

In Search of Telomeric Fusions in Telomere Mutants of *Tetrahymena thermophila*

Arun George Paul*

Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Summary

The ends of eukaryotic chromosomes are protected by nucleoprotein structures called telomeres consisting of GC-rich repeat sequences. Studies have shown that mutated telomeres are detrimental to the cell, causing chromosomal fusions and increased genomic instability (Dandjinou *et al.*, 1999). Previous research in our lab on *Tetrahymena thermophila* (*T. thermophila*) has shown a severe anaphase arrest (Kirk *et al.*, 1997) and extensive loss of telomere sequences in response to telomeric DNA mutations. In other eukaryotes, telomere loss results in telomeric fusions (Tong *et al.*, 2001). Therefore, in our telomeric mutants I hypothesize the existence of telomeric fusions. In order to test our hypothesis, I developed a polymerase chain reaction (PCR) assay that can detect telomere fusions. Primers were designed from the sequences just adjacent to the telomere called telomere-associated sequences (TAS). I designed a positive control to ensure that our assay could overcome the typical difficulties encountered in amplifying long GC-rich PCR products. Clones of *T. thermophila* TAS sequences were cut and ligated to mimic potential *in vivo* fusion events. PCR on my positive control demonstrated that our assay can amplify telomeric fusions up to 841 bp. Preliminary assays of the mutant DNA did not amplify any fusion products. However, reamplification of the products from PCR on digested DNA yielded products. Southern blot analysis of these products using a telomeric probe showed signals that indicate fusions. However, sequence analysis showed the signals to be artifacts produced by the reamplification. The study suggests that micronuclear fusions up to 841 bp may not exist in our mutants and if they do, the number of such fusion events is beyond the amplification limit of my assay.

Introduction

The Beginning of the End: The World of Telomeres

Milestones of Telomere History

In the field of modern molecular biology, telomere is a common word that resonates with the terms ageing and cancer. By definition, telomeres are the end sections of chromosomes. Although the era of contemporary molecular biology starts with Watson and Crick's discovery of DNA in the early 50s of the twentieth century, the history of telomeres goes further back. The concept of telomeres originated from a set of simple

genetic experiments in the early part of the twentieth century by legendary cytologists Herman Muller and Barbara McClintock in 1938.

The Terminal Gene Hypothesis by Herman Muller

The first person to notice the uniqueness of chromosomal ends was Herman Muller (Muller, 1938). When observed under a microscope with high magnifying power, chromosomes, the genetic material of cells, appear as linear structures with no apparent difference at their tips. Muller changed this idea in 1938 through his classical study, which for the first time created artificial mutation in chromosomes by exposing fruit flies (*Drosophila melanogaster*) to X-rays. His mutants demonstrated several chromosomal aberrations, such as translocations, deletions, and reversions throughout the chromosomes, except in the very ends (Muller, 1938). This intriguing observation led him to propose that chromosomal ends have a specialized structure (Muller, 1938). In his classical lecture at the Woods Hole Marine Biological Laboratory, he concluded that "... the terminal gene must have a special function that of sealing the end of the chromosome, so to speak, and that for some reason a chromosome cannot persist indefinitely without having its ends thus sealed." (Muller, 1938).

The Chromosome Healing Hypothesis by Barbara McClintock

After Muller, Barbara McClintock, another veteran cytologist and Nobel Laureate further developed the idea of the specialized chromosomal ends by demonstrating their potential function as a healer for aberrations in the ends. In her classical study, she observed that broken chromosomes fused to form dicentric chromosomes by a process called breakage-fusion-bridge cycle (McClintock, 1938). However, to her surprise the ends of the chromosomes were never subjected to this process and she called this process "chromosome healing" (McClintock, 1941). Two decades later, Muller gave the term 'telomere' to the specialized chromosomal ends (Muller, 1962).

Telomeres and End-Replication Problem

The interest in chromosomal ends or telomeres further expanded when Olovnikov (1971) and Watson (1972) independently proposed the end-replication problem. DNA polymerases synthesize DNA only in the 5' to 3' direction (Alberts, 1997). Therefore, the lagging strand of the replication fork is created in discontinuous segments also known as Okasaki fragments, where an RNA primer initiates each segment formation (Alberts, 1997). However, at telomeric ends, there is no sequence for the primer to bind and therefore after each division the chromosomes get shorter (Alberts, 1997). This issue became known as the end-replication problem (Alberts, 1997) and was fascinating for the time of Olovnikov and Watson, since, the mechanism that single-celled organisms and germ cells used to maintain their chromosome length after each cycle of replication was unknown. However, the solution that nature had evolved to solve this problem remained a mystery for another decade.

* The report of the investigation was undertaken as a Senior Thesis with Dr. Karen Kirk in the Department of Biology.

Discovery of Telomeric DNA by Elizabeth Blackburn

Probably the most fascinating part of telomeric history was the discovery of the uniqueness of telomeric DNA. In 1978, Elizabeth H. Blackburn and Joseph G. Gall showed that in *T. thermophila*, unlike normal DNA sequences consisting of nucleotides sequences with no apparent pattern, telomeres consists of short repeats of GGGGTT (Blackburn and Gall, 1978). After her discovery, similar results were reported from several organisms, such as yeast (Shampay and Blackburn, 1984), *Arabidopsis thaliana* (*A. thaliana*) (Richards and Ausubel, 1988), and humans (Moyzis *et al.*, 1988). The discovery of telomeric DNA by Blackburn, besides being fascinating for its uniqueness also intrigued scientists, since it provided an intellectual foundation upon which novel hypotheses that explain the end replication problem can be made.

Research for the last two decades, since the discovery of telomeric DNA by Blackburn and Gall (1978) has further expanded our understanding of telomere structure. By definition, telomeres are now considered as DNA-protein complexes. Either component is vital for the proper functioning of the telomeres.

Telomeric DNA: Studies have shown that telomeric repeats do not vary much between different species (McEachern *et al.*, 2000). For example, a number of species, such as *Aspergillus nidulans* (Bhattacharya *et al.*, 1997), have the vertebrate telomere sequence TTAGGG. The reason why there are similar telomere sequences between diverse species is still an intriguing aspect of telomeres. Potential explanations include the conservation of DNA binding domains between the telomere maintenance proteins of different species (McEachern *et al.*, 2000). Although telomeric sequences are similar between several species, they can also vary in size and type. The number of telomeric repeats ranges from several thousands, as in humans, which consist of ~60,000 TTAGGG sequences, to a few hundred GGGGTT repeats, as in the macronucleus of *T. thermophila* (Kirk and Blackburn, 1995). Another significant variation observed in telomeric tracts is the existence of more than one type of repeat in the same chromosome (McEachern *et al.*, 2000). For instance, micronuclear telomeres of *T. thermophila* have an internal G4T3 telomeric tract (0.6 -1.0 kb) along with the external G4T2 tract (1.4 - 2.1 kb; Kirk and Blackburn, 1995). In some cases, such as with *Saccharomyces cerevisiae* (*S. cerevisiae*), *Saccharomyces pombe* (*S. pombe*) (Shampay and Blackburn, 1984) and Chlorarachinophyte algae (Gilson and McFadden, 1995), the complete set of telomere sequences also can vary between the chromosomes.

Telomere Protein Components: The protein components of telomeres, also known as telomeric proteins, form parts of the telomeric nucleo-protein complex and are well characterized in humans, mouse, yeast, and ciliated protozoa (McEachern *et al.*, 2000). They are differentiated on the basis of the type binding to telomeric sequences. The two main classes of telomeric proteins are the single and double-stranded DNA binding proteins. They are well characterized in *S. cerevisiae* (Klobutcher *et al.*, 1981; Shore and Nasmyth, 1987 & Larson *et al.*, 1994), *S. pombe* (Copper *et al.*, 1997 & Vassetzky *et al.*, 1999) and humans (Broccoli *et al.*, 1997; Chong *et al.*, 1995). The significance of these proteins for the stability of

telomere structures (Conrad *et al.*, 1990), telomere length regulation (Lustig *et al.*, 1990; Steensel and de Lange, 1997), and the requirement of having an optimal amount of these proteins for telomere maintenance and stability (Steensel *et al.*, 1998) has been well demonstrated.

Discovery of Telomerase by Carol Greider and Elizabeth Blackburn

A decade after the discovery of telomeric DNA by Blackburn (1985), she also formulated the solution to the end replication along with her graduate student Carol Greider by the discovery of the enzyme terminal transferase (telomerase) in *T. thermophila* (Greider and Blackburn, 1985). They showed that telomerase is a ribonucleoprotein complex with an RNA template called telomerase RNA later called TER (Greider and Blackburn, 1987) with complimentary sequences to the respective telomere sequence (Lentz *et al.*, 1990). Telomerase uses RNA as the template to synthesize novel telomere repeats (Fig. 1). The synthesis of telomeres is conducted by telomerase Reverse Transcriptase (TERT), the reverse transcriptase that forms the enzymatic core of the telomerase, which is characterized in several species, including *T. thermophila* (Collins and Gandhi, 1998) and humans (Lingner *et al.*, 1997 & Nakamura *et al.*, 1997).

Even though eukaryotic telomere length is maintained by telomerase, non-telomerase based mechanisms or alternate lengthening of telomeres (ALTs) also exist. For example, both *S. cerevisiae* (Lundblad and Blackburn, 1993 & Mangahas *et al.*, 2001) and *K. Lactis* (McEachern and Blackburn, 1996) is shown to maintain their telomeres without telomerase through chromosomal recombination pathways (Bechter *et al.*, 2003). Another example is *Drosophila melanogaster*, where telomeres are extended by insertion of specific DNA sequences called retro-transposons (Biessmann and Mason, 1997).

The Telomeres-Ageing-Cancer Connection

The link between telomeres, telomerase, and the end-replication problem also resulted in the formulation of novel ideas regarding ageing and cellular immortality. Ageing refers to the limited replicative potential of the cell. This phenomenon was first demonstrated by Hayflick and Moorehead (1961) in skin fibroblast cells, which had a replicative potential of 50 cell divisions. In 1989, the connection between telomeres and ageing was first demonstrated in *S. cerevisiae*, where a defect in telomere elongation resulted in cellular senescence (Lundblad and Szostak, 1989). The study showed that in the absence of telomerase, chromosomes shortened after each cycle of replication and lead to chromosome instability that subsequently activates the programmed cell death machinery (Lundblad and Szostak, 1989).

Cancer refers to uncontrolled cell growth or the ability of the cells to bypass ageing. Conceptually, cells with active telomerase will maintain their telomere length and will therefore be immortal. In fact, several studies have demonstrated that cells will become immortal and cancerous, when telomerase is active (Greider, 1998; Multani *et al.*, 1999). In general, telomerase is inactive in all somatic cells (Harley *et al.*, 1990; Kim *et al.*, 1994) and therefore their telomeres will shorten as we age (Allsopp *et al.*, 1992). The exceptions are germ line cells (Kim *et al.*, 1994) and

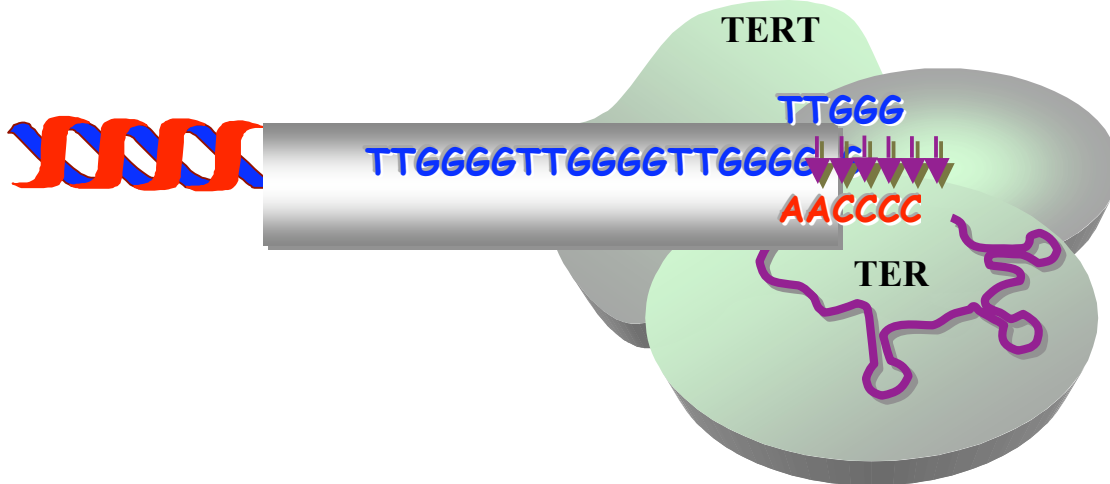


Figure 1. Model for Telomere repeat addition by *T. thermophila* telomerase

The structure of telomerase consists of TER (RNA component) and TERT (catalytic core). The TERT acts as a reverse transcriptase and uses the TER for the *de novo* addition of telomeric repeats using the telomerase RNA template (picture by Kirk (1996).

embryonic cells (Kilian *et al.*, 1998) that need to maintain their telomere length. Many scientists also propose that telomerase activity is an excellent marker for certain type of cancers (McKenzie *et al.*, 1999). Interestingly, this topic has been further investigated due to the recent discovery of shortened telomeres and their corresponding shortened life spans in cloned organisms (Shiels *et al.*, 1999).

Discovery of the D-Loop-T-Loop

After the discovery of telomeric DNA, telomerase, and consequently the revelation of the telomere-ageing-cancer connection, one of the most fascinating discoveries of chromosome biology is the telomere-loop or t-loop. The first hint for this discovery came from Henderson and Blackburn (1989), who showed that the end of *T. thermophila* telomeres consists of a 3' single stranded overhang. In humans, the G-overhang consists of 75-300 bp of TTAGGG repeats (Wright *et al.*, 1999). A decade later, the examination of telomere structure using electron microscopy by Griffith and de Lange (1999) showed that the very end of telomeres form a large loop of ~10-20 kb. The study also showed that the 3' overhang of the telomere invades the double stranded DNA and base pairs with a homologous telomeric strand to form a smaller loop (~100-200 bp) called the D-loop (Fig. 2; adapted from Grieder, 1999). Further studies have revealed that the D-loop provides a binding site for the telomere binding protein TRF2 and stabilizes the loop structure (Grieder, 1999). Structurally, the present model proposes that both the t-loop and the D-loop provide binding sites for several telomere-binding proteins (Grieder, 1999).

The discovery of the t-loop with proteins bound to it was so profound that it immediately proposed an architectural model to the mechanisms that protected telomeres from being degraded or fused. The model also explained how telomere shortening is linked to senescence and apoptosis (Evans and Lunblad, 2000). When telomere length becomes shorter than the minimal amount (~300-500 bp) to form the t-loop, proteins like TRF1 and TRF2 will not bind to it. This, in turn will expose the free telomeric ends to DNA degradation (Evans and Lunblad, 2000), which can lead

to apoptosis in a p53-dependent DNA-damage response pathway (Evans and Lunblad, 1999).

Although the discovery of the t-loop-D-loop was intriguing, classical telomere biology was explained completely on the basis of linear telomeres. Conceptually, therefore it was necessary to propose a mechanism that exposed telomeres to telomerase by uncapping the telomere protein-loop structure (Grieder, 1999). The present model suggests that the t-loop complex and its proteins exist as a feedback system that regulates telomere length by controlling the availability of telomeres to telomerase (Dubrana *et al.*, 2001). The model proposes the existence of two physical states for telomeres—a closed and open state (Dubrana *et al.*, 2001). During the open state, DNA-binding proteins in general forms a high order complex that prevent the accessibility of telomeres to telomerase and vice-versa in closed state (Dubrana *et al.*, 2001).

The Puzzle of Double-Strand Breaks

Although, several of the telomere puzzles have been resolved, there is one that still fascinates and haunts telomere researchers from McClintock's time. In 1941, McClintock demonstrated the phenomenon of 'chromosome healing,' where the ends of the chromosomes behaved differently from ends created by chromosomal breakage. When chromosomes break, the free ends are known as double-strand breaks (DSBs), which are repaired by a mechanism known as non-homologous recombination (NHEJ) (Blackburn, 2001). The major molecular players of the NHEJ pathway are the Ku heterodimer, Sir family of proteins and the Mre11p-Rad50-Xrs2 complex (Bertuch and Lundblad, 1998). Being the ends of the chromosomes, telomeres often become uncapped during replication and are structurally the same as DSBs (Blackburn, 2001). However, they do not fuse by the repair mechanisms activated by NHEJ, since most cells possess an unknown molecular machinery that prevent telomeres from being identified as DSBs (Blackburn, 2001).

Initially, scientists thought of the existence of a special mechanism that inactivates NHEJ machinery on telomeres. Paradoxically, the NHEJ protein Ku is

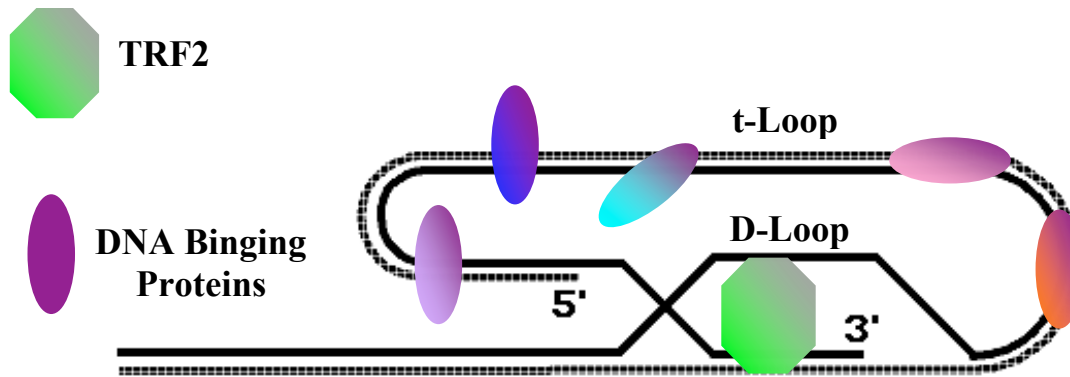


Figure 2. Telomere t-Loop-D-Loop (adapted from Grieder, 1999)

The figure shows the current model for the looping end of telomeres (Grieder, 1999). The model proposes that both the t-loop and D-loop provide binding sites for several telomere-binding proteins (Grieder, 1999). Studies have shown that the stability of the D-loop depends on the binding of TRF2.

also found to be a telomere binding protein (Haber, 1999) that is essential for nuclear organization of telomeres (Laroche *et al.*, 1998), telomere length maintenance (Gasser and Susan, 2000) and most importantly for telomere protection from DNA degradation (Baumann, 2000), fusions (Hsu *et al.*, 2000). DNA cross-linking assays and immunofluorescence have demonstrated that Ku can bind to telomere heterochromatin and to telomere repeats (Hsu, 1999). When there is a DSB, Ku gets delocalized from telomeres and activates the NHEJ repair machinery (Gasser and Susan, 2000).

To make matters more complicated, similar to Ku mutations, mutations in other NHEJ proteins including Mre11p (Gasser and Susan, 2000) and DNA-Pkcs (Gilley *et al.*, 2001) can also result in telomere length impairment. This suggests that the DNA repair machinery for DSBs and telomere maintenance overlaps at several stages. Studies have also shown that this enigmatic sharing of the DSB repair and telomere maintenance is conserved from yeasts to humans (Gasser and Susan, 2000). Nevertheless, no one knows why the same molecular machinery repairs telomeres and DSBs.

Many of the insights to the puzzle associated with the overlap in the molecular mechanisms that repair DSBs and maintain telomere length came from the genetic experiments on yeast (Gasser, 2000). They showed that the Ku heterodimer, Sir family of proteins and the Mre11p-Rad50-Xrs2 complex that are necessary for DSB and telomere maintenance are regulated by the S-phase checkpoint proteins Tel1p (yeast) or ATM (humans) (Craven *et al.*, 2002). This discovery quickly provided a hypothetical solution to the "DSB-Telomere-NHEJ" dilemma. The study suggested that it is advantageous for the cell to have the same machinery acting differently for both DSBs and telomere dysfunction and therefore maintaining chromosome stability, since, both events takes place during or after DNA replication (Gasser and Susan, 2000).

The other major question in the field of telomeres and DNA repair is the ways and means by which the cell differentiates open-ended telomeres from DSBs. Answers to this question also came from the genetic studies on yeast have that led to the discovery

of two NHEJ proteins Taz1 (Ferreira *et al.*, 2001) and NEJ1 (Liti *et al.*, 2003). Taz1 is a telomere binding protein, whereas NEJ1 works with NHEJ ligases LIF1 and DNL4 (Liti *et al.*, 2003). Both interact with telomeres and prevent them from being treated as DSBs (Liti *et al.*, 2003). Although, extensive research is being done in this field, to this day, the detailed molecular and cellular processes underlying the DSB-telomere dilemma or McClintock's 'chromosome healing' phenomenon is still a mystery.

Telomeres and Cell Cycle

In addition to the protective function, telomeres have recently been found to play an important role in meiosis. The potential link between telomeres and cell cycle was first demonstrated by Chikashige *et al.*, (1994), who showed the clustering of telomeres during prophase in *S. cerevisiae*. Further investigation of this link demonstrated that alteration of telomere sequences (Kirk *et al.*, 1997) or deletion of telomere associated meiotic proteins like NDJ1 (Conrad *et al.*, 1997) or Tam1 (Chua and Roeder, 1997) can result in abnormal phenotype and growth patterns. Within the last seven years, several other links between telomeres and meiosis had been characterized such as the nucleolus-associated clustering of telomeres before the formation of synapses (Armstrong *et al.*, 2001) and the requirement of functional telomeres for successful chromosomal alignments (Funabiki *et al.*, 1993) and for the integrity of spindles (Liu *et al.*, 2002)

Current Status of Telomere Biology and Our Lab's Goal

Within the last two decades, telomeres have thus emerged as one of the most well studied fields of cell and molecular biology. The t-loop and its structural modifications on a timely basis for telomere maintenance has been one of the major focuses of research for the last five years (Evans *et al.*, 1999). The complexity of telomerase structure, its regulation and its associate components other than hTERT and TER are also being investigated extensively (Evans *et al.*, 1999). Along with research on telomerase mediated telomere maintenance, scientists are also investigating ALTs in yeasts and *Drosophila* (Evans *et al.*, 1999). Initial studies on telomeres were based completely on

single celled eukaryotes like *T. thermophila*. However, the last decade has also witnessed an expansion by including worms (*C. elegans*) and plants (*Arabidopsis thaliana*) (Evans *et. al.*, 1999). And as previously mentioned, the connection of DNA repair pathways, cell cycle progression and telomeres is also a growing area of extensive research (Gasser *et. al.*, 2000).

Our lab's goal is to study the DNA component of telomeres in *T. thermophila*. Most recent studies in the lab have been focused on telomeres and cell cycle. A common way to approach this is by altering the telomere sequence using a telomerase with a mutated RNA template sequence that can create sequence specific mutations at the telomeric termini (Yu *et. al.*, 1990). The different *T. thermophila* mutants created in our lab are 43A, 43AA & 44+AA and are so called because of the specific telomeric sequence alterations (Petcherskaia *et. al.*, 2003). Previous studies by Kirk *et. al.*, (1997) on these mutants showed an arrest at anaphase with absence of chromosome segregation. We have further characterized the cell's response to the mutation and have investigated the molecular basis for the defect. Examination of whether the cell cycle continues despite the block in anaphase demonstrated a dramatic increase in the proportion of micronuclei with condensed chromatin, as well as an increase in the proportion of cells with an intact mitotic spindle apparatus (Christ, 2002). Further investigation of the 43AA mutant by southern blot analysis revealed that telomere sequence mutations have also resulted in extensive degradation of the micronuclear telomeric DNA (unpublished).

My Senior Thesis Project

The purpose of my study was to further extend the investigation of the molecular basis of anaphase arrest we observed in our telomere mutants. Although, we have demonstrated extensive micronuclear telomere degradation, we do not know whether the degraded DNA is treated as DSBs. I predicted that the mutations we induced in the telomeric DNA inhibited the binding of the terminal telomere binding proteins thereby destabilizing the t-loop-D-loop cap structure of telomeric ends. Therefore, the exposed telomere would be subject to the activity of exonucleases resulting in extensive DNA degradation, as we have observed. However, we are unaware of the extent, to which the DNA has been degraded and we predict the existence of three types of degraded chromosomes (Fig. 3). Several studies have shown that shortened telomeres (McEachern and Iyer, 2001) and sequence specific telomeric mutations (McEachern *et. al.*, 2000) can result in telomere fusions. The formation of fusions might occur because degraded telomere is no different from DSBs and will, therefore be modified by the NHEJ machinery leading to the fusion of chromosomes. Therefore, I hypothesize that in our *T. thermophila* telomere mutants, the extensive micronuclear DNA degradation resulted in the formation of telomeric fusions. Based on previous studies in the lab (unpublished), I have excluded existence of the fusions of non-degraded telomeres (Fig. 3) and hypothesize the existence of three types of fusions (Fig. 3).

In order to test our hypothesis of telomere fusions, we investigated the possibilities of several candidate techniques used in other studies to detect chromosomal fusions, such as pulse field gel electrophoresis (Liti, 2003), polymerase chain reaction (PCR) (Hackett *et. al.*, 2001; Chan *et. al.*, 2003), and

fluorescent *in situ* hybridization (FISH) (Espejel *et. al.*, 2002). However, pulse field gel electrophoresis and FISH assay cannot be applied because they are incapable of specifying telomeric fusions. In addition, our lab lacks the technical facilities to conduct pulse field gel electrophoresis, and FISH antibodies specific to the *T. thermophila* have not been developed. Therefore, we developed a PCR assay that can detect telomeric fusions. Primers were designed from the sequences just adjacent to the telomere of *T. thermophila* called telomere-associated sequences (TAS) (Fig. 3), which were cloned by Kirk *et. al.*, (1995). We also designed a positive control to ensure that our assay could overcome the typical difficulties encountered in amplifying long GC-rich PCR products. PCR on the positive control demonstrated the ability of our assay in detecting telomeric fusions up to 841 bp. Among the different types of fusions listed in Fig. 3, we are capable of detecting Types II and III, since our primers are designed from TAS sequences. Although, preliminary assays of the mutant DNA did not amplify any fusion products, reamplification of the products from PCR on digested DNA yielded potential fusion bands. However, sequencing of these bands showed them to be non-telomeric sequences.

Results

Creation of Artificial Telomere Fusions

To investigate the existence of telomeric fusions, I used PCR with primers designed from TAS sequences. Unfortunately, telomeres are notoriously difficult to amplify by PCR because a) the high GC content of telomeres can prevent the double stranded template of the PCR from being denatured completely thereby preventing the efficient binding of the primers and enzymes and b) telomeres can inhibit PCR by the formation of secondary structures. Therefore, to test the potential of our PCR assay in detecting telomere fusions, we developed a positive control by creating artificial fusions and testing our primers on it. To accomplish this, we used the *T. thermophila* TAS sequences cloned by Kirk *et. al.*, (1995). We cut out the TAS sequences with telomeric tracts by restriction digest with Sac I and EcoR V and were ligated to create artificial fusions. The TAS sequences I selected were called TAS A & TAS C, with sizes 296 bp and 511 bp, respectively (Fig. 4). TAS A has 9 G4T3 and 23 G4T2 repeats, and TAS C has 37 G4T3 and 26 G4T2 repeats (Fig. 4). The expected sizes of the different fusions of TAS C & TAS A were 606 bp, 841 bp, and 1056 bp for the artificial telomeric fusions types: a) TAS A & TAS A, b) TAS C & TAS C, and TAS C & TAS A, respectively (Fig. 4).

The next goal of the project was to test the artificial fusions with the primers we designed from the TAS sequences. We conducted PCR on the artificial fusions using the respective primers and the products were run on a 1.5% agarose gel. The gel (Lane 1, Fig. 5) shows two bands of expected sizes for the artificial fusions A & A11 (606bp) and C & A (841bp). However, we were unable to amplify the C & C fusion band. This suggests the existence of a potential size barrier for the PCR assay. We predict this to be due to the longer stretches of the telomere tracts of TAS C & C artificial fusion, when compared to the other two types of artificial fusions (Fig. 4), thereby making the PCR vulnerable to all the issues that is related to the amplification of GC-rich sequences.

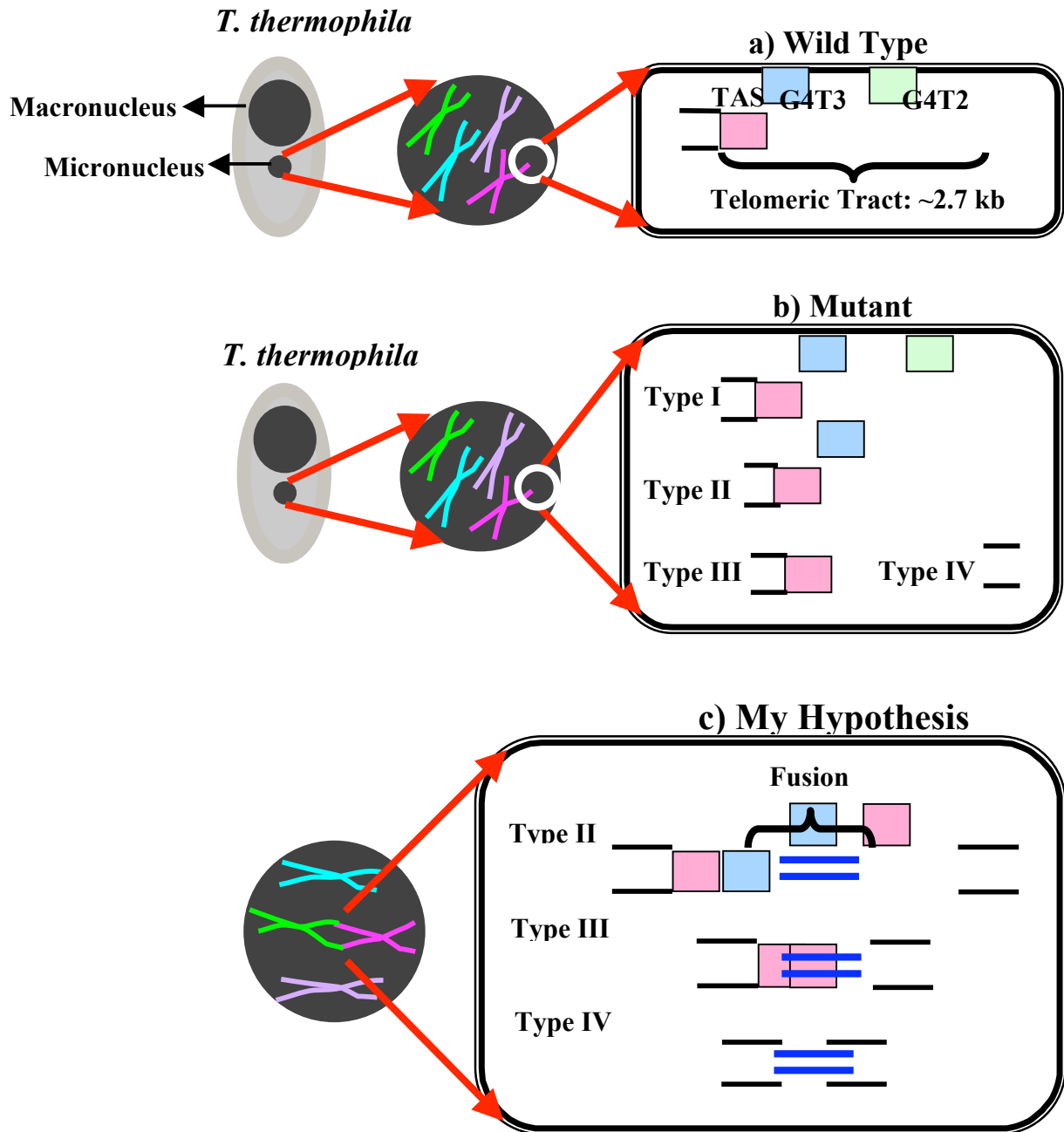


Figure 3. Different types of fusions that can potentially exist. The figure of *T. thermophila* was adapted from Asai et. al., (2000) The wild type micronuclear DNA (a) consists of a telomeric tract of size ~2.7 kb with the internal and external telomeric tracts G4T3 and G4T2, respectively. In our *T. thermophila* telomere mutants, previous studies have suggested the types of degraded telomeres potentially existing (b), based on which I hypothesized the existence of three types of chromosomal fusions (c). Among these (c), we are searching for Type II and Type III, since our primers are designed from the TAS sequence.

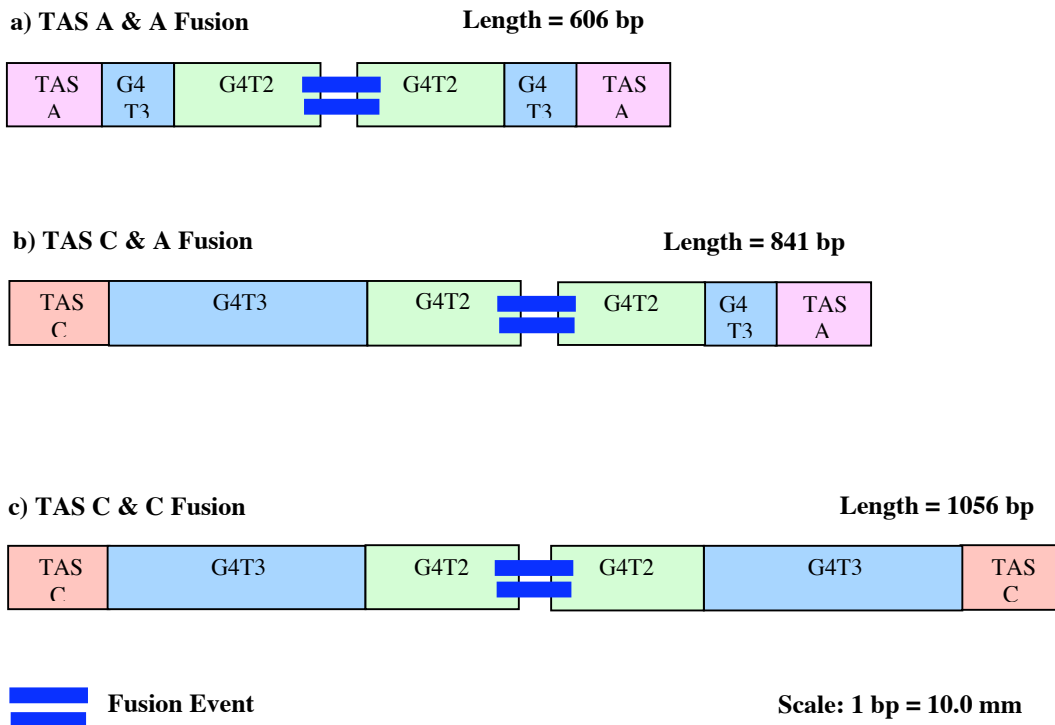


Figure 4. Types of Artificial Telomere Fusions

TAS sequences with telomeric tracts were cut out by restriction digest with TAS A and TAS C having a size of 296 bp and 511 bp, respectively. They were ligated to create three types of fusions : a) TAS A & A Fusion (606 bp), b) TAS A & C Fusion (841 bp), and c) TAS C & C Fusion (1056 bp). The sizes of the images are scaled to 10 mm for 1 bp.

Optimization of the PCR Assay

To determine the minimum amount of the artificial fusions and optimal annealing temperature that are required for our PCR assay, different dilutions (1:10, 1:100 & 1:1000) of the artificial fusions were tested using TAS C and TAS A primers (Materials & Methods) at temperatures ranging between 56°C and 58°C (Fig. 5). The PCR products were run on a 1.5% agarose gel (Fig. 5) and it shows the ability of the PCR in amplifying telomeric fusions: a) A & A (606 bp), b) C & A (841 bp) at annealing temperature 56°C from all the three dilutions (Fig. 5; Lanes 1, 2, & 3). But at 58°C, the efficiency of the PCR to detect fusions from the 1:1000 dilution dramatically decreased (Fig. 5; Lanes 5, 6, and 7). This optimization experiment indicated that our assay was most efficient, with minimal non-specific binding at an annealing temperature of 56°C and with a template concentration of 1:10 or 1: 100 of the artificial fusions (Fig. 5; Lanes 1 & 2).

Although, my assay was successful in detecting two artificial fusions with expected sizes (Fig. 5), the PCR also amplified several bands of unexpected sizes. The prevalence of these bands was less dramatic with decrease in the concentration of the artificial fusion (Lanes 1, 2, 3, 5, 6, & 7; Fig. 5), suggesting that they were due to the non-specific binding of the primers. However, the existence of non-specific products questions the specificity of the PCR products. Therefore, we conducted a nested PCR using primers PRX TAS C and PRX TAS A. These primers were designed 3' to the primers TAS A and TAS C. As expected, the nested PCR demonstrated a decrease in

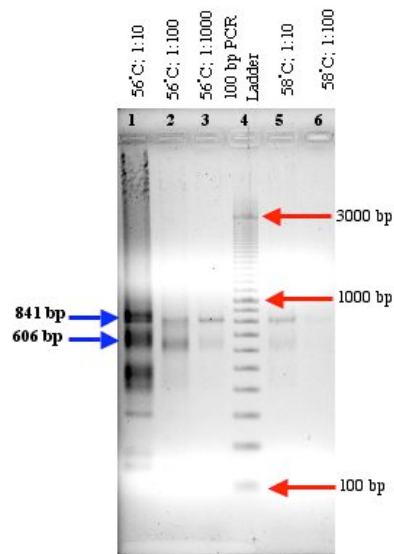


Figure 5. Thermal & Volumetric Optimization of the PCR assay

PCR was conducted on the different dilutions of the A & C artificial fusions at annealing temperatures 56.00C & 58.00C and was run on an agarose gel. The content of each lane is labeled with the respective annealing temperature and template dilution used for each PCR. The gel shows the A & A and A & C fusions with expected sizes 841 bp and 606 bp (blue arrows), but lacks the C & C fusion band of size 1056 bp.

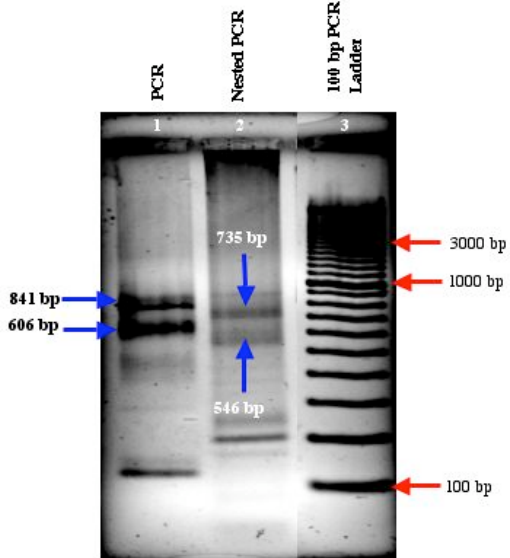


Figure 6. Nested PCR

PCR was conducted on the 1:10 dilution of the C & A ligates at 56.00C. One μ l of the product was then used for the nested PCR. Both products were run on an agarose gel. The content of each lane is as follows, Lane 1: PCR on 1:10 dil. of C & A ligate at 56.00C, Lane 2: Nested PCR, Lane 3: 100 bp PCR Ladder.

the sizes of the PCR products we predicted to be artificial (Fig. 6; Lanes 1 & 2). The C & A (841bp) and A & A fusion products (606bp) show a decrease of 94 bp and 140 bp to give a 735 bp and a 546 bp fragment, respectively (Fig. 6; Lanes 1 & 2). In addition, the non-specific bands produced by the assay were not reproducible and those that did reappear did not drop down (Fig. 6), thereby further confirming the specificity of our assay.

43AA genomic DNA can inhibit the efficiency of our PCR assay

My assay succeeded in detecting artificial telomere fusions. However, I anticipated that when repeated in the presence of genomic DNA, the PCR efficiency would decrease. This is because genomic DNA can act as a competitor by providing multiple sites for primer binding or can inhibit the reaction due to its complex structure and sequence complexity. Therefore, it was necessary to test the PCR efficiency in the presence of genomic DNA. I aimed to mimic the *in vivo* situation, where my assay needs to amplify the fusions from a large amount of genomic DNA. Ultimately, my goal was to estimate the minimum number of fusions the assay requires to amplify it from the maximum amount of the genomic DNA. I addressed this issue in two steps.

Firstly, I was interested in examining the minimal amount of 43AA genomic DNA that could compete or inhibit the PCR reaction. Therefore, PCR was conducted on 1:10 dilution of the artificial fusion mixed with different dilutions (Materials & Methods) of the 43AA genomic DNA. The products were run on a 1.5% agarose gel (Fig. 7). The absence of any bands in Lane 1 (Fig. 7) demonstrated that either the genomic DNA itself or an unknown factor present with the genomic DNA inhibited PCR drastically. However, as we decreased the amount of genomic DNA in serial dilutions, the artificial fusion bands of expected sizes (841 bp & 606 bp) were amplified (Lanes 2 through 7;

Fig. 7). Between the different dilutions (Lanes 2 through 7), the PCR efficiency was similar, since the intensity of the fusion bands (841 bp & 606bp) was invariant (Lanes 2 through 7; Fig. 7). This suggested that the competitive/inhibitory roles of either the genomic DNA or the unknown factor associated with the genomic DNA was decreased by dilution. The experiment also shows that the maximum amount of genomic DNA that potentially may not affect PCR efficiency and still can be used as a PCR template is a 1:10 dilution, although smaller dilutions needs to be considered.

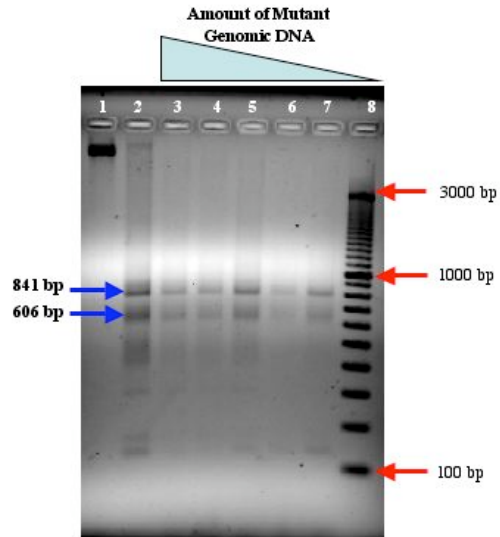


Figure 7. Diluted 43AA Genomic DNA did not affect PCR efficiency

PCR was conducted on the different dilutions of the 43AA genomic DNA along with C & A ligate at 56.00C and was run on an agarose gel. Lane 9 consists of 10 μ l of the 100 bp PCR Ladder. Lanes 2 to 7 consist of products from PCR on 1:10 dilution of the artificial fusion mixed with: non-diluted genomic DNA (Lane 1), 1:10 (Lane 2), 1:100 (Lane 3), 1:1000 (Lane 4), 1:10,000 (Lane 5), 1:100,000 (Lane 6), and 1:1,000,000 (Lane 7) dilutions of the 43AA genomic DNA.

Secondly, I estimated the minimum amount of artificial fusions that the assay required as templates in the presence of 1:10 dilution of genomic DNA. PCR was conducted on non-diluted artificial fusion and on different dilutions of the artificial fusions in the presence of 1:10 dilution of 43AA genomic DNA. The gel for the PCR products (Fig. 8) shows successful amplification of fusions (841 bp & 606 bp) from non-diluted and 1:10 dilution of the artificial fusion in the presence of genomic DNA. However, the PCR was unable to amplify any fusions from dilutions above 1:10 (Fig. 8; Lanes 4 through 7). Thus, I demonstrated that my assay had a concentration limit to successfully amplify telomeric fusions. The experiment shows that 1:10 dilution of the artificial fusion was the minimum amount of the artificial fusion that could give rise to a fusion product by PCR in the presence of 1:10 dilution of 43AA genomic DNA (Fig. 8).

Considering the results of the two experiments (Fig. 7 & Fig. 8), I anticipated the molecular problems caused by the inhibitory roles of genomic DNA and the minimum number of fusions that are required for our assay to amplify. The first experiment (Fig. 7) suggests that any dilutions less than 1:10 of genomic DNA can inhibit the PCR. The second

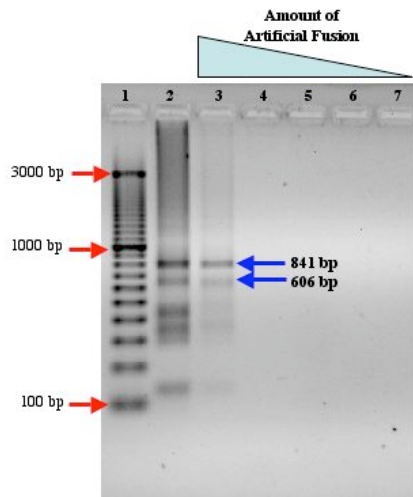


Figure 8. 43AA Genomic DNA did not inhibit PCR on 1:10 dilution of artificial fusions

PCR was conducted on the different dilutions of C & A ligate along with 1:10 dilution of 43AA genomic DNA at 56.00C and was run on an agarose gel. Lane1 consisted of 100bp PCR Ladder. Lanes 2 to 7 consist of products from PCR on 1:10 dilution of the 43AA genomic DNA mixed with: non-diluted (Lane 2), 1:10 (Lane 3), 1:100 (Lane 4), 1:1000 (Lane 5), 1:10,000 (Lane 6), and 1:100,000 (Lane 7) dilutions of the artificial fusion.

experiment (Fig. 8) shows that any dilution above 1:10 might by pass the minimum number of template fusions required for the PCR to amplify. Based on these two results, I concluded that to yield the maximum efficiency of the PCR, I should use 1:10 dilution of the 43AA genomic DNA to investigate the existence of telomeric fusions

PCR on 43AA DNA does not give any PCR Products

Our PCR assay was able to detect fusions up to 841 bp long. Therefore, I anticipated the possibility of detecting fusions from the 43AA genomic DNA within this size range. For PCR, I used a 1:10 dilution of 43AA genomic DNA, since, non-diluted genomic DNA demonstrated to be inhibitory to PCR. All the primers combinations possible from the different primers (TAS A, TAS B, TASC, TAS D, TAS E, and TAS F) were used for the PCR. However, we did not see any products (data not shown). Similar kinds of results were seen when PCR was conducted on 1:1000 dilution of 43AA genomic DNA (data not shown).

PCR on digested 43AA DNA does not give any PCR Products

The absence of fusion products from 43AA genomic DNA by PCR suggested several possibilities, such as: a) micronuclear telomeric fusions may not exist, b) the size of the telomeric fusions existing are larger than the amplification potential of our PCR assay, and c) the genomic DNA itself is inhibiting the efficiency of the PCR due to its sheer size. To resolve last issue, I decided to digest the 433AA genomic DNA with the enzyme Mse I. We anticipated that the digestion would reduce the secondary structure formation of the genomic DNA, if it were the causative agent for reducing PCR efficiency, thereby increasing its availability for PCR. Another issue that concerned the assay was the low GC content and short lengths of the

primers we used (Table 1). Therefore, we designed new primers (Last 3 rows; Table 1) with longer size and high GC content that could anneal to the template for longer periods thereby increasing the efficiency of PCR.

PCR was then conducted on the digested 43AA genomic DNA using the new primers. The positive control consisted of the artificial fusion with digested non-diluted 43AA genomic DNA. The PCR did not detect any fusion products (Fig. 9; Lane 3 through 8), as we did not see any bands. However, PCR on the artificial fusions in the presence of non-diluted 43AA DNA did show the expected band (Fig. 9; Lane 1). This was an encouraging result. Because, previous parts of this study had shown the inability of the assay to detect artificial fusions in the presence of non-diluted genomic DNA (Fig. 7, Lane 2). This suggests that the digestion of the DNA and the new primers had indeed increased the efficiency of the PCR. The absence of any telomeric fusion bands from mutant genomic DNA might be because of their low numbers.

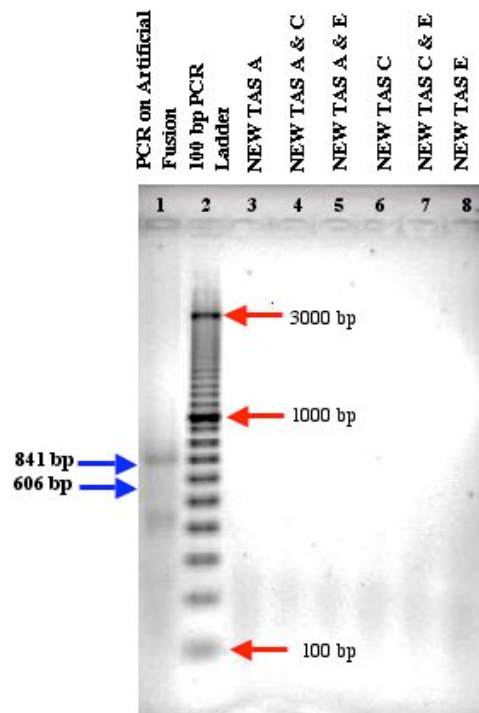


Figure 9. PCR on digested 43AA genomic DNA does not give products

PCR was conducted on Mse I digested 43AA genomic DNA and was run on an agarose gel. Newly designed primers with high GC content and longer size were used (Materials & Methods). Annealing Temperature: 65.00C. Lanes 1 & 2 consists of PCR on 1:10 dilution of artificial fusion with primers NEW TAS A & NEW TAS C and 100 bp PCR Ladder, respectively. Lane 3 to 9 consist products from PCR on Mse I digested 43AA genomic DNA with the "NEW TAS" primers: Lane 3: A alone, Lane 4: A & C, Lane 5: A & E, Lane 6: C alone, Lane 7: C & E, Lane 8: E alone.

Detection of Potential Fusions After the Reamplification of the PCR Products

The initial set of experiments had demonstrated that my PCR assay requires a certain number of telomeric fusion events as the minimum number to amplify them. Therefore, a potential reason for the absence of any products by PCR on 43AA genomic DNA (Fig. 9) might

have been the low number of fusion events. To resolve this problem, the PCR products from 43AA genomic DNA were reamplified by a second cycle of PCR. I predicted that the additional recycling should yield the potential fusion bands that are amplified to the PCR's minimum template level by the initial 30 cycles. However, I also anticipated the amplification of a number of non-specific products due to non-specific binding of the primers during reamplification. Therefore, the experiment was repeated on wild type (WT) genomic DNA, which according to our hypothesis does not possess any telomeric fusions.

Surprisingly, PCR yielded products from both WT (Fig. 10; Bottom row) and 43AA genomic DNA (Fig. 10; Top row) templates. Lanes 4, 7, and 10 in each row (Fig. 10) are followed by the corresponding reamplification products. The reamplification of the positive control (Fig. 10; Lane 4; Row 1) shows several non-specific bands. Overall, each of the lanes containing the reamplification products demonstrates extensive smearing suggesting the amplification of several non-specific products. Interestingly, none of the products from WT DNA template is of the same size as the ones from 43AA genomic DNA suggesting that either telomeric fusions exist in low number in both WT and 43AA genomic DNA or that the PCR products are due to the non-specific binding of the primers at different sites of the template DNA.

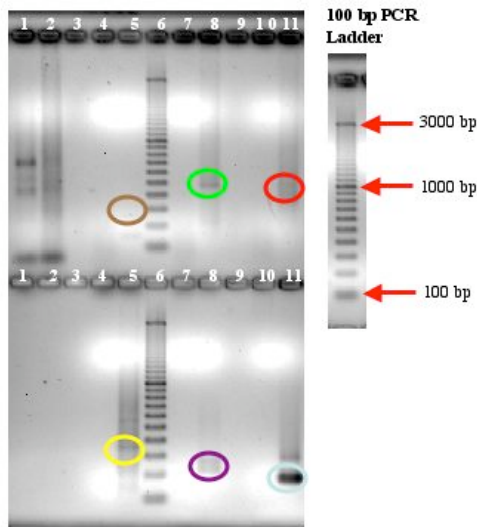


Figure 10. Reamplification of PCR on digested 43AA genomic DNA give products

One μ l of the product from the PCR on 1:10 dil. of digested 43AA genomic DNA was used for reamplification by another cycle of PCR. The positive control (Lane 3) was also reamplified (Lane 4). Lanes 4, 7, and 10 consist of products from PCR on Mse I digested 43AA genomic DNA with primers: NEW TAS A, NEW TAS C, and NEW TAS E, respectively. Lanes 5, 8, and 11 are the products of the reamplification PCR conducted on the contents in Lanes 4, 8, and 11, respectively. The contents in the bottom row are similar to the top row except that the template used was wild type DNA and the positive control was not conducted again.

In order to confirm whether the bands were due to telomeric fusions, we conducted southern blot analysis on the gel from Fig. 10 (Fig. 11) using a telomeric probe specific to micronuclear telomere sequence G4T3 (Fig. 4). The southern blot exhibited strong telomeric (G4T3) signals at those sections of the gel (Fig. 10; Lanes 6, 9,

& 12) that corresponds to the reamplification products from both WT (Fig. 11; Bottom Row) and 43AA genomic DNA (Fig. 11; Top Row). A closer analysis shows that the intensity of the signals increases at those points in the Southern Blot (Fig. 11) where a potential fusion band was observed in Fig. 10. The strong presence of G4T3 signals further suggests that the PCR products from both WT and 43AA genomic DNA might be fusions.

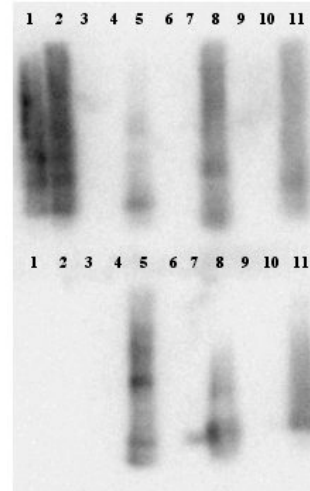


Figure 11. Southern Blot Analysis of the Reamplification Products

Southern Blot was conducted on the gel from Fig. 10 using a G4T3 probe. The annealing and wash temperatures were 55.00C and 60.00C, respectively. The presence of signals in reamplifications lanes in both Row 1 (43AA DNA) and Row 2 (Wild type) suggests either the presence of too much background or the presence of fusions in both type of DNA.





For further verification, it was decided to sequence the PCR products. The experiment was repeated. In both the top and the bottom rows (Fig. 12), lanes 8, 11, and 14 consist of the reamplified PCR products. Compared to Fig. 10, we observed extra bands for each of the reamplification reaction. I decided to clone those PCR products that do not repeat themselves in the WT row (Bottom; Fig. 12). I also avoided products less 200 bp long, since they could either be formed due to primer dimers or due to the non-specific binding of the primers. Arrows with specific colors indicate the PCR products selected for cloning (Fig. 12). The names and sizes of these products are summarized in Table 3, with respect to their colored arrow. Figure 13 shows successful cloning and sequencing of the fusion products Mut. A1, Mut. C1, and, WT. A1 (Figure 13). The respective primers used are matched to the cloned sequences (Figure 13). And lastly, sequencing of plasmids to which the PCR products Mut. A2, Mut. E1, WT. A2, and WT. C1 were cloned showed the absence of any inserts (Data not shown). The sequencing results suggests none of the PCR products we sequenced were telomeric fusions, thereby demonstrating them to be formed by the non-specific binding of the primers during the reamplification cycle.

Discussion

My study demonstrates that the PCR assay I developed can amplify telomere fusions up to 841 bp. The absence of any fusion bands from PCR on mutant

genomic DNA suggests that telomere fusions up to 841 bp may not exist in the telomere mutants we created.

Table 3. The names and sizes of potential fusion bands from Figure 12

Type					in a double-stranded DNA molecule, G and C are bound by three hydrogen bonds and the A and T are bound by two hydrogen bonds and therefore, the		
Name	Mu t. A1	Mu t. A2	Mu t. C1	Mu t. E1	WT. A1	WT. A2	WT. C1
~Size (bp)	369	320	263	280	334	250	360

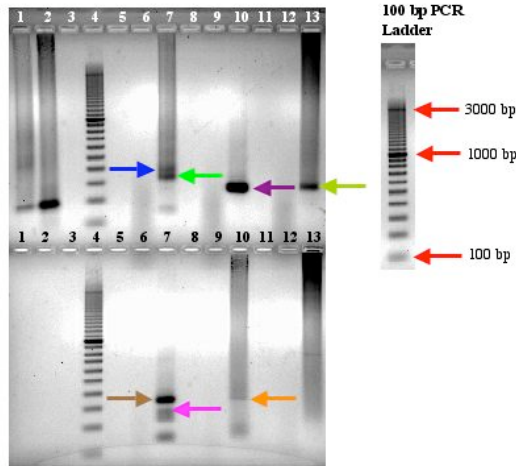


Figure 12. Reamplification of PCR on digested 43AA genomic DNA

The contents of the gel (above) is similar to Fig. 10. Potential fusion bands that are present in either rows were selected for cloning. The names and sizes of these bands are matched to the respective arrow and are summarized in the Table 2 (below).

If they do exist, I predict the number of such fusion events to be below the amplification limit of our assay. The possibilities of telomeric fusions larger than 841 bp cannot also be ruled out, since, our assay has a size barrier of ~841 bp.

Telomeres: the Nemesis of PCR and the Significance of a Positive Control

PCR is one of the most significant and useful techniques of biotechnology. It is a fast and efficient method to amplify known DNA sequences. However, in my study, I demonstrated that telomeric fusions are DNA templates that are extremely difficult to be detected by PCR. The PCR assay demonstrated its ability to detect telomere fusions with sizes 606 bp & 841 bp, but it failed to amplify the TAS C & C fusion, which has a size of 1056 bp. From a molecular perspective, studies have shown that the efficiency of PCR is affected by several parameters such as template complexity, and template sequence, primer sequence and stability, polymerase type, buffer type, (Innis, 1999). Among these, one or more of factors may provide insights to the inherent size barrier of our assay as summarized below.

GC-Content of the template: The high GC content of telomeres might be one of the primary reasons for this.

thermal energy required to denature GC rich strands for PCR are much higher than conventional DNA sequences (Moreau *et al.*, 1994). The issue of GC content worsens, when telomeric sequences need to be amplified by PCR, since, it consists of tandem GC repeats (Pherson, 2000). To resolve this issue, most studies recommend elevated denaturation temperature of 98°C from conventional conditions of 95°C to ensure efficient denaturation of long stretches of telomeres (Pherson, 2000). Although, I used 98°C as the denaturation temperature, during the primer binding and extension phases of PCR, the reaction temperature lowers to ~60°C. This might have affected the PCR efficiency due to potential of single stranded telomeric sequences to form secondary structures such as hairpins as demonstrated by Henderson *et al.*, (1987) and Gualberto *et al.*, (1992) that can lead to premature primer binding and the inhibition of the DNA polymerase from synthesizing new DNA.

Size and GC Content of the Primer: I also propose that the primers I designed (Table 1) might have contributed to the inefficiency of the PCR assay in amplifying the 1056 bp artificial fusion. If the size of the template to be amplified is long, the respective primers must stay on its binding site for a longer time period, which is chiefly determined by high GC content and large size of the primers (Beasley *et al.*, 1999). However, the primers I designed were short (20-22 bp) and low in GC content (25-60%) that may have resulted in premature primer binding.

Primers and Secondary Structures: In addition to GC content and size, studies have shown that the ability of the primers to form secondary structures, like self-duplexes, can also lead to premature primer-template binding (Breslauer *et al.*, 1986 & Kolmodin and Williams, 1997). Table 1 shows that several of the primers had secondary structure formation properties indicating its potential along with the other factors in inhibiting the PCR. Although, the primers TAS C and TAS A were able to amplify the telomeric fusions of 606 bp and 841 bp, either of them could also form self-duplexes. Therefore, it is possible that their primer binding ability is competing with the secondary structure formation, in which self-duplex formation might be the dominating while amplifying the 1056 bp telomeric fusion. Consequently, at a fixed time period, the number of primers available for efficient primer-template binding would be lower resulting in the amplification of less number of the 1056 bp telomeric fusions.

T_p and T_m of Primers: The other factor that may have played a role in reducing the efficiency of the PCR is

the drastic difference T_p and T_m . Several studies have shown that if $T_m > 3^{\circ}\text{C}$ than T_p , the primer with higher

```

Sequence of Mut.A1
5'
CGTCCGAACAACCTCAAAGTTGTGAGGAACG CAGCCAACAGCCAAAAGG
TTCTGAGGAACGCAGCCAACAGCCAAAAGGTCCTGAGGAACGCAGCCAACAG
CCAAAAGGTTCTGAAGAACGCAGCCAACAACCAAAAAGGTTCTGAGGAAGGCA
GCCAGCAGCCAAAAGGTTCTGAGGAACGCAGTCAACAGCCAAAAGGTTCTGA
GGAACGTAGCCAACAGCCAAAAGGTTCTGAGGAAGGCAGCCAACAGTCAAA
AGGTTCTGAGGAACGCAGCCAACAGCCAAAAGGTTCTGAGAAAACGCGTTCCT
CACAACTTGAGGTTGTCGGAACGGCG CGTTCCTCACAACTTTGAGGTTG
TTCGGAACG
3'

Sequence of Mut.C1
5'
GTTCCGAACAGCCACTAGGTTGTGAGGAACG TAAAAATCTAAAATAGAGC
GTAGCAAACAAGCAAGCAAGCATCAATAAAAATCAAACCAATCTATATA
GACAAAGGAAGAGAGAAAAAAAATAATGAAAAAAGTTGGAAAAACAAAG
GAATGATAAAGAAAGAAACAAATAATTGTAATTTGAATTGATCTATGATG
TATTCATAAGAGAATGAAAATTA CGTTCCTCACAACTAGTGGCTGTTCCG
AAC
3'

Sequence of WT.A1
5'
CGTCCGAACAACCTCAAAGTTGTGAGGAACG CGTTCCTCAGAACCCTTGG
CTGTGGCTGCGTTCCTCAGAACCTTTGACTGTTGGCTGCCCTCCTCAGAACCT
TTGACTGTTGGCTACGTTCCCTCAGAACCTTTGCTGTTGGCTGCGTTCCTCAG
AACCTTTGGTTGCTGGCTGCCCTCCTCAGAACCTTTGGCTGTTGGCTGCGTTC
CTCAGAACCTTTGGCAGTTGGCTGCGTTCCTCAGAACCTTTGGCTGTTGGCT
GCGTTCCTCAGAACCTTTGGCTGTTGGCTG CGTTCCTCACAACTTTGAGGTT
GTTCCGAACG
3'

```

Figure 13. Sequences of Reamplified PCR Products

The PCR products selected from Fig. 12 were cloned and sequenced and they showed to be non-telomeric. The sequences of Mut. A1, Mut. C1, and WT.A1 are matched to the primers used (bolded & underlined), while the sequences of Mut. A2, Mut. E1, WT. A2, and WT. C1 showed no inserts in their respective plasmids.

T_m will anneal to secondary priming sites at the lower temperature optimal for annealing of the second primer (Cheng and Kolmodin, 1997). The difference between T_p and T_m were more than 3°C for all the primers I designed suggesting that the secondary binding of the primers might have competed with the primary binding leading to a decrease in the PCR amplification. The presence of the non-specific products further confirms the secondary binding properties of the primers. All these factors may have negatively affected the efficiency of the PCR in amplifying the 1056 bp artificial fusion. Therefore, in the later parts of the study (Fig. 5- Fig. 9), longer primers with high GC content were used.

Enzyme Fidelity: Although, the enzyme (ThermAce) is shown to be a high fidelity enzyme (Barnes, 1992) that is able to amplify GC-rich templates of size (Barnes, 1994), the study has not demonstrated the enzyme's ability in amplifying large telomeric tracts. In addition, the enzyme have an inherent limit of ~850 bp for amplifying telomeric tracts, which also might have affected the efficiency of our PCR assay. Studies have also shown that the efficiency of the enzyme can vary with changes in the concentrations of MgCl_2 (Innis *et al.*, 1990 & Erlich *et al.*, 1989) and other buffers such as EDTA in the PCR reaction mixture (Zangenberg *et al.*, 1999). The buffers and the different salts present in reaction mixture determine the ionic strength and buffering capacity required for the fidelity of the enzyme (Zangenberg *et al.*, 1999). However, we have not examined the effects of changing the salt concentrations of the buffer in product specificity.

Due to the afore-mentioned reasons, it was very crucial to create artificial telomere fusions as positive control upon which our PCR assay could be tested. This is because, if the assay cannot demonstrate its ability to amplify telomeric fusions from the positive control, a negative result from the real experiment on mutant genomic DNA cannot be used to differentiate between the absence of fusions and the limitations of the PCR assay.

Genomic DNA's Inhibitory Properties against PCR

Our goal was to detect fusions from mutant genomic DNA and our positive control was capable of amplifying telomeric sequences up to 841 bp. Nevertheless, from the view point of PCR, the reaction scenarios of using the positive control and genomic DNA as templates are drastically different. When using artificial fusions as templates, the primers and the enzymes are faced with only the DNA sequences ranging from 606 bp to 1056 bp or more created by multiple ligation reactions. Conversely, the genomic DNA is the collection of the entire genome of *T. thermophila*, which is in the order of 2×10^5 kb for the micronucleus (Yao *et al.*, 1974) and 9×10^6 kb for the macronucleus (Woodard *et al.*, 1972). Consequently, there are several factors the PCR needs to overcome to detect fusions from genomic DNA in addition to those already mentioned (Cheng and Kolmodin, 1997). Firstly, the complexity of genomic DNA in the reaction can provide numerous non-specific binding sites for the primers (Cheng and Kolmodin, 1997). And secondly, the tendency of genomic DNA to undergo intra-molecular rearrangements, such as super coiling (Alberts, 2002), formation of self-duplexes

(Alberts, 2002) and formation of secondary structures (Alberts, 2002) can lead to premature primer binding, inaccessibility of the primers to their binding sites and withdrawal of DNA polymerase (Cheng and Kolmodin, 1997). In addition to the problems faced by PCR with genomic DNA, my assay also needs to overcome the difficulties of amplifying telomeric sequences. Several studies have demonstrated the difficulty in amplifying telomeric sequences from genomic DNA (Hackett *et al.*, 2001; Chan *et al.*, 2003).

Therefore, to create an ideal positive control, it was crucial to test the efficiency of our assay in detecting telomeric fusions in the presence of genomic DNA. By doing so, I mimicked a "real" experiment, where the PCR needs to amplify telomeric fusions from genomic DNA. I conducted PCR on different mixtures of the artificial fusion and the mutant genomic DNA that varied in the amount of each reaction. The experiment suggested to us the minimum amount of the artificial fusion the PCR assay required to amplify it in the presence of the maximum amount of the genomic DNA. When the amount of genomic DNA was decreased by serial dilutions, the assay succeeded in detecting artificial telomeric fusions from its 1:10 dilution. Furthermore, when the experiment was repeated with different dilutions of the artificial fusion in the presence of the genomic DNA, the assay was unable to detect artificial fusions from dilutions above 1:10. These results clearly demonstrate the inhibitory effects of the genomic DNA.

When we did the "real" experiment on 43AA genomic DNA with no addition of artificial fusions, we did not see any products (data not shown). This might be because one or more of the reasons that have been discussed. Similar to our results, other studies (Moreau *et al.*, 1994) have also shown reduction in the efficiency of PCR on telomeric DNA due to its ability to form secondary structures. Several studies have solved the inhibitory roles of genomic DNA on PCR by digesting it with an endonuclease (Hackett *et al.*, 2001; Chan *et al.*, 2003). Theoretically, the concept works on the premise that digestion of genomic DNA will reduce the formation of secondary structures and increases the accessibility of the primers to the binding sites. I decided to conduct PCR on 43AA genomic DNA digested with Mse I because: a) it can digest AT-rich templates like that of *T. thermophila* and b) it does not possess any restriction sites 5' to the primers we had designed thereby preventing the digestion of potential fusions that could be detected by our assay. Along with digested DNA, I also designed longer primers with higher GC content as recommended by other studies (Hackett, 2001; Chan, 2003). PCR on MseI digested DNA with the new primers also did not yield any products. However, the experiment shows that the usage of digested DNA or the new primers or both had increased the efficiency of the assay. Because PCR on digested and non-diluted 43AA genomic DNA with the new primers yielded the expected products, whereas the same experiment with non-digested and non-diluted 43AA DNA with the old primers did not.

Frequency of Fusion Events in our *T. thermophila* telomere mutants

Although usage of digested genomic DNA increased the efficiency of the PCR, the assay did not detect any fusion bands. One possible cause for this might have been the absence of the optimal number of fusions in mutants that the assay requires to amplify. In fact, studies in *S. cerevisiae* (Chan *et al.*, 2003 and Liti *et*

al., 2003) and *Arabidopsis thaliana* (Riha *et al.*, 2003) have shown that the number of telomeric fusions events that occur due to telomeric mutations are very small, although mutated telomeres are highly recombinogenic (McEachern and Iyer, 2001). These studies have suggested that in *S. cerevisiae* and *A. thaliana*, proteins such as NEJ1 and Ku, respectively prevents NHEJ-dependent telomere fusions. In my study, if telomere fusions are created by the activation of NHEJ machinery that was activated to repair our mutant's degraded telomeres, adjacent molecular pathways using proteins with similar functions as NEJ1 might also be in work to prevent NHEJ, resulting in low numbers of fusions.

Therefore, we decided to reamplify our PCR products. Reamplification of the PCR yielded products from both 43AA genomic DNA. Surprisingly, the PCR amplified products from wild type DNA yielded too. Southern blot analysis of these bands showed strong signals in those lanes containing the reamplification products. This suggested to us either that telomeric fusions might be present in both wild type and 43AA genomic DNA in low number, or that what we observed were artifacts of reamplification. However, cloning of these bands showed them to be non-telomeric sequences. From the perspective of PCR, the cloning result was not surprising, since, studies have demonstrated the formation of unwanted non-specific products when the number of PCR cycles is increased due to the non-specific primer binding (Innis *et al.*, 1990 & Erlich *et al.*, 1989). The strong signals observed in the Southern Blot might be due to the presence of the non-specific binding of the telomeric probe to the background DNA. Interestingly, such signals were not seen in those lanes containing non-reamplified PCR products, which suggest that the reamplification has produced a large amount of artifacts that provides non-specific binding sites to the telomeric probe.

Summary & Future Research

In conclusion, I have created a PCR assay that can amplify telomeric fusions. Nevertheless, due to the several factors that have been discussed, the assay also has a size limit of 841 bp. The absence of any PCR products suggests that either telomeric fusion up to 841 bp may not exist or their numbers are below the amplification potential of the assay. Nevertheless, when compared to other studies (Chan *et al.*, 2003), this study demonstrates its potential in detecting telomeric fusions by PCR that are much larger than the conventional telomeric fusions detected by PCR, which is between 100-150 bp (Chan *et al.*, 2003).

Our assay was unable to detect artificial fusions above 841 bp. Therefore, further studies should be conducted on the existence of larger telomeric fusions and non-telomeric fusions. To further increase the efficiency of the assay, varying the concentrations of MgCl₂ (Innis, 1990 & Erlich, 1989) of the PCR buffer and the application of co-solvents such as dimethyl sulfoxide (DMSO) (Winship, 1989), formamide (Sarkar, 1990), glycerol (Landre, 1995), and tetramethylammonium chloride (TEMAC) (Hung, 1990) to melt secondary structures are options that could be explored. Technique wise, pulse field gel electrophoresis is a potential method that could be applied to investigate the existence of chromosomal fusions, although it cannot differentiate between chromosomal and telomeric fusions.

For future research, we are also interested in exploring whether telomeric fusions exist in the macronucleus. Previous studies in the lab have shown that the sequence specific telomeric mutations had not resulted in the macronuclear telomeric degradation unlike the micronucleus. However, whether macronuclear telomeric fusions exist or not in our mutants remain unanswered. Conceptually, the same assay could be applied for the investigation using primers designed from the macronuclear TAS sequences.

The study by Kirk *et al.*, (1997) have demonstrated that sequence specific telomeric mutations display an acute mitotic chromosome segregation defect at anaphase, which eventually result in cell death. Nevertheless, further research must be conducted to explore whether the mutants are undergoing apoptosis or some other kind of cell death mechanism such as necrosis. In addition, the molecular mechanisms activated by the mutations that caused the cell death are also another area that needs to be examined. Further characterization of the mitotic defect observed by Kirk *et al.*, (1997) in our lab established more evidence for the anaphase arrest (Christ, 2002) and extensive micronuclear DNA degradation (unpublished) by monitoring the progression of cell cycle. In these mutants, we are also interested in the examining molecular indicators such as the over expression of anaphase and checkpoint specific proteins.

Experimental Procedures

Creation of Artificial Fusions

pUC19 plasmids containing *T. thermophila* TAS sequences with telomeric tracts were cloned by Kirk *et al.*, (1995). Frozen clones of TAS A and TAS C were thawed at room temperature for a few seconds and inoculated into LB/Amp cultures (1.0% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 1.0% Sodium Chloride, 1% Ampicillin in sterile nano pure H₂O). The pUC19 plasmids were isolated (Qiagen Plasmid Purification Kit; Valencia, CA;) and digested with Sac I (New England Biolabs; Beverly, MA), and EcoR V (New England Biolabs; Beverly, MA) as recommended by Kirk *et al.*, (1995). The reaction recipe consists of 62% sterile nano pure water, 10% NEB Buffer 2 (New England Biolabs; Beverly, MA), 10% BSA (New England Biolabs; Beverly, MA), 10% pUC19 plasmids, 4% of Sac I and EcoR V in a total reaction volume of 20 μ l. The restriction products were extracted (QIAquick Gel Extraction Kit by Qiagen; Valencia, CA) from a 1.5% agarose gel and joined by ligation to create artificial fusions. The ligation reaction consisted of 1 μ l of T4 DNA ligase (Invitrogen, Carlsbad, CA), 1 μ l of the respective templates, 1 μ l of ligation buffer, and 4 μ l of sterile nano pure water at 14°C for ~14hrs.

Polymerase Chain Reaction (PCR)

A PCR assay was designed to amplify telomeric fusions. In order to denature the telomeric templates and any secondary structures, the PCR assay we designed has a higher denaturation temperature (98°C; Pherson, 2000) and longer denaturation time (3 min), when compared to the conventional denaturation temperature and time of 95°C and 1 minute. The melting temperatures of the primers were 65.8°C and 67.26°C and therefore the annealing temperature was maintained between 56°C–58°C. The size of micronuclear telomeres is ~6.0 kb (Kirk *et al.*, 1995). To amplify such large fusion fragments of size above 3.0 kb, the extension time of the PCR was maintained at 7 minutes. And finally, a special enzyme called ThermAce (Invitrogen; Carlsbad, CA) that can amplify GC-rich templates and stable at higher denaturation temperatures and longer extension times was used for the PCR. The amounts of the buffers, primers, sterile nano pure water and template used were adapted from the ThermAce Enzyme Kit. The PCR was conducted in a MJ Research PTC-200 Peltier Thermal Cycler with denaturation at 98°C for 3.0 minutes, annealing

temperature was maintained 10°C less than the primer melting temperature) for 30 cycles, extension at 72°C for 7.0 minutes and final extension at 72°C for 10.0minutes.

Design of Primers

The primers were designed from the *T. thermophila* TAS sequences (Table 1). The 11 primers were labeled as TAS A, TAS B, TAS C, TAS D, TAS E, and TAS F (Table 1). To conduct nested PCR, sequences 3' to the primers TAS A and TAS C were used to develop PRX TAS A and PRX TAS C (Table 1). The last three primers (NEW TAS A, NEW TAS C, and NEW TAS E) were created with higher GC-content and melting temperature to increase the efficiency of the PCR (Table 1) and were labeled as per the name of the respective TAS sequence used (Table 1). The annealing temperature was calculated using the algorithm developed by Wu *et al.*, (1991) and the melting temperatures were calculated using the Mac Vector Software.

DNA Gel Electrophoresis

For a 1.5% gel, 1.5 g of agarose were dissolved in 100 ml of 1X TBE (54.0 g of Tris Base, 27.5 g of Boric acid and 20 ml of 0.5 M EDTA {pH 8.0} in 1.0 L of nano pure H₂O) buffer by microwaving. The agarose solution was then poured into the gel tray containing the appropriate gel comb and was allowed to solidify. The gel tray was then kept on the gel box, and enough 1X TBE was added to cover the gel. The respective DNA solutions were added to each well with loading dye. The gel was run at the appropriate voltage (~95-120V) and time until the loading dyes were seen next to positive end of the gel. The gels were then stained with ethidium bromide and analyzed by BioRad Versa Doc Imaging System and Quantity One Software.

Transformation of *T. thermophila* and Isolation of Genomic DNA

Transformation of *T. thermophila* was performed by electroporation as described by Gaertig and Gorovsky (1992). Cell lines CU428.1 were grown in PPYS-PSF media (2% Protease peptone, 0.2% Bacto-Yeast Extract, 0.003% Sequestrene with 500 μ l of PSF {45 ml of sterile nano pure water was added to a vial of lyophilized Penicillin/Streptomycin (Sigma, St. Louis, MO)} followed by 5 ml of Fungizone; Gibco) at 30°C, while shaking at 100 rpm for 48 hours. The cells were then washed twice in 10 mM Tris-HCl buffer (pH 7.4), combined in equal ratios, and shaken at 150 rpm at 30°C for another 12–18 hours to prevent the cells from pairing until a desired time. Cells were washed again twice 10.5 hours later in 10 mM Hepes buffer (pH 7.4). The cells were then transformed with plasmid DNA containing the desired telomerase RNA template mutation (43AA) purified from previously transformed *Escherichia coli* cells using a BTX Electroporation System/Electro Cell Manipulator 600 at settings previously determined by Gaertig and Gorovsky (1992). Electroporated cells were drugged with paromomycin (Sigma Chem Co., St. Louis, MO) at a final concentration of 120 μ g/ml to select for transformed cells.

One ml of the transformed CU428.1 strains was inoculated in 50 ml of PPYS-PSF media in a 250 ml conical flask for ~48 hrs at 30°C. The 43AA genomic DNA was then isolated (by Christina Christ '03, Lake Forest College, IL, USA) from the cultures using the Genomic DNA Maxi Isolation Kit supplied by Qiagen (Valencia, CA) and was run on a 1.5% agarose gel to check the presence of the genomic DNA.

For creating the ideal positive control, PCR was conducted on different mixtures of the artificial fusions and the 43AA genomic DNA. The concentrations of their different dilutions are summarized in Table 2.

Restriction Digestion of 43AA DNA

The 43AA DNA was digested with the restriction enzyme Mse I (New England Biolabs; Beverly, MA). The digestion reaction consisted of 10% NEB Buffer 2, 10% BSA, 7.5% Mse I, 25% template and 47.5% of sterile nano pure water in a total reaction volume of 20 μ l at 37°C for ~24hrs. Mse I was selected as restriction enzyme because of two reasons. Firstly, MseI's

restriction site is ideal for templates that are AT- rich like *T. thermophila* genome. Secondly, none of the TAS sequences

possessed a restriction site for MseI according to sequence analysis conducted by Mac Vector software.

Table 1: The names, sequences, melting temperatures, length & GC content of the primers

Primer Name, Sequence (3'-5') & Ability to form Secondary Structures: Hair Pin, Primer Dimer and Self Duplex	Length	Annealing Temperature (°C)	Melting Temperature (°C)	% GC
TAS A: AATAGGTATAGATTAGTACTGGC Forms Self-Duplex	23	67.26	48.8	34.7
PRX TAS A: ATGATTGCGAAGCATCATTAAC Form hairpin and Self Duplex	22	65.80	59.1	25.0
TAS B: AAATGATTTCTAAGTGATTATTAAC Forms Self-Duplex	25	65.80	50.5	20.0
TAS C: AAAATATTTATGGAAAAGTACAGG Forms Self-Duplex	24	65.80	53.1	25.0
PRX TAS C: GCCACTAGGTTGTGAGGAAC Forms Self-Duplex	26	67.26	57.2	55.0
TAS D: CGCAGAGCGAACTATTAATCC Form hairpin and Self Duplex	21	67.26	59.0	47.6
TAS E: GTTCCGAACAGCCAACAGCC Forms Self-Duplex	20	68.72	65.3	60.0
TAS F: CGCATAGTGATTATTAACCAGC Forms Self-Duplex	22	67.26	57.3	40.9
NEW TAS C: GTTCCGAACAGCCACTAGGTTGTGAGGAACG Form Hairpin and Self Duplex	31	92.08	75.1	54.8
NEW TAS E: CAGCCAACAGCCAAAAGTTCTGAGGAACG Form Hairpin and Self Duplex	30	89.16	75.9	53.3

Table 2: Concentrations of the different dilutions of artificial fusions and 43AA genomic DNA

Type	Non-Diluted (µg/ml)	1:10 Dilution (µg/ml)	1:100 Dilution (µg/ml)	1:1,000 Dilution (µg/ml)	1:10,000 Dilution (µg/ml)	1:100,000 Dilution (µg/ml)	1:1,000,000 Dilution (µg/ml)
C & A Ligase	205	20.5	2.05	2.05×10^{-1}	2.05×10^{-2}	2.05×10^{-3}	2.05×10^{-4}
43AA Genomic DNA	1215	121.5	12.15	1.215	1.215×10^{-1}	1.215×10^{-2}	1.215×10^{-3}

Southern Blotting

The PCR products to be analyzed were run on a 1.5% agarose gel and the gel was used for the following procedures:

a) *Alkali Transfer gel Treatment Protocol*- The gel was treated with 0.125 M HCl for 10.0 minutes for depurination (Amersham Biosciences, Piscataway, NJ). Enough 0.125 M HCl was added to a tray containing the gel to cover it. After complete depurination, as indicated by the change in the color of the bromophenol dye from blue to yellow, the gel was submerged for 30 minutes in fresh denaturation buffer (87.66 g of NaCl and 20 g of NaOH in 1.0 L of nano pure H₂O) for denaturation

(Amersham Biosciences). The content of the gel was then transferred to a Hybond-N⁺ membrane by Capillary Blotting (Amersham Biosciences). The blot (Hybond-N⁺ membrane) was then used for hybridization with a telomeric probe.

b) *Hybridization* - The blot was pre-hybridized by submerging in hybridization buffer (1 mM EDTA, 7.0% of 1 M Na₂HPO₄ (pH 7.2), 7.0% of 20.0% SDS and 1% BSA) in a tray at 37°C for 1 hr (Church and Gilbert, 1984). For hybridization, the blot was added to a hybridization jar containing the probe (Mix 1.0 µl of oligonucleotide {5' end radiolabeled oligonucleotide, 5'-(GGGGTTT)₃, 6.0 µl of sterile nano pure H₂O, 1 µl of 10X

buffer, 1 μ l of 32 P label, and 1 μ l of kinase at 37°C for 20 minutes) mixed with 3 ml of hybridization buffer (Church and Gilbert, 1984) and was rotated gently in a hybridization oven at 55°C (Kirk et al., 1995) for ~16 hours.

c) *Washing & Analysis*- After hybridization, the blot was washed with wash buffer (1 mM EDTA, 40mM of 1 M Na₂HPO₄ (pH 7.2), and 1% SDS in nano pure H₂O) five times at 60°C for 5 minutes each. The blot was then analyzed for telomeric signals using the BioRad Personal Molecular Imager and Quantity One Software.

Cloning & Sequencing Fusion Fragments

The cloning of the fusion fragments was conducted using the TA Cloning Kit (Invitrogen, Carlsbad, CA). PCR products to be sequenced were cut out (QIAquick Gel Extraction Kit by Qiagen; Valencia, CA), ligated to pUC19 plasmids (Invitrogen) and were used for transforming One Shot[®] competent *Escherichia coli* (*E. coli*) cells (Invitrogen, Carlsbad, CA) by heat shocking. The plasmid DNA was then isolated (Qiagen Plasmid Purification Kit; Valencia, CA) and verified for the presence of the insert by restriction digest using EcoR V (New England BioLabs; Beverly, MA) as recommended by Invitrogen. The reaction recipe for the digestion consists of 76% sterile nano pure water, 10% NEB Buffer 3 (New England BioLabs; Beverly, MA), 10% BSA (New England BioLabs; Beverly, MA), 10% pUC19 plasmid, and 4% of EcoR V in a total reaction volume of 20 μ l. Plasmids with the inserts were sequenced at the Sequencing Facility at University of Chicago, IL, USA.

Acknowledgement

I would like to express my deepest gratitude to my advisor Prof. Karen E. Kirk with whom I conducted this study. I would like to thank my student mentors Jennifer McGuire and Christina Christ for their training and intellectual assistance and my colleague Daniel McNabney who also helped me with various experiments. I show my earnest appreciation to my Senior Thesis Committee members Prof. Douglas B. Light and Prof. Ronald Miller for their commitment and patience in critiquing this manuscript. I also like to express my special thanks to Prof. Douglas B. Light for helping me write this Senior Thesis, when my advisor was away due to sickness. In addition, I thank Sara Herrera, Keith Hartley, and Prof. Shubhik K. DebBurman for peer reviewing this manuscript. Lastly, but not the least, I am grateful to God (if he/she/it exists) in the development of this project.

Disclaimer

Eukaryon is published by students at Lake Forest College, who are solely responsible for its content. The views expressed in Eukaryon do not necessarily reflect those of the College.

References

Alberts Bruce, Alexander J, Julian L, Martin R, Keith R, Peter W. 1997. Molecular Biology of the Cell. 4th ed. Garland Science Taylor & Francis Group, New York: p. 263, 437.

Allsop RC, Vazri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, and Harley CB. 1992. Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. USA*, 89, 10114-10118.

Armstrong SJ, Christopher HF, and Gareth H. Jones. 2001. Nucleolus-associated telomere clustering and pairing precede meiotic chromosome synapsis in *Arabidopsis thaliana*. *Journal of Cell Science*, 114, 4207-4217.

Asai, DJ, and James DF. 2000. Methods in Cell Biology, Vol. 62. Academic Press, New York: p. 138, 417.

Baumann P, and Cech TR. 2000. Protection of telomeres by the Ku protein in fission yeast. *Mol. Biol. Cell*, 11, 3265-3275.

Barnes WM. 1992. The Fidelity of Taq Polymerase Catalyzing PCR is Improved by an N-terminal Deletion. *Gene*, 112, 29-35.

Barnes WM. 1994. PCR Amplification of up to 35-kb DNA with High Fidelity and High Yield from Bacteriophage Templates. *PNAS*, 91, 2216-2220.

Beasley EM, Richard MM, David RC, and Laura CL. Statistical Refinement of Primer Design Parameters." Edited by Innins, Michael A., David H. Gelfand, and John J. Sninsky (1999). PCR Application, Protocols for functional genomics. Academic Press, New York, 55-72.

Bechter OE, Zou Y, Shay JW, and Wright WE. 2003. Homologous recombination in human telomerase-positive and ALT cells occurs with the same frequency. *EMBO Rep.*, 12, 1138-1143.

Bertuch A and Victoria L. 1998. Telomeres and double-strand breaks: trying to make ends meet. *Trends in Cell Biology*, 8, 339-342.

Bhattacharya A and Blackburn EH. 1997. *Aspergillus nidulans* maintains short telomeres throughout development. *Nucleic Acids Research*, 25, 1426-1431.

Biessmann H. and Mason J.M. 1997. *Drosophila* telomere elongation. *Chromosoma*, 106, 53-67.

Blackburn EH. 2001. Switching and Signaling at the Telomeres. *Cell*, 106, 661-673.

Blackburn EH, and Joseph GG. 1978. A Tandem Repeated Sequence at the Terminal of the Extrachromosomal Ribosomal RNA Genes in *Tetrahymena*. *J. Mol. Biol.*, 120, 33-53.

Breslauer KJ, Frank R, Blocker H, and Marky LA. 1986. Predicting duplex stability from the base sequence. *PNAS*, 83, 3746-3750.

Broccoli D, Smogorzewska A, Chong L, and de Lange T. 1997. Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat. Genet.*, 17, 231-235.

Chan, Simon WL and Blackburn EH. 2003. Telomerase and ATM/Tel1p Protect Telomeres from Nonhomologous End Joining. *Mol. Cell*, 11, 1379-1387.

Chen Phil and Blackburn EH. 1998. Two Types of Telomeric Chromatin in *Tetrahymena thermophila*. *JMB*, 280, 327-344.

Cheng Suzanne and Kolmodin LA. 1997. XL PCR amplification of Long Targets from genomic DNA. PCR Cloning Protocols. edited by Bruce A. White. Humana Press, New Jersey, 17-29.

Chikashige Y, Ding DQ, Funabiki H, Haraguchi T, Mashiko S, Yanagida M, and Hiraoka Y. 1994. Telomere-led premeiotic chromosome movement in fission yeast. *Science*. 264, 270-273.

Chong L, van Steensel B, Broccoli D, Erdjument-Bromage H, Hanish J, Paul Tempst, and de Lange T. 1995. A human telomeric protein. *Science*, 270, 1663-1667.

Christ Christina. 2002. Cell Cycle Defects in *Tetrahymena thermophila* telomere mutants. Senior Thesis, Biology Department, Lake Forest College, IL, 60045.

Chua PR and Shirleen GR. 1997. Tam1, a telomere-associated meiotic protein, functions in chromosome synapsis and crossover interference. *Genes & Development*, 11, 1786-1800.

Church GM and Gilbert W. 1984. Genomic Sequencing. *PNAS*, 81, 1991-1995.

Collins Kathleen and Leena G. 1998. The reverse transcriptase component of the *Tetrahymena* telomerase ribonucleoprotein complex. *Proc. Natl. Acad. Sci. USA.*, 95, 8485-8490.

Conrad MN, Jocelyn H, Wright A, Wolf J, and Virginia AZ. 1990. RAP1 Protein interacts with Yeast telomeres In vivo: Overproduction alters telomere structure and decreases chromosome stability. *Cell*, 63, 739-750.

Conrad Michael N, Ana MD and Michael ED. 1997. Ndj1p, a Meiotic Telomere Protein Required for Normal Chromosomes Synapsis and Segregation in Yeast. *Science*, 276, 1252-1255.

Copper JP, Nimmo ER, Allshire RC, and Cech TR. 1997. Regulation of telomere length and function by a Myb-domain protein in fission yeast. *Nature*, 385, 744-747.

Craven RJ, Patricia WG, Margaret D, and Thomas DP. 2002. Regulation of Genome Stability by TEL1 and MEC1, Yeast Homologs of the Mammalian ATM and ATR Genes. *Genetics*, 161, 493-507.

Dandjinou AT, Dionne I, Gravel S, LeBel C, Parenteau J, and Wellinger RJ. 1999. Cytological and functional aspects of telomere maintenance. *Histo. Histopathol.* 14, 517-524.

Dubrana K, Severine P and Susan MG. 2001. Turning telomere off and on. *Nucleus and gene expression*, 281-289.

- Erllich HA. ed. 1989. PCR Technology, Principles and Applications for DNA Amplification. Stockton, New York.
- Espejel Silvia, Sonia F, Sandra RP, Simon DB, Juan CC, and Maria AB. 2002. Mammalian Ku86 mediates chromosomal fusions and apoptosis caused by critically short telomeres. *THE EMBO Journal*, 21, 2207-2219.
- Evans SK, Alison AB, and Victoria L. 1999. Telomeres and telomerase: at the end, it all comes together. *Trends Cell Biol.*, 9, 329-331.
- Evans SK and Victoria L. 2000. Positive and negative regulation of telomerase access to the telomere. *J. Cell Sci.*, 113, 3357-3364.
- Ferreira MG and Julia PC. 2001. The Fission Yeast Taz1 Protein Protects Chromosomes from Ku-Dependent End-to-End Fusions. *Mol. Cell*, 7, 55-63.
- Funabiki H, Iain H, Satoru U, and Mitsuhiro Y. 1993. Cell-Cycle-dependent Specific Positioning and Clustering of Centromeres and Telomeres in Fission Yeast. *The Journal of Cell Biology*, 121, 961-976.
- Gaertig J and Gorovsky MA. 1992. Efficient mass transformation of *Tetrahymena thermophila* by electroporation of conjugants. *PNAS*, 19, 9196-9200.
- Gasser SM. 2000. A Sense of the End. *Science*, 288, 1377-1379.
- Gilley D, Hiromi TM, Prakash H, Akihiro K, Gloria CL, Mitsuo O, and David J. Chen. 2001. DNA-PKcs is critical for telomere capping. *PNAS*, 98, 15084-15088.
- Gilson P and Geoffrey IM. 1995. The chlorarachniophyte: a cell with two different nuclei and two different telomeres. *Chromosoma*, 103, 635-641.
- Greider CW and Blackburn EH. 1985. Identification of a Specific Telomere Terminal Transferase Activity in *Tetrahymena* Extracts. *Cell*, 43, 405-413.
- Greider CW and Blackburn EH. 1987. The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell*, 51, 887-898.
- Grieder CW. 1998. Telomerase activity, cell proliferation, and cancer. *PNAS*, 95, 90-92.
- Greider CW and Blackburn EH. 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature*, 337, 331-337.
- Greider CW. 1999. Telomeres Do D-Loop-T-Loop. *Cell*, 97, 419-422.
- Griffith JD, Laurey C, Soraya R, Rachel MS, Alessandro B, Heidi M and de Lange T. 1999. Mammalian Telomeres End in a Large Duplex Loop. *Cell*, 97, 503-514.
- Gualberto A, Patrick RM and Walsh Kenneth. 1992. Nuclei acid specificity of a vertebrate telomere-binding protein: evidence for G-G base pair recognition at the core-binding site. *Genes & Development*, 6, 815-824.
- Haber JE. 1999. Sir-Ku-itous Routes to Make Ends Meet. *Cell*, 97, 829-832.
- Hackett JA, David MF, and Carol WG. 2001. Telomere Dysfunction Increases Mutation Rate and Genomic Instability. *Cell*, 106, 275-286.
- Harley CB, Futcher AB, and Carol WG. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature*, 345, 458-460.
- Hayflick L, and Moorehead PS. 1961. The limited in vitro lifetime of human diploid strains. *Experimental Aging Research*, 25, 614-636.
- Henderson ER and Blackburn EH. 1989. An overhanging 3' terminus is a conserved feature of telomeres. *Mol Cell. Biol.*, 9, 345-348.
- Henderson ER, Hardin CC, Wlak SK, Tinoco I Jr. and Blackburn EH. 1987. Telomeric DNA Oligonucleotides form novel intramolecular structures containing guanine-guanine base pairs. *Cell*, 51, 899-908.
- Hsu HL, Gilley D, Blackburn EH, and Chen DJ. 1999. Ku is associated with the telomere in mammals. *PNAS*, 96, 12454-12458.
- Hsu HL, David G, Sanjeev AG, Hande MP, Beth A, Sahn HK, Gloria CL, Judith C, Terumi KS, and David JC. 2000. Ku acts in a unique way at the mammalian telomere to prevent end joining. *Genes & Development*, 14, 2807-2812.
- Hung T, Mak K, and Fong K. 1990. A specificity enhancer for polymerase chain reaction. *Nucleic Acids Res.*, 18, 4953.
- Innis MA, Gelfand DH, Sninsky JJ, and White TJ, eds. 1990. PCR Protocols. A Guide to Methods and Applications. Academic, San Diego, CA.
- Innis MA, Gelfand DH, and Sninsky JJ, eds. 1999. PCR Applications: Protocols for Functional Genomics. Academic Press, San Diego, 3-22.
- Kilian A, Heller K, and Kleinhofs A. 1998. Development patterns of telomerase activity in barley and maize. *Plant Mol. Biol.*, 37, 621-628.
- Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello, GM, Wright WE, Weinrich, SL, and Shay JW. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science*, 266, 2011-2015.
- Kirk KE, and Blackburn EH. 1995. An unusual sequence arrangement in the telomeres of the germ-line micronucleus in *Tetrahymena thermophila*. *Genes & Development*, 9, 59-71.
- Kirk KE, Harmon BP, Reichardt IK, Sedat J, and Blackburn EH. (1997). Block in anaphase chromosome separation due to a telomerase template mutation. *Science*, 275, 1478-1481.
- Klobutcher LA, Swanton MT, Donini P, and Prescott DM. 1981. All genized DNA molecules in four species hypotrichs have the same terminal sequence and an unusual 3' terminus. *Proc. Natl. Acad. Sci. USA*, 78, 3015-3019.
- Kolmodin LA, and Williams JF. 1997. PCR: Basic Principles and Routine Practice. PCR Cloning Protocols. edited by Bruce A. White. Humana Press, New Jersey, 3-16.
- Landre PA, Gelfand DH, and Watson RH. 1995. The use of co-solvents to enhance amplification by the polymerase chain reaction. PCR Strategies, Academic, San Diego, CA, 3-16.
- Larson GP, Castanotto D, Rossi JJ, and Malafa MP. 1994. Isolation and functional analysis of a *Kluyveromyces lactis* RAP1 homologue. *Gene*, 150, 35-41.
- Lentz DS and Blackburn EH. 1990. Functional Evidence for an RNA Template in Telomerase. *Science*, 247, 546-552.
- Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, and Cech TR. 1997. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*, 276, 561-567.
- Liti G and Edward JL. 2003. NEJ1 Prevents NHEJ-Dependent Telomere Fusions in Yeast without Telomerase. *Mol. Cell*, 11, 1373-1378.
- Liu L, Maria AB and David LK. 2002. Requirement of functional telomeres for metaphase chromosome alignments and integrity of meiotic spindles. *EMBO reports*, 3, 230-234.
- Lundblad V, Szostak JW. 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell*, 57, 633-643.
- Lundblad V, Blackburn EH. 1993. An alternative pathway for yeast telomere maintenance rescues est1-senescence. *Cell*, 73, 347-360.
- Lundbald V. 1999. Telomeres Keep on Rapping. *Science*, 288, 2141-2142.
- Lustig AJ, Stephen K and David S. 1990. Involvement of the Silencer and UAS binding protein RAP1 in regulation of Telomere Length. *Science*, 250, 549-553.
- Mangahas JL, Mary KA, Lisa S, and Virginia AZ. 2001. Repair of Chromosome ends after Telomere Loss in *Saccharomyces*. *Molecular Biology of the Cell*, 12, 4078-4079.
- McClintock B. 1938. The fusion of broken chromosome ends of sister half-chromatids following chromatid breakage at meiotic anaphases. *Missouri Agricultural Experiment Station Research Bulletin*, 290, 1.
- McClintock B. 1941. The stability of broken ends of chromosomes in *Zea mays*. *Genetics*, 26, 234.
- McEachern MJ and Blackburn EH. 1996. Cap-prevented recombination between terminal telomeric repeat arrays (telomere CPR) maintains telomeres in *Kluyveromyces lactis* lacking telomerase. *Genes Dev.*, 10, 1822-1834.
- McEachern MJ, Anat K, Blackburn EH. 2000. Telomeres and their control. *Annu. Rev. Genet.*, 34, 331-358.
- McEachern MJ, Shilpa I. 2001. Short Telomeres in Yeast Are Highly Recombinogenic. *Molecular Cell*, 7, 695-704.
- McEachern MJ, Shilpa I, Tracy BF and Blackburn EH. 2000. Telomere fusions caused by mutating the terminal region of telomeric DNA. *PNAS*, 97, 11409-11414.
- McKenzie KE, Christopher BU and Saraswati S. 1999. Applications of telomerase research in the fight against cancer. *Molecular Medicine Today*, 5, 114-122.

- Moreau A, Duez C, and Dusart J. 1994. Improvement of GC-rich template amplification by inverse PCR. *BioTechniques*, 17, 232-234.
- Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, and Wu JR. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A*, 85, 6622-6626.
- Muller HJ. 1938. The remaking of chromosomes. *Collecting Net* 8, 182.
- Muller HJ. Studies in Genetics. Indiana University Press, Bloomington, IL, 1962.
- Multani AS, Ozen M, Sen S, Mandal AK, Price JE, Fan D, Randinsky R, Ali-Osman F, Von Eschenbach AC, and Pathak S. 1999. Amplification of telomeric DNA directly correlates with metastatic potential of human and murine cancers of various histological origin. *International Journal of Oncology*, 15, 423-429.
- Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, and Cech TR. 1997. Telomerase catalytic subunit homologs from fission yeast and human. *Science*, 277, 955-959.
- Olovnikov AM. 1971. Principle of marginality in template synthesis of polynucleotides. *Doklady of Akademii Nauk SSR*, 201, 1496-1499.
- Petcherskaia M, McGuire J, Pherson JM, and Kirk KE. 2003. Loss of cap structure causes mitotic defect in *Tetrahymena thermophila* telomerase mutants. *Chromosoma*, 111, 429-437.
- Pherson J. 2000. Study of Mutant Telomeres and Telomerase in *Tetrahymena thermophila*. Senior Thesis, Biology Department, Lake Forest College, IL, 60045.
- Richards EJ and Ausubel FM. 1988. Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell*, 53, 127-136.
- Riha K and Shippen DE. 2003. Ku is required for telomeric C-rich strand maintenance but not for end-to-end chromosome fusions in *Arabidopsis*. *PNAS*, 100, 611-615.
- Sarkar G, Kaplner S, and Sommer SS. 1990. Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Res.*, 18, 7465.
- Shampay J and Blackburn EH. 1984. DNA Sequences of telomeres maintained in yeast. *Science*, 247, 154-157.
- Shiels PG, Alexander JK, Keith HS, Campell DW, Ian W, Alan C, and Angelika ES. 1999. Analysis of telomere lengths in cloned sheep. *Nature*, 399, 316-317.
- Shore D and Nasmyth K. 1987. Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. *Cell*, 51, 721-732.
- Steensel B and de Lange T. 1997. Control of telomere length by the human telomeric protein TRF1. *Nature*, 385, 740-743.
- Steensel B and Smogorzewska A and de Lange T. 1998. TRF2 protects human telomeres from end-to-end fusions. *Cell*, 92, 401-413.
- Tong WM, Prakash MH, Peter ML, and Zhao QW. 2001. DNA Strand Break-Sensing Molecule Poly (ADP-Ribose) Polymerase Cooperates with p53 in Telomere Function, Chromosome Stability, and Tumor Suppression. *Molecular and Cellular Biology*, 21, 4046-4054.
- Vassetzky NS, Florence G, Christine B, Susan MG, and Eric G. 1999. Taz1p and Teb1p, two telobox proteins in *Schizosaccharomyces pombe*, recognize different telomere-related DNA sequences. *Nucleic Acids Research*, 27, 4687-4694.
- Watson JD. 1972. Origin of concatameric T7 DNA. *Nature New Biology*, 239, 197-201.
- Winship PR. 1989. An improved method for directly sequencing PCR amplification material using dimethyl sulfoxide. *Nucleic Acids Res.*, 17, 1266.
- Wirght WE, Tesmer VM, Liao ML, and Shajy JW. 1999. Normal human telomeres are not late replicating. *Exp. Cell Res.*, 251, 492-499.
- Woodard J, Kaneshiro E, and Gorovsky MA. 1988. Cytochemical studies on the problem of macronuclear subnuclei in *Tetrahymena*. *Genetics*, 70, 251.
- Wu DY, Ugozzoli L, Pal BK, Qian J, and Wallace RB. 1991. The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. *DNA Cell Biol.*, 10, 233-238.
- Yao MC, and Gorovsky M. 1974. Comparison of the sequences of macronuclear and micronuclear DNA of *Tetrahymena pyriformis*. *Cell*, 12, 121-132.
- Yu GL, Bradley JD, Attardi LD, Blackburn EH. 1990. In vivo alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature*, 344, 126-132.
- Zangenberg G, Saiki RK, and Reynolds R. 1999. Multiplex PCR: Optimization Guidelines. Edited by Innins, Michael A., David H. Gelfand, and John J. Sninsky. PCR Application, Protocols for functional genomics. Academic Press, New York, 73-94.