands were due to a non-complete digestion, rather than a mixed cell culture. The Southern blot with the *LYS2* 5' probe shows several partial digestion bands greater than 5 kb and the endogenous *LYS2* at 5 kb (Figure 15B). In addition, recombination bands in lanes 1, 2, and 4-13 are the same length, between 2.59 kb and 2.83 kb, as the bands seen in the C$_4$A$_2$ *hdf1Δ* Southern blot probed with the disrupted *ADH4*. The identical recombination band lengths in both blots supports identification of the recombination product. This confirms that the additional bands are likely due to partial digestion and not a mix of cells. Additional recombination bands were shown in lanes 3, 4, 9, and 11. The length of these bands is either 1.1 kb (lanes 3 and 9) or 0.9 kb (lanes 4 and 11). This distance represents the length between the *LYS2* 5' probe and the BglII cut site at the 5' end of the *LYS2* gene. Interestingly, lane 3 only shows a band at 1.1 indicating that the BglII digestion went to completion.

SUMMARY AND CONCLUSIONS

In wild-type cells, endogenous telomeres normally repress $C_{1-3}A$ direct-repeat

recombination (Stavenhagen and Zakian, 1998). The results in this study indicate that

hdf1Δ cells with shorter telomeres have a 9.4-fold increase in the rate of $C_{1-3}A$ direct-

repeat recombination rate when compared to wild-type cells. This increase is specific to

$C_{1-3}A$ direct repeat tracts as *hdf1Δ* strains harboring control C_4A_2 tracts showed

recombination rate comparable to the rate of wild-type cells. Also, recombination rates

were similar in *hdf1Δ* cells grown at permissive and non-permissive temperatures.

Specific recombination pathways were studied in $C_{1-3}A$ *hdf1Δ* cells to determine if the

direct-repeat recombination is dependent on a *RAD1* or a *RAD52* recombination pathway.

The recombination rates from double knockout *hdf1Δrad1Δ* and *hdf1Δrad52Δ* cells

indicated that *hdf1Δ* cells show a preference for the *RAD1* recombination pathway. In

addition, examination of the *hdf1Δrad1Δrad52Δ* triple mutant strain suggested that $C_{1-3}A$

direct-repeat recombination requires at least one of these recombination pathways for

survival. The requirement for at least one recombination pathway is similar to what is

observed in wild-type cells (Stavenhagen and Zakian, 1998). Additionally,

recombination rates were determined in three strains containing longer telomeres. Using

three different strain backgrounds: cells transformed with a high-copy plasmid containing

300 bp of excess $C_{1-3}A$ DNA, a strain harboring a *pif1-m2Δ* mutation, and a *rif1Δrif2Δ*

double mutant, $C_{1-3}A$ recombination was not substantially enhanced. This result suggested that long telomeres have only a minor influence on recombination between $C_{1-3}A$ direct repeats. Genomic Southern blots revealed the physical products of recombination events in $C_{1-3}A$ and C_4A_2 $hdf1\Delta$ cells. Southern blots for both $C_{1-3}A$ $hdf1\Delta$ and C_4A_2 $hdf1\Delta$ strains showed that the recombination events in the presence of $hdf1$ are similar to those observed in $HDF1$ cells following the excision of the $URA3$ gene. In both $C_{1-3}A$ and C_4A_2 strains, recombination events that occurred through elimination of the intervening DNA including $URA3$ were confirmed by genomic Southern blots. The Southern blots indicated that recombination occurred via an excision event.

The increase in recombination detected in the absence of Hdf1p does not account for the total repression of $C_{1-3}A$ direct repeat recombination. In $HDF1$ cells there is an approximately 20-fold reduction in $C_{1-3}A$ direct repeat recombination near the telomere, and this repression is primarily due to the absence of $RAD52$-dependent recombination events (Stavenhagen and Zakain, 1998). In $hdf1\Delta$ cells, less than a 10-fold increase in $C_{1-3}A$ direct repeat recombination was detected. Because the $C_{1-3}A$ recombination rate in $hdf1\Delta$ cells did not return to C_4A_2 wild-type levels, an $HDF1$ mutations only results in a partial derepression on recombination. Furthermore, the increase occurred primarily via a $RAD1$-dependent pathway. Together these data indicate that the increase in $C_{1-3}A$ direct repeat recombination occurs through a different pathway than the repression of recombination in wild-type cells suggesting that there are two pathways that regulate recombination between yeast telomeric direct repeats.

Similar recombination rates for $C_{1-3}A$ *hdf1Δ* cells grown at permissive and non-permissive temperatures indicated that recombination and temperature sensitivity are not facilitated by the same mechanism. Previous studies with *hdf1Δ* mutants show that they do not grow well at $37^{O}C$ (Boulton and Jackson, 1996). Additional studies propose that the absence of *HDF1* along with elevated temperatures leads to cell death for the vast majority of the cell culture and survival for a few cells facilitated by a mechanism similar to telomerase-independent recombination (Fellerhoff *et al.*, 2000). Our experiments showed that the increase in recombination rates between $C_{1-3}A$ *hdf1Δ* cells grown at permissive and non-permissive temperatures are nearly identical. These similar recombination rates suggest that the mechanism responsible for survivor formation during growth at higher temperatures is different from the primary recombination pathway for $C_{1-3}A$ *hdf1Δ* cells. Furthermore, temperature resistant *hdf1Δ* survivors used in a previous study rely on *RAD52* for survival (Fellerhoff *et al.*, 2000). In contrast, the increase in recombination in $C_{1-3}A$ *hdf1Δ* mutants in this study showed a preference for the *RAD1* pathway. The additional insult to the cells at the $37^{O}C$ growth temperature had no effect on the rate of initiation or stability of recombination intermediates. One alternative possibility is that all the *hdf1Δ* cells that were sensitive to the non-permissive temperature died and were not recovered for the recombination assay. If many of the *hdf1Δ* cells died, it would suggest that telomeres are not shortened consistently in the overall culture, which is different from observations with telomerase-negative cells (Lundblad and Blackburn, 1993; Singer and Gottschling, 1994).

Because temperature does not seem to influence recombination rate, the loss of telomere length in *hdf1Δ* cells may be one reason for the increase in direct-repeat

recombination. Studies using strains with telomerase mutations show that cells with shorter telomeres have an increase in recombination rate (Ford and Stavenhagen, submitted). The proposed model for survivors with an inactive telomerase suggest that in response to telomere shortening, Y' subtelomeric fragments are amplified and rearranged via telomere recombination. Following this rearrangement, telomerase negative survivors are produced (Lundblad and Blackburn, 1993). Studies on *EST1*, which encodes a telomerase accessory factor, show that mutant survivors use the *RAD52* recombination pathway to maintain telomere length and viability (Lundblad and Blackburn, 1993). Similarly, *TLC1*, which encodes the essential RNA component of telomerase, also results in survivors that are *RAD52* dependent (Singer and Gottschling, 1994; Ford and Stavenhagen, submitted). Mutations in the *RAD1* recombination pathway in *tlc1Δ* strains have no effect on survivor formation (Le *et al.*, 1999; Ford and Stavenhagen, submitted). In contrast, the *hdf1Δ* strains used in this study preferred a *RAD1* rather than a *RAD52* recombination pathway for survival. Because the increase in $C_{1-3}A$ direct repeat recombination in *hdf1Δ* cells occurred via a different pathway than observed in strains lacking telomerase and because the increase did not result in recombination rates equal to the rates determined in control strains, the repression on telomere direct repeat recombination cannot be totally dependent on length maintenance.

The increase in $C_{1-3}A$ direct repeat recombination through a specific recombination pathway in *hdf1Δ* cells may instead be attributed to the removal of Hdf1p at the end of the telomere. One role of *HDF1* is to serve as a protective cap preventing nucleolytic degradation and recombinogenic activities at the telomere (Polotnianka *et al.*, 1998). Additionally, when Hdf1p is bound to the telomere, subnuclear localization of

telomeres is also maintained (Laroche *et al.*, 1998). Because Hdf1p plays an essential role in maintaining subnuclear localization, its function may also extend beyond the end of the chromosome to the internal $C_{1-3}A$ tracts. Furthermore, when Hdf1p is bound to the end of the telomere it may have an additional protective role, functioning as a repressor of internal direct-repeat recombination. Once Hdf1p is no longer bound to the end of the chromosome, there could be a loss of telomere structure that leads to an increase in direct-repeat internal recombination. Previous experiments show that the telomere is able to silence gene expression proximally along the chromosome (Stavenhagen and Zakian, 1998). Likewise, a similar effect of the telomere on $C_{1-3}A$ direct repeat recombination may also occur.

In addition to binding at the telomere, Hdf1p may also bind directly to the internal direct repeats. Furthermore, the increase in $C_{1-3}A$ direct repeat recombination observed in *hdf1Δ* cells may be attributed to the exposure of the internal telomeric DNA to recombination proteins when Hdf1p is no longer bound. Although it is unknown if Hdf1p binds directly to telomere direct repeats in the cassette system, other features of *HDF1* suggest that may bind to internal telomere tracts. Specifically, Hdf1p binds in a sequence-independent manner at the junction between single and double stranded telomeric DNA at the end of the chromosome (Feldmann and Winnacker, 1993). Hdf1p also binds internally at the site of double stranded breaks facilitating repair through the nonhomologous end-joining pathway (NHEJ) (Critchlow and Jackson, 1998). Taken together, the evidence that Hdf1p is known to bind to telomeric DNA and to double stranded breaks suggests that Hdf1p may also bind to the internal telomeric direct repeats in the cassette. Furthermore, *hdf1Δ* cells may have had an increase in recombination rate

because recombination proteins had access to the internal direct repeats. Additionally, the increase in recombination rate in *hdf1Δ* cells may be due to the inactivation of recombination in the NHEJ recombination pathway. NHEJ may repress other recombination pathways, so in *hdf1Δ* cells the *RAD1* recombination pathway could facilitate recombination. Further investigation of the proteins bound to internal direct-repeat cassette DNA is needed to determine if Hdf1p binds directly.

There is the additional possibility that it is not the removal of *HDF1* either at the telomere or internal repeat tracts that causes an increase recombination, but instead the absence of another protein bound to *HDF1*. Both Sir3p and Sir4p have been identified as binding Hdf1p, *in vivo* (Tsukamoto *et al.*, 1997). However, previous studies using the *URA3* cassette to measure recombination have shown that both *sir3Δ* and *sir4Δ* mutants lead to a decrease in recombination rate (Stavenhagen and Zakian, 1998). This result lends further support that it is the removal of *HDF1* itself and not any of the associated SIR proteins that causes an increase in recombination rate.

Because it is unknown whether the increase in recombination rate seen in $C_{1-3}A$ *hdf1Δ* cells is due to shorter yeast telomeres or a loss of *HDF1* specific telomere interactions, recombination rates of other shorter telomere strains should be determined. One possible target for a recombination study is *TEL1*. *TEL1* is an advantageous target because its elimination results in shorter telomeres, but appears to be defective in cell cycle checkpoints rather than telomere binding (Lustig and Petes, 1986; Weinert *et al.*, 1994). Additionally, *tel1* mutants have a higher rate of chromosome loss and meiotic recombination (Greenwell *et al.*, 1995). After determining the recombination rate and pathway for *tel1Δ* $C_{1-3}A$ cells with the *URA3* cassette system, the results could then be

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compared to the data from *hdf1Δ* cells. If there is no change in recombination rate in *tel1Δ* cells, then the effect in *hdf1Δ* is independent of telomere length. If there is an increase in recombination rate, recombination pathways could be examined. A similar dependence on *RAD1*, would suggest that short telomeres trigger similar type recombination events. However, if *tel1Δ* cells are not dependent on *RAD1*, it would indicate that the absence of the *HDF1* specific telomere binding protein rather than shortened telomere length is responsible for the increase in *RAD1* recombination rate. Finally, if *tel1Δ* recombination increases via a *RAD52* pathway, it could be inferred that multiple recombination pathways can be triggered by mutations that effect telomere length.

The results of this study give additional clues to the mechanism of recombination as an alternative method of maintaining telomere length. The new questions about telomere binding proteins suggest a continued direction for this project. In addition, the answers to these questions may also provide the clues needed to determine why cells have a telomerase-independent mechanism for maintaining telomere length. Finally, because *S. cerevisiae* cells are similar to cancer cells in their methods of maintaining telomere length, the knowledge gained from the yeast recombination pathway may some day be applied to the treatment of cancer.

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