TELOMERE DYNAMICS AND END PROCESSING

IN MAMMALIAN CELLS

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DEDICATION

Dedicated to my parents Joseph and Randa;
My twin pillars without whom I could never stand.

TELOMERE DYNAMICS AND END PROCESSING IN MAMMALIAN CELLS

by

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DISSERTATION

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TELOMERE DYNAMICS AND END-PROCESSING

IN MAMMALIAN CELLS

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Telomeres are repetitive DNA sequences that end in single-stranded 3' overhangs. With each cell division, normal human cells lose a small amount of telomeric DNA due to the end-replication problem and the action of an unidentified nuclease. In order for tumor cells to divide indefinitely, they maintain telomere length by expressing the enzyme telomerase.

The end structure of mammalian telomeres is not very well understood. Two assays were developed using ligation and PCR amplification to identify the terminal

nucleotides of both the C-rich and G-rich telomeric strands in human cells. The results showed that ~ 80 % of the C-strands terminate precisely in AT**C**-5', demonstrating that the nuclease resection of the C-strand post replication is specific for a single nucleotide. In contrast, the last base of the G-strand in normal human cells was less precise with 70% of the ends being TA**G**-3', TT**A**-3' or GT**T**-3'. An enrichment for the TA**G**-3' end was noted in cells that express telomerase.

A series of nucleases were tested for their involvement in specifying the last base of C-strands and the results indicated that none of those nucleases were responsible for telomere-end resection. Inhibiting the normal function of most telomere binding proteins altered normal telomere function, however only one protein (POT-1) influenced last base specificity. Knocking down POT-1 in normal and tumor cells randomized the last base of the C-strand. These finding have important implications for the processing events that act on the telomere ends and they will help identify the nuclease that resects the chromosome ends.

In the second part of this study, the dynamics of telomerase action in mammalian cells was examined. Using a PCR-based, single telomere-length measurement assay (STELA) we showed that telomerase adds an average of 250-nucleotides per end in one replication cycle. Cell cycle studies showed that while the telomeres on the Xp chromosome replicated in early S-phase their elongation by telomerase took place during late S/early G2 phase. Therefore, in mammalian cells telomerase action is not coupled to DNA replication. These studies will provide much needed information for exploiting our knowledge of telomere biology for telomerase-based therapeutic purposes.

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LIST OF ABBREVIATIONS

ALT	Alternative Lengthening of Telomeres
bp	Base pair(s)
BrdC	5-Bromodeoxycytidine
BrdU	5-Bromodeoxyuridine
cDNA	Complementary DNA
CMV	Cytomegalovirus
DAPI	4', 6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic Acid
dsRNA	Double-stranded RNA
FISH	Fluorescence *in situ* Hybridization
HR	Homologous Recombination
hTERT	Human Telomerase Reverse Transcriptase (Protein Component)
hTR	Human Telomerase RNA (template RNA Component)
ITAS	Internal Telomerase Assay Standard
kb	Kilobase Pair(s)
M1	Mortality Stage 1
M2	Mortality Stage 2
Mb	Megabase Pair(s)
mRNA	Messenger RNA
NHEJ	Non Homologous End Joining
PARP	Poly ADP-Ribose Polymerase

PCR	Polymerase Chain Reaction
ReDFISH	Replicative Detargeting Fluorescence *In Situ* Hybridization
RNA	Ribonucleic Acid
RNAi	RNA interference
RNP	Ribonucleoprotein
RT-PCR	Reverse Transcription PCR
siRNA	Short interfering RNA
shRNA	Short hairpin RNA
STELA	Single Telomere Length Analysis
SV40	Simian Virus 40
tet	Tetracycline
TRAP	Telomeric repeat amplification protocol
TIF	Telomere-dysfunction-induced foci
TRF	Telomere restriction fragment

CHAPTER ONE

General Introduction and Literature Review

THE HISTORY OF TELOMERES AND TELOMERASE

Linear chromosomes of all eukaryotic cells end in a special structure named 'telomere', derived from the Greek; *telos* (end) and *meres* (part) (shown in fig. 1-1). Throughout the years, telomeres have received considerable attention and the field has advanced enormously, especially when altered telomere structure or function was linked to disease. The impact of telomere/telomerase on cancer has been elucidated, the influence of telomeres on aging has been recognized and the consequence of telomere/telomerase dysfunction in genetic diseases such as Dyskeretosis Congenital and Werner syndrome has been established. Since telomeres were identified in 1978 (Blackburn and Gall, 1978), much information about the telomere end structure has been characterized in yeast, ciliates and mammals. A growing number of specific telomere binding proteins with great implication on telomere function have been identified. The telomerase ribonucleoprotein enzyme was purified, its protein and RNA component cloned and substantial work has been done to understand its regulation.

The telomere story started almost 65 years ago when Hermann Muller (1938) and Barbara McClintock (1941) using cytogenetic analysis demonstrated that the end of the chromosomes are different from double stranded breaks produced with ionizing radiation.

Broken chromosome ends often heal by fusing to each other to generate chromosome rearrangement and further damage. However chromosome ends, termed “natural ends” by Barbara McClintock which would be later called “telomeres”, remain intact and do not fuse to broken ends.

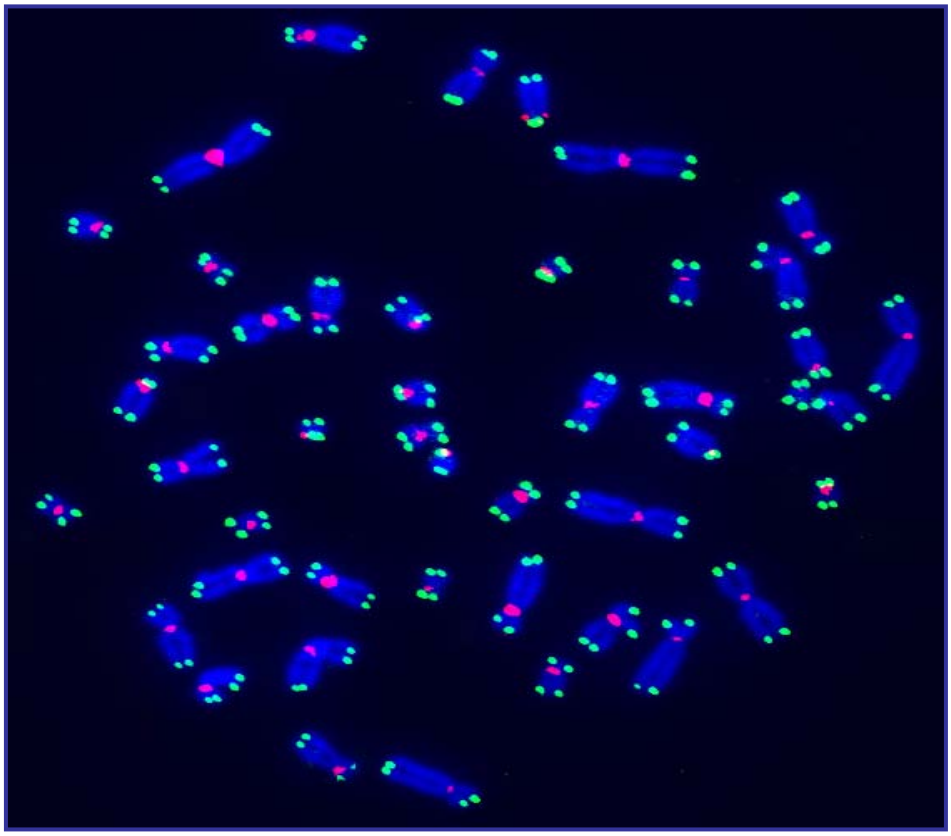


Figure 1.1- A metaphase spread of a normal human male BJ fibroblast cell. Chromosomes in Blue (DAPI stain). Telomeres in Green (PNA telomeric probe). Centromeres in Red. (Courtesy of Ying Zou)

While the mystery of chromosome ends awaited molecular explanation, two other problems were portrayed and required molecular understanding. The first was presented by Leonard Hayflick and Paul Moorhead in 1961 when they showed that normal human

diploid fibroblasts do not grow indefinitely in culture. Such cells are programmed for a limited lifespan and they grow until they reach the "Hayflick limit" at which point they senesce and stop dividing. They proposed the existence of a replicometer (counter of replication) whose molecular basis was unknown at the time, that kept track of the number of divisions a given cell had undergone (Hayflick and Moorhead, 1961). Previously, in 1953 Watson and Crick published the double helix structure of deoxyribonucleic acid (DNA) (Watson and Crick, 1953) in the 50's and the 60's the biochemistry of the DNA replication machinery was unraveled (Lark, 1969; Richardson, 1969), and eukaryotic chromosomes were shown to consist of linear DNA molecules. These discoveries paved the way to the description of the end replication problem. In 1971, Alexey Olovnikov, a Russian theoretical biologist, proposed that with each round of cell division DNA at the very end of the chromosome could not be fully copied (Olovnikov, 1971; Olovnikov, 1973). One year later, James Watson proposed that chromosomes lose DNA from the ends because the DNA polymerase can not completely replicate the 3' end of a linear DNA molecule (Watson, 1972). If not compensated for, this phenomenon would eventually result in the loss of vital information (genes) at the chromosome end. The molecular answers to these questions were found to be the telomeres that cap the ends of the chromosome, block end-end fusion, protect chromosomes ends from loss of vital information and constitute the molecular basis for the replicometer of the Hayflick Limit.

In 1978, Elizabeth Blackburn determined the telomere sequence of the terminal DNA structure by examining the ends of extra chromosomal ribosomal DNA genes in *Tetrahymena thermophilia* and found that telomeres consisted of approximately 50

tandem repeats of CCCCAA/GGGGTT (Blackburn and Gall, 1978). In the early 80's other eukaryotic organisms were shown to have similar tandemly repeated G-rich sequences at their chromosome ends (Shampay et al., 1984). The human telomeres sequence was shown to contain repetitive (TTAGGG)n sequences (Moyzis et al., 1988) (de Lange et al., 1990). In the following year, the (TTAGGG)n repeat was found to be conserved among 91 different vertebrates (Meyne, 1989; Morales et al., 1999).

The enzymatic activity compensating for the end-replication problem was discovered by Carol Greider, a graduate student in the lab of Elizabeth Blackburn, when she demonstrated an activity that synthesized de novo telomeric repeats onto the *Tetrahymena* telomere ends (Greider and Blackburn, 1985). They called that enzyme terminal transferase or telomerase. Later on, telomerase was shown to contain an RNA component that specifies the repeats (Greider and Blackburn, 1989; Yu et al., 1990) that is closely associated with the protein component TERT (telomerase reverse transcriptase). Telomerase counteracts telomere shortening and confers on *Tetrahymena* the ability to divide indefinitely. With respect to human telomere/telomerase, it was known at the time that cancer cells are immortal, and thus must have a mechanism similar to *Tetrahymena* to counteract the end-replication problem and allow them to divide indefinitely. In 1989 Gregg Morin was able to detect telomerase activity in cell extract of tumor cells (Hela) (Morin, 1989).

Substantial evidence linking telomeres and replicative senescence was presented in 1990 when Calvin Harley and Carol Greider showed that telomeres do shorten significantly when normal human cells age and reach their Hayflick limit (Harley et al.,

1990). In 1989 Woody Wright and Jerry Shay proposed a mortality stage 1 and mortality stage 2 model and in 1992 they summarized the relationship between telomere shortening, aging and cancer (Wright et al., 1989; Wright and Shay, 1992). They proposed that as cells divide in culture, their telomeres shorten gradually until a subset of the telomere-ends are short. At that point the cells hit Mortality Stage 1, where they stop dividing and undergo replicative senescence. Only the cells that have blocked cell cycle checkpoints (altered p53 or p16/Rb pathway) can proliferate further and lose more telomeres. However those cells will eventually lose telomeres on most of their ends and reach a second proliferative blockade (crisis or Mortality Stage 2) that is characterized by a balance between cell division and apoptotic cell death. Cancer cells can overcome this M2 only by reactivating telomerase to replenish their telomeres (85-90%) or by engaging the ALT (alternative lengthening of telomere) pathway that is based on telomere recombination (10% of tumors) (Bryan et al., 1997; Kim et al., 1994).

In 1994, a simple yet highly sensitive PCR-based assay (TRAP= Telomere Repeat Amplification Protocol) was developed that can detect telomerase activity in a small number of human cells. Using this assay, Kim and coworkers, showed that telomerase is active in all cancer-derived cell lines and 85% of primary human cancers, but was absent from adjacent normal cells (Kim et al., 1994; Shay, 1997). This study introduced telomerase as a target for cancer diagnosis and anti-cancer therapy and placed the telomere field on the map of cancer research. Telomerase is expressed in the testes, in certain types of adult pluripotent stem cells, and in peripheral blood lymphocytes, however the level of telomerase activity in those cells is less than what is found in cancer

cells (Wright et al., 1996) and their telomeres are much longer. Thus telomerase could be an effective target for tumor therapy. To that extent, various strategies including hTERT vaccines/immunotherapy, telomerase RNA inhibitors (antisense to the template region), and hTERT-based oncolytic viruses are under investigation and some anti-telomerase cancer therapies are in preclinical and clinical trials (reviewed in (Shay and Wright, 2002).

It was not until mid to late 90's that the RNA and the protein component of human telomeres were cloned. The RNA component termed hTR for human telomerase RNA was cloned in 1995 (Feng et al., 1995) and the protein component named hTERT (for human telomerase reverse transcriptase) was cloned in 1997 (Harrington et al., 1997b; Nakamura et al., 1997). hTR is expressed in all cells, but hTERT is expressed only in cells with telomerase activity (Ducrest et al., 2002; Takakura et al., 1998). Scientists from the biotech company Geron were able to reconstitute telomerase activity using *in vitro* translated hTERT and *in vitr*o transcribed hTR proving that those were in fact the essential core components of telomerase (Weinrich et al., 1997). In an attempt to reinforce the impact of telomere shortening on cell senescence, Bodnar et al., in a landmark paper overexpressed hTERT in telomerase-negative normal human cells and discovered that those "telomerized" cells can now grow indefinitely and exceed the Hayflick limit for growth (Bodnar et al., 1998). This study confirmed the causal relationship between telomere shortening and cellular senescence. Nevertheless, the question remains as to whether telomere driven replicative senescence is involved in organismal aging. The experimental evidence pinpointing the role of replicative senescence in organismal aging remains controversial and unproven. However, some studies show a correlation between

donor age and *in vitro* lifespan of cells (Schneider and Mitsui, 1976), and fibroblasts from patients with premature aging syndromes (Werner and Progeria) have an extremely short lifespan in culture (Ouellette et al., 2000b).

REPLICATIVE SENESCENCE

Almost 45 years ago Leonard Hayflick and Paul Moorhead demonstrated that normal human diploid cells in culture have a limited lifespan and do not grow indefinitely (1961). In a well-controlled experiment, they mixed young female cells and old male cells in culture and monitored their growth. After their control culture of unmixed male cells quit dividing, they examined the mixed cultures and discovered that it consisted only of female cells. In fact, the male cells in the mix culture remembered their age and stopped dividing when they reached their inherent limit (Hayflick and Moorhead, 1961). This was later termed the Hayflick Limit and was strongly challenged at the time by many scientists especially since it contradicted the accepted wisdom of the time set by Alexis Carrel, the French Nobel laureate who showed in 1921 that cells in culture are immortal (Carrel et al., 1921). Hayflick implied the existence of a counting model that kept tract of the number of divisions cells undergo in culture before they senesce. That counting mechanism or the replicometer as used by Hayflick was inherent in the cells since cryogenically frozen cells kept tract of the number of times they divided before they were frozen. In 1975, Woodring Wright, who was Hayflick's student at the time, showed that the replicometer emanates from the nucleus of the cells (Wright and Hayflick, 1975). 15 years later, Calvin

Harley made the initial connection between replicative senescence and telomeres when he showed that telomeres do shorten as human fibroblasts approach the Hayflick limit (Harley et al., 1990). Senescent cells are characterized by altered morphology, increased granularity and enlarged nuclei, and they are arrested at G1 phase. Most importantly, they have altered levels of gene expression (Shelton et al., 1999).

In 1992 Jerry Shay and Woody Wright integrated their Mortality Stages 1 and 2 model with telomeres, aging and cancer. As cells divide, their average telomere length shortens and when a subset of the 92 telomeres (around 10) become sufficiently short, mortality stage 1 antiproliferative mechanism would be in effect. The critically short end would activate a DNA damage checkpoints (d'Adda di Fagagna et al., 2003; Zou et al., 2004b) by activating the p53 and Rb pathways. Cells that have a compromised p53 and Rb pathway checkpoint are able to further divide up to the point where most telomeres are extremely short, and the second block to proliferation is reached. This is referred to as Mortality Stage 2 and is characterized by chromosome end-end fusion, genomic instability and most cells undergo undergoing apoptotic cell death. Only the rare cell that can activate telomerase or engage ALT is able to escape M2 (Bryan et al., 1997; Kim et al., 1994).

Many recent experiments confirm the idea that telomeres constitute the molecular counter or the replicometer that determines the proliferative potential of normal cells and signal replicative senescence (Allsopp et al., 1995; Allsopp and Harley, 1995; Allsopp et al., 1992; Vaziri et al., 1994). A key experiment that corroborated the consequence of telomere length on replicative aging was done by Bodnar et al., (Bodnar et al., 1998) when

ectopically expressed telomerase prevented telomere shortening of normal human fibroblasts, was able to bypass replicative senescence and led to the immortalized normal diploid human cells. Short telomeres trigger a DNA damage signal and become associated with gamma H2AX, 53 BP and other DNA repair factors (d'Adda di Fagagna et al., 2003; Zou et al., 2004b). This DNA damage signal leads to the block in cell proliferation. Another study showed that when TRF2 (a telomere-repeat binding factor) is overexpressed in normal cells, replicative senescence was delayed markedly and the telomere length of TRF2 overexpressing cells at senescence was significantly shorter than the telomere length of normal cells at senescence (Karlseder et al., 2002). This led to the following question: is telomere length alone what dictates senescence or is it disruption of telomere-end structure that goes along with short telomeres that constitutes the signals for senescence? This question still awaits an answer.

The vast majority of normal human cells, with the exception of germ line cells, proliferative stem cells of renewal tissues and some immune cells, lack telomerase activity and as such would display replicative senescence (Wright et al., 1996). This is sharp contrast to cancer cells that are telomerase positive and can grow indefinitely (Kim, Piatyszek et al. 1994). This leads to a fundamental question regarding the significance of replicative senescence. Why do our cell age? Why did evolution favor replicative senescence as opposed to allowing all cells to grow indefinitely? One theory put forward viewed replicative senescence as an anti-cancer protection mechanism and an additional line of defense set by our cells to protect against tumorigenesis. A normal cell requires at least 4 -6 mutations to become malignant. Since the rate of spontaneous mutation is low,

each mutant cell needs to proliferate for many doublings to reach a population size large enough for the next mutation to occur, so each mutation takes up an average of 20-30 doublings. If our cells are programmed to live for 60-80 doublings, then most cancer cells with a few mutations will reach the Hayflick Limit before they become fully malignant. As such, replicative senescence would block the progression of premalignant cells and only the cells that can escape the Hayflick Limit by activating the expression of hTERT would have a chance to become malignant. Some *in vivo* evidence in support of this theory came from studies done on premalignant lesion in naevi, whereby premalignant cells were shown to stain positive for β-galactosidase, a marker for senescence (Michaloglou et al., 2005).

While replicative senescence is strictly telomere driven, some cells in culture stop dividing while their telomeres are relatively long and this is due to stress imposed on them by inadequate culture conditions. This is referred to as stress-induce senescence or "stasis" and leads to the accumulation of non-dividing senescent-like cells in culture. Proliferation of such cells cease independently of how many times they have divided in culture. Telomerase cannot immortalize cells in stasis. Mouse embryonic fibroblasts (MEF), which do express telomerase and as such would not be expected to undergo replicative senescence, are an example of cells that hit stasis after few doublings in culture (Parrinello et al., 2003).

Telomere-driven replicative aging is probably an ancient strategy with deep roots in the evolutionary tree and utilized by orders as diverse as Primates (Steinert et al., 2002), and Artiodactyla (Gomes et al., unpublished data). However lagomorphs (Forsyth et al.,

2005), rodents (Parrinello et al., 2003) and other orders have cells that possess telomerase activity and long telomeres and as such do not exhibit replicative senescence. This suggests that there might be a trade-off between using telomeres to counting cell divisions versus having longer telomeres.

THE END REPLICATION PROBLEM

In 1971 Alexi Olovnikov first suggested that a small amount of DNA from the ends of the chromosomes is lost with each cell division (Olovnikov, 1971). In an independent study, James Watson predicted the end replication problem based on T7 phage replication (Watson, 1972). What does the end-replication problem entail? According to the semi-conservative model of DNA replication, the two strands of eukaryotic chromosomal DNA are copied by leading and lagging strand synthesis that are strongly coupled in space and time (fig. 1-2). DNA replication is mediated by a complex protein machinery that is loaded at each replication fork to unwind the parental strands and synthesize the two progeny strands simultaneously and in the 5' to 3' direction. The leading strand synthesis is continuous and in the direction of the fork movement. However, the lagging strand synthesis is discontinuous and runs in the opposite direction of the fork. Lagging strand synthesis involves the repeated synthesis of oligonucleotide primers, which are then elongated into short DNA chains known as Okazaki fragments. Linear leading strand synthesis is continuous and polymerase could copy the chromosome to the very end of the telomere to generate a blunt ended DNA. Alternatively, the

replication machinery could fall off before it reaches the end generating a 5'C-rich overhang. As DNA synthesis continues, each Okazaki fragment is processed whereby the RNA primer is removed and the fragment is ligate to the next one. The removal of the RNA primer from the last Okazaki fragment will leave a gap of either about 10-14nt (if the last primer was placed at the very end of the chromosome) or up to 300 nt (if the last primer is randomly placed with respect to the end of the chromosome). The result of that would be the shortening of the chromosome end by up to 300nt/division and this is what is referred to as the end-replication problem. Recent studies have shown that the final product of leading strand synthesis also has a significant 3'-overhang, predicting the existence of a nuclease that resects the 5' end of the chromosome, augmenting the end replication problem further. Whether overhangs of lagging strand synthesis are processed further upon the removal of the last RNA primer is yet to be determined.

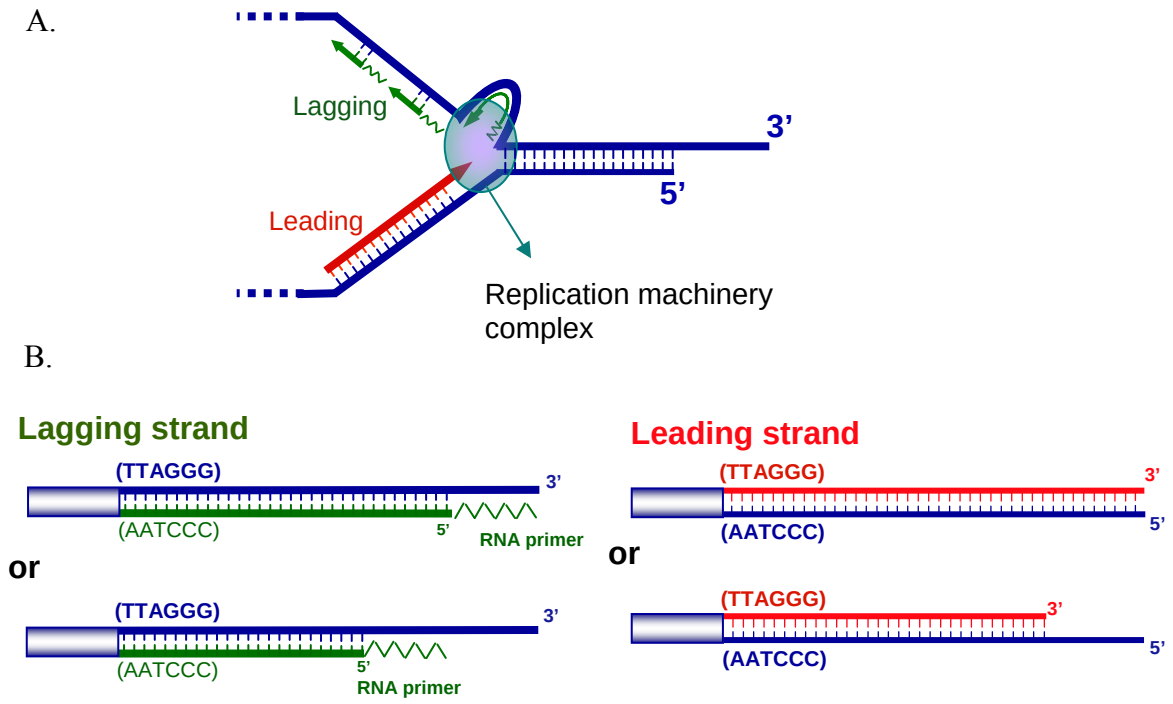


Figure 1.2- DNA replication and the end replication problem. A) Unwinding of the DNA strands will allow the replication machinery to sweep through. Leading strand synthesis is continuous, while lagging strand synthesis is discontinuous, made of fused Okazaki fragments. B) As the replication machinery is progressing through the telomere end, it will generate two structurally different ends. The products of lagging strand synthesis have 3'G-rich overhangs while products of leading strand synthesis are initially either blunt ended telomeres or telomeres possessing 5'C-rich overhang.

TELOMERE END ARCHITECTURE

Mammalian telomeres consist of many kilobases of 5'-TTAGGG / 3'-AATCCC DNA repeats (de Lange et al., 1990; Meyne et al., 1989)that are bound by specialized proteins, forming a unique end-structure (For review de Lange, 2005). The G-rich strand (TTAGGG) extends beyond the double stranded region to form a single stranded 3'overhang that is believed to be important for telomere-structure and proper functioning (Greider, 1999; Griffith et al., 1999; Makarov et al., 1997; Wellinger et al., 1996; Wright et al., 1997). Ultrastructural studies at the Electron Microscopy level have shown that the 3' G-rich overhang does not always exist as a free extension in cells, but is often tucked back into the preceding double stranded region to form a lariat like structure the "t-loop" (Griffith et al., 1999). T- loops can be present on both ends of a chromosome, suggesting that the leading DNA strands (initially blunt-ended or possessing a 5' C-rich extension) is resected by an unidentified nuclease in order to generate the 3' G-overhang that is required for T-loop formation.

T-loops, which were first discovered by electron-microscopy analysis of human telomeric DNA (fig. 1-3), have been found to exist at the termini of micronuclear chromosomes of *Oxytricha nova* (Griffith et al., 1999; Murti and Prescott, 1999), at the telomeres of *Trypanosome brucei* (Munoz-Jordan, 2001; Munoz-Jordan et al., 2001), *Pisum sativum* (Cesare et al., 2003), mouse and chicken (Nikitina and Woodcock, 2004), as well as at the ends of linear mitochondrial DNA of the yeast *Candida parapsilosis* (Tomaska et al., 2002). T-loops present an evolutionary ancient means of telomere

maintenance that may have used factors that are involved in recombination-dependent replication (de Lange, 2004). Their size range from 1 kb in trypanosome to up to 50 Kb in peas, while human T-loop size is variable. Telomere-binding proteins help maintain the stability of this structure, especially TRF2, which has been shown to bind the T-loop juncture (Griffith et al., 1999; Stansel et al., 2001). As long as the overhang is longer than 9 nucleotides, it is of no consequence on T-loop formation, at least in an *in vitro* setting (Griffith et al., 1999; Stansel et al., 2001). Key information about t-loop formation, structure and significance, including their detailed base structure, how and when are they form during the cell cycle, and how telomerase action is affected by t-loop formation is largely unknown. What is evident at this point is that the very end of the chromosome folds back, allowing the single-stranded telomeric 3' overhang to invade and hybridize with a region of the double-stranded telomere repeat, resulting in three-stranded D-Loop formation (Griffith, Comeau et al. 1999). Some segment of the C-strand may also invaded, resulting in a Holiday junction. The T-loop conformation may prevent telomere ends from being recognized as DNA damage and may block the DNA-damage response.

Overhangs constitute an important structure of the ends that is involved in proper telomere function, T-loop formation and setting the telomere shortening rate, yet information about their generation, especially in mammalian cells remains scarce. Most of the information we know about telomere end structure derives from model organisms (yeast and ciliates), in which genetic and structural studies are more easily undertaken (Fan and Price, 1997; Jacob et al., 2003; Jacob et al., 2001; Wellinger et al., 1993a; Wellinger et al., 1993b).

Saccharomyces cerevisiae overhang length is regulated in a cell cycle dependant fashion (Dionne and Wellinger, 1996). Overhangs that are > 30 nt are generated in late S phase in a telomerase independent manner (Wellinger et al., 1993a; Wellinger et al., 1993b). Then they are processed to give a final length of 12-14 nucleotides throughout the rest of the cell cycle (Larrivee et al., 2004). This implies that some sort of machinery with nuclease activity must act on the overhangs at the end of S-phase to process it to a length of 12-14 nucleotides. Yeast G-strand termini show no base specificity (Forstemann et al., 2000), while their C-strand termini have not been directly determined. While the processing machinery and the nuclease identity is still largely unknown, some protein complexes have been shown to affect overhang length. Studies have shown that Cdc13, a protein that binds to the single-stranded G-rich overhang, controls overhang length by blocking extensive C-strand resection (Booth et al., 2001). Nevertheless, the mechanistic details of the protection are still unknown. The Ku 70/80 heterodimer, whose main role is to repair double stranded breaks via NHEJ, has been shown to regulate overhang length also by protecting the end from excessive nuclease resection (Gravel et al., 1998). Furthermore, the MRX complex encoded by MRE11, RAD50 and XRS2 affect G-rich overhangs, yet the Rad50 nuclease activity is not responsible for the resection (Larrivee et al., 2004; Takata et al., 2005). So far the only nuclease demonstrated to have a direct role in overhang generation is Dna2, which was reported to be the nuclease that is responsible for C-strand resection in fission yeast (Tomita et al., 2004) but this has not been extended to other organisms.

Ciliates have short and very abundant telomeres that facilitate analysis. Studies by Price and coworkers have shown that the overhangs in *Euplotes* are generated with precise terminal nucleotides at both ends (GGTTTTG**G**-3' at the G-rich strand and AAAACC**C**-5' at the C-rich strand) and the length of the overhang is always 14nt (Fan and Price, 1997). Extensive studies characterizing mechanisms of overhang processing have also been done in *Tetrahymena*. Instead of ending in GGGTT**G**-3' as would be expected if the terminus is generated by dissociation of telomerase during the translocation step, most *Tetrahymena* telomeres end in TGGGG**T**-3'. *Tetrahymena* C-rich strands end with CAACC**C**-5' or CCAAC**C**-5'. This suggests that overhang generation is mediated by two separate processing steps; one cleaves the G strand and the other resects the C strand, and both steps are distinctively terminated at a specific base (Jacob et al., 2003; Jacob et al., 2001).

Multiple assays have been developed to measure G-rich overhang lengths in mammalian cells. Electron-microscopy based studies (Huffman et al., 2000; Wright et al., 1997) as well as molecular techniques (Telomere-Oligonucleotide Ligation Assay or T-OLA (Cimino-Reale et al., 2001), Telomeric Overhang Protection Assay (Chai et al., 2005), G-tail telomere Hybridization Protection Assay (Tahara et al., 2005) and in gel hybridization (Dionne and Wellinger, 1996) showed that human telomeres have variable overhang lengths ranging from 35-300nt in length and are present on both strands following DNA replication. While some studies proposed that overhang erosion in old cells constitutes the molecular signal that triggers replicative senescence (Stewart et al., 2003), additional data disproved that theory (Celli and de Lange, 2005; Chai et al., 2005;

Keys et al., 2004). The current view is that overhangs are important for optimal telomere structure (T-loop formation) and function (chromosome end protection), affect telomere-shortening rates and are maintained at senescence. Certain reports have invoked the idea that leading and lagging telomeres might be created differently. This is based on studies that inhibited DNA-PKs in human cells and showed that telomeric fusion preferentially involved the products of only leading DNA strands (Bailey et al., 2001). Inhibiting Werner activity resulted in telomere loss on lagging strands, (Crabbe et al., 2004) and altering TRF2 function resulted in leading strand telomeric overhang loss (Zhu et al., 2000; Zhu et al., 2003). Chai et al (2006) separated leading from lagging telomeric DNA and measured their overhang lengths. Their results showed that in normal cells lagging daughter strands have longer overhang than leading daughters. When telomerase is expressed, leading strand overhangs were elongated to be about the same size as lagging overhangs, while lagging strand overhangs remained unchanged. This suggests overhang processing to a certain extent is different between leading and lagging ends, and that following telomerase extension, the overhangs might be filled-in by a mechanism similar to lagging-strand synthesis (Chai et al., 2006a).

The machinery of telomere end processing and overhang generation in human cells is still widely unexplored. The identity of the nuclease that resects the overhang is unknown, the factors that regulate its activity and specify the overhang length are unspecified, and information on its temporal regulation is also lacking. An increasing number of telomere-associated proteins have been identified. A set of nucleases/helicases have been characterized in mammalian cells. Determining which nuclease (s) act on the

end and what factors regulate its activity remains on of the biggest challenges in the telomere field.

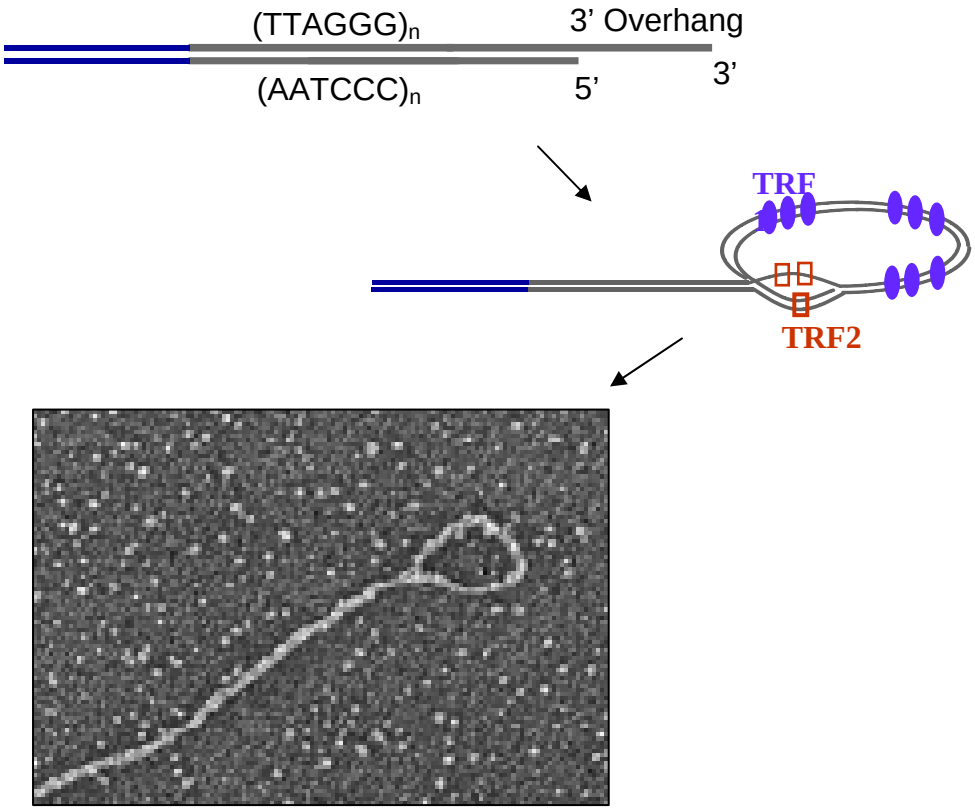


Figure 1.3- Human t-loop architecture. A) t-loop formation whereby, the extended 3' G-rich overhang invades the preceding double stranded region creating the d-loop structure. The t-loop is stabilized by telomere-binding protein like TRF2 and TRF1. B) Visualization of the T-loop structure of a telomere end by electron microscopy (by courtesy of Dr. Griffith) (Muñoz-Jordán et al., 2001)

THE PROTEIN COMPONENT OF MAMMALIAN TELOMERES

Vertebrate telomeres are composed specialized TTAGGG repeats whose length is inherited and varies between species. In humans, the length of the telomere ranges from 2-30 Kb (de Lange et al., 1990; Meyne et al., 1989). Within one given cell, the telomere length on different chromsomes is heterogeneous. The DNA part of the telomeres is bound by specialized DNA-binding proteins and other associated factors to form a higher-order structure (fig. 1-4). Some of these proteins are telomere-specific, while others participate in different cellular processes as well (reviewed in de Lange, 2005; Smogorzewska and de Lange, 2004)

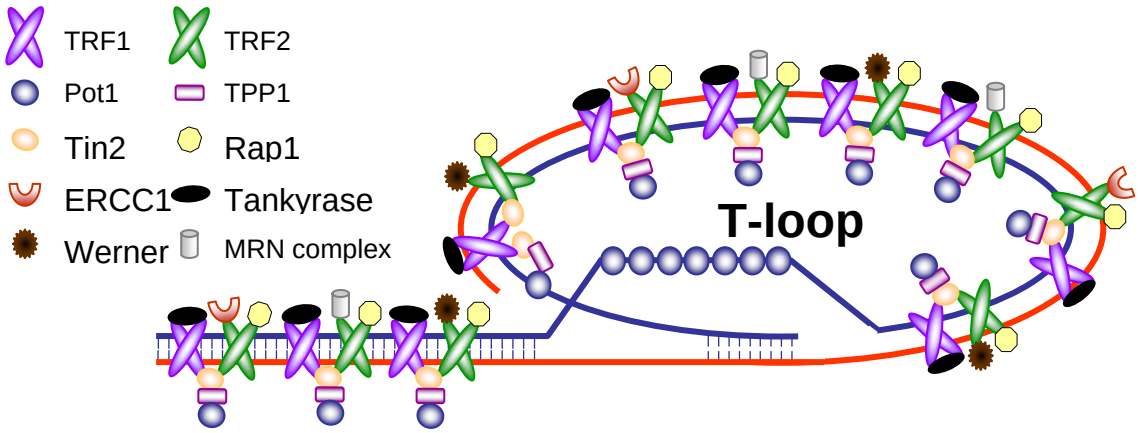


Figure 1.4- The telomeric DNA and protein complex. The telomeric DNA is associated with a set of telomere-associated proteins, some of them bind directly to the DNA and others are associated with them.

The basic telomeric protein complex is a six-member telomere-specific complex containing TRF1, TRF2, hRap1, Tin2, Tpp1 (PTOP/Tint1/PIP2), and POT1. The complex is bound to the DNA *via* TRF1 and TRF2 binding to the duplex region, while POT1 can also bind to the single stranded overhang. The six-member complex, or shelterin is involved in forming proper telomere end structure (T-loop formation and end processing), controlling telomere length by recruiting and regulating telomerase activity, and protecting the chromosome end from the DNA damage checkpoint and repair machinery (de Lange, 2005; Smogorzewska and de Lange, 2004).

TRF1 (Telomere Repeat Factor1) was the first human telomere binding protein to be identified (Zhong et al., 1992). It exists as a homodimer, contains three domains (an acidic region and the amino terminal end, a dimerization domain and two myb domains at the carboxy terminal) and binds with great specificity to the duplex region of the telomere (Bianchi et al., 1997; Bianchi et al., 1999; Chong et al., 1995). Human TRF1 undergoes alternative splicing to generate a smaller protein "Pin2", that is missing 20 amino acids form the linker region (Shen et al., 1997). Using a SELEX software, Bianchi et al., (1999) showed that TRF1 binds to 5' YTAGGGTTR-3' region (Bianchi, Stansel et al. 1999). The dimer of TRF1 is proposed to generate bending of the telomere to allow T-loop formation (Bianchi, Smith et al., 1997). TRF1 is also involved in telomere-length homeostasis in telomerase-positive cells (Smogorzewska et al., 2000; van Steensel and de Lange, 1997). Mouse knockout models of TRF1 are embryonic lethal, suggesting it may also have other functions or is essential for telomere structure (Karlseder et al., 2003).

Another telomere double stranded DNA binding protein that was identified by sequence homology to TRF1 is TRF2 (Broccoli et al., 1997). TRF2 is important for T-loop formation, and is possibly involved in tucking the G-overhang into the preceding double-stranded telomeric DNA (Griffith et al., 1999; Stansel et al., 2001; Yoshimura et al., 2004). TRF2 has a key role in protecting the telomere end from the DNA damage response (Celli and de Lange, 2005). Overexpression of a dominant negative TRF2 lead to the complete loss of G-rich overhang on 50% of the chromosome ends producing massive chromosomal instability, increasing end-to-end fusion, ATM and p53 mediated apoptosis or senescence (Karlseder et al., 1999; van Steensel et al., 1998) and a DNA damage response exemplified by the accumulation of TIFs (telomere-induced foci) containing 53 BP1, Nbs1, and P-ATM (d'Adda di Fagagna et al., 2003; Takai et al., 2003). TRF2 protected the G-rich overhangs by blocking the ability of ERCC1/XPF endonuclease to clip the overhang at the juncture of single to double stranded DNA (Zhu et al., 2003). This is compromised when a dominant negative form of TRF2 is expressed. TRF1 and TRF2 are key components of the six-member complex.

Tin2 establishes the bridge between TRF1 and TRF2 that forms the base of the six-member telomere complex. It stabilizes TRF1 on telomere, and protects it from tankyrase driven degradation. Tin2 overexpression leads to telomere shortening in telomerase positive cells probably due to the accumulation of six-member complex on telomeres (Ye and de Lange, 2004). TRF2 interacts with hRap1, the human orthologue of yeast Rap1. hRap1 has 3 domains, a BRCT domain, an RCT domain (Rap1 carboxy domain) that mediates TRF2 interaction and contains the nuclear localization signal and a Myb domain.

Unlike the yeast protein, hRap1 does not use its Myb domain to directly bind to the DNA; instead it relies on TRF2-interaction for its telomere localization. hRap1 is a negative regulator of telomere length. Furthermore, hRap1 is important in telomere-length heterogeneity, whereby overexpressing deletion mutants lacking the BRCT or the Myb domain decreased the overall heterogeneity of telomeres (Li and de Lange, 2003; Li et al., 2000)

The single-stranded overhang is bound by several heterogeneous nuclear ribonucleoproteins (hnRNPs) (Ishikawa et al., 1993) and POT1 (protection of telomeres 1), which is also a part of the six-member complex (Baumann and Cech, 2001). hPOT1 was identified by sequence homology with the ciliate telomere binding protein TEBPα. It contains two OB (oligonucleotide/oligosaccharide) folds that it utilizes in order to bind to the G-rich overhang with great specificity. In vitro binding assays showed that POT1 binds specifically to the 5'- (T)TAGGGTTAG-3' sequence and the crystal structure of Pot-1 indicated that the first OB fold binds firmly to the first 6 nucleotides, and the second fold protects the terminus that would end in TAG-3' (Lei et al., 2002; Lei et al., 2003; Lei et al., 2004; Lei et al., 2005; Loayza et al., 2004). However, the DNA binding domain is not the only means by which Pot1 interacts with the telomere, since overexpressing a Pot1 delta OB fold that lacks the DNA binding domain still localizes to the telomere (Loayza and De Lange, 2003). In fact, POT1 is recruited to the telomere through the six-member complex by its interaction with TPP1, the link between POT1 and Tin2 (Houghtaling et al., 2004; Liu et al., 2004a; Ye et al., 2004). Pot1 is proposed to function downstream of TRF1 to regulate telomere length in telomerase positive cells (Loayza and De Lange,

2003). The crystal structure analysis, together with *in vitro* assays, suggested that the localization of Pot1 on its preferred sequence could regulate telomerase action at the end of the telomere (Kelleher et al., 2005; Lei et al., 2005).

TPP1 (PTOP/PIP1/TINT1) is the last factor of the six-member complex to be purified and it functions in recruiting Pot1 to the six-member complex. Low levels of TPP1 leads to a telomere elongation phenotype that is consistent with diminished levels of POT1 at the ends (Houghtaling et al., 2004; Liu et al., 2004a; Ye et al., 2004).

There are a number of telomere associated proteins that are not solely involved in telomere biology including tankyrase, WRN and BLM helicases, Rad51, Artemis, DNA-PKcs, Ku 70, ERCC1/XPF, the Mre11 complex and other factors (for review (de Lange, 2005).

Artemis is a 5'-3' exonuclease with some endonuclease activity that is involved in NHEJ and VDJ recombination. Artemis deficiency can be detected in some RS-SCID humans. Artemis-deficient Murine ES cells displayed high levels of genomic instability with marked telomeric fusion (Rooney et al., 2003; Rooney et al., 2002). However, the precise function of Artemis at the telomere is yet to be characterized.

Werner syndrome patients have a premature aging phenotype due to the mutation in the WRN RecQ helicase (Ozgenc and Loeb, 2005). Fibroblasts from Werner Syndrome patients have slightly shorter telomere length and reach senescence faster than normal fibroblasts (Baird et al., 2004; Ouellette et al., 2000a). WRN interacts with TRF2 (Machwe et al., 2004; Opresko et al., 2002) and POT1 (Opresko et al., 2005) and is important for telomere replication, especially for lagging strand synthesis (Crabbe et al.,

2004) since it is resolves G-quartets (Johnson et al., 2001). BLM helicase also functions at the telomeres and fibroblasts from Bloom syndrome patients age faster (Baird et al., 2004; Ouellette et al., 2000a). The BLM protein is associated with telomere-containing PML bodies in ALT cells (Watt et al., 1996).

Tankyrase 1 is a TRF1 specific PARP (poly-ADP ribose polymerase). Ribosylation of TRF1 by tankyrase primes it for degradation and removes it from the telomeres. Under normal conditions this is blocked by the interaction of TRF1 with Tin2, that stabilizes the former at the telomeres (Smith and de Lange, 1999; Smith and de Lange, 2000; Smith et al., 1998).

An important function of telomere binding proteins and the six-member complex in particular is to hide the telomere end from the DNA repair machinery. However, one surprising aspect of telomere biology is that many DNA repair factors involved in NHEJ, HR and base excision repair are important for normal telomere function and maintenance. The Ku70/80 proteins are recruited to the telomere by TRF1 and TRF2 and interact with telomerase (Chai et al., 2002; Song et al., 2000). Their deficiency leads to telomere fusion, presumably by altering the structure of the 3' G-rich overhang (Hsu et al., 2000). Similarly, DNA-dependent protein kinase (DNA-PKc) inhibition leads to telomeric end-end fusion (Espejel et al., 2002; Espejel et al., 2004; Goytisolo et al., 2001). The MRN complex associates with telomeres through its interaction with TRF2. Mre11 and Rad50 are bound to the telomeres throughout the cell cycle, whereas NBS1 is recruited during S phase (Zhu et al., 2000).

A structure-specific endonuclease, excision repair cross-complementing 1 / Xeroderma pigmentosum F (ERCC1/XPF) is a nucleotide excision repair protein and a component of the telomeric TRF2 complex. It cleaves on the 5' side of bubble structures containing damaged DNA, and cuts DNA duplexes adjacent to a 3' single-stranded DNA flap (de Laat et al., 1998). Proteins functioning upstream in the DNA damage sensing and signaling cascade such as ATM, and ATR have been found to have a great impact on telomere integrity as well as telomere length regulation. ATM has not been shown to directly interact with telomeric DNA, however it binds to TRF2. Interestingly, TRF2 binds to the S1981 site of ATM, the site that is phosphorylated in response to damage (Karlseder et al., 2004).

The telomeres, which serve in differentiating chromosome ends from internal DNA strand breaks, could be recognized as DNA breaks under certain circumstance. For example, telomeres could be recognized as DNA breaks and recruit DSB repair proteins when they are extremely short, when their integrity is compromised by deleting telomere binding proteins, or for a short time after they replicate. A recent study showed that in every G2 phase of the cell cycle telomeres trigger a transient DNA damage response and recruit Mre11, Phosphorylated NBS1 and ATM (Verdun et al., 2005). Presumably this response is required for processing of the telomere end and to form t-loops. Short telomeres are recognized as DNA breaks since they associate with DNA damage repair markers: phosphorylated histone H2AX, 53BP1, and NBS1 (d'Adda di Fagagna et al., 2003; Zou et al., 2004b). When cells are close to senescence, some of their telomeres would become sufficiently short and trigger a DNA damage response eventually causing

cell cycle arrest (Zou et al., 2004b). Furthermore, when telomeres are rendered dysfunctional, such as when TRF2 is inhibited, checkpoint pathways are triggered. 53BP1, p-ATM, NBS1, Rif1 and Rad17 are recruited to the telomere to form TIFs (telomere dysfunctional induced foci) and the DNA damage sensors would eventually block cell cycle progression (Zhu et al., 2000; Zhu et al., 2003). The function of TRF2 in maintaining telomere structure and integrity has been carefully examined. Knocking out TRF2 enabled the NHEJ pathway to gain access to the end and lead to an ERCC1/XPF – mediated loss of 3'overhangs (Zhu et al., 2000; Zhu et al., 2003). Ultimately, this generates chromosome end fusions driven by DNA ligase IV (Celli and de Lange, 2005; Wang et al., 2004). Overexpressing a mutant form of TRF2 lacking the amino terminal basic domain TRF2 delta B, permits recombination to take place a the base of the T-loop, creating telomere circles and causing telomere rapid deletion events (Celli and de Lange, 2005; Wang et al., 2004). Thus some of TRF2 normal function is to hamper NHEJ and HR at the telomere end and maintain telomere integrity.

THE TELOMERASE RIBONUCLEOPROTEIN COMPLEX

The solution to the end-replication problem was revealed upon the discovery of telomerase activity (Greider and Blackburn, 1985; Greider and Blackburn, 1989). Human telomerase uses the catalytic protein component (reverse transcriptase enzyme hTERT) and its associated RNA component (hTR or hTERC) as a template to add TTAGGG repeats onto the 3' end of chromosomes and counteract telomere loss after each round of replication (Greider and Blackburn, 1985; Greider and Blackburn, 1989). Telomerase activity is detected in almost all established tumor cell lines, in ~85-90% of primary tumor biopsies and is absent from adjacent normal cells (Kim et al., 1994). The telomerase RNA (TR or TER) component is present in a wide variety of organisms including yeast, ciliates and vertebrates, but its sequence has greatly diverged throughout evolution (Chen et al., 2000; Lingner et al., 1997; Romero and Blackburn, 1991; Romero and Blackburn, 1995). The template region of all TRs is conserved and is made of a single stranded region (11bp for hTR) that permits Watson-crick base pairing with the telomere end and then allows the synthesis of one repeat (Greider and Blackburn, 1985; Greider and Blackburn, 1989). The telomerase reverse transcriptase (TERT) catalytic subunit protein sequence is conserved among different species and contains different domains involved in catalysis, dimerization, RNA binding, recruitment to telomeres and nuclear localization (Cech et al., 1997; Harrington et al., 1997b; Lingner et al., 1994; Nakamura and Cech, 1998; Nakamura et al., 1997). Telomerase binds to the telomere end, and then undergoes

progressive cycles in which it elongates the end by adding one GGTTAG repeat, and then translocates to the end of the newly added repeat, repeating this cycle until it dissociates from the end (fig. 1-5) (reviewed in(Cong et al., 2002; Kelleher et al., 2002).

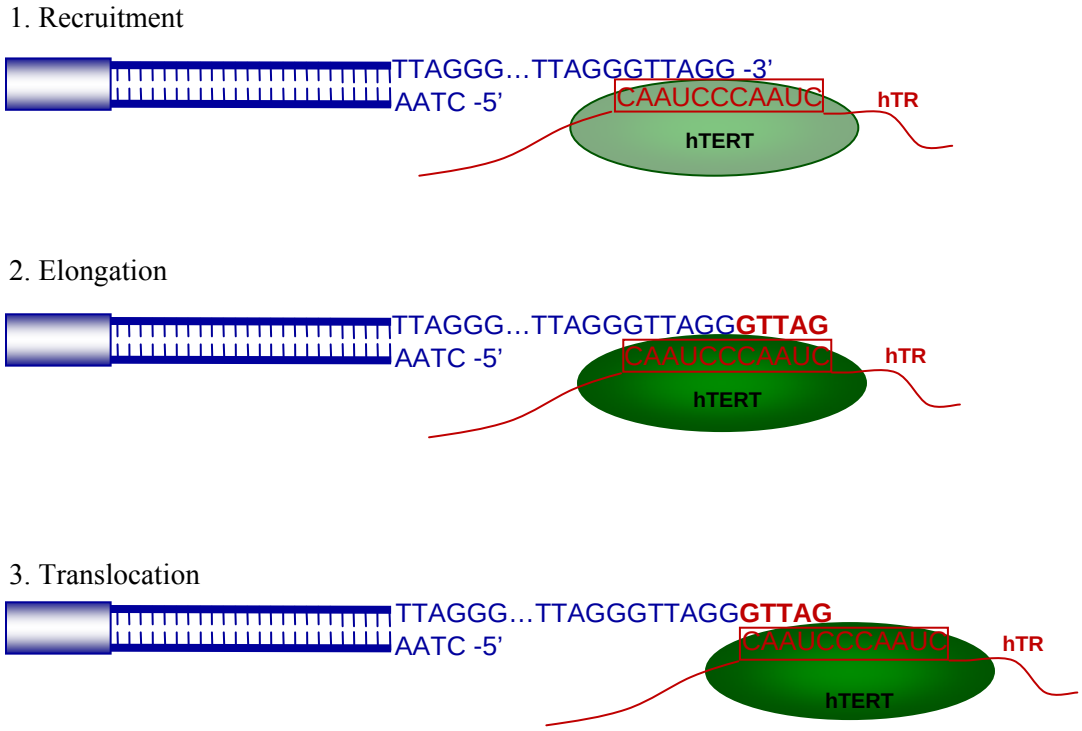


Figure 1.5- The dynamics of telomerase action. Telomerase is recruited to the telomere and the template region of hTR aligns to the telomere end. Then the catalytic subunit (hTERT) of telomerase elongates the telomere by adding bases complementary to the hTR template region. Finally the complex translocates and the elongation step is repeated.

Human telomerase has a mass of over 1mDa and is associated with many proteins, including snoRNA binding proteins (Dragon, 2000; Mitchell, 1999), heterogeneous nuclear ribonucleoproteins (hnRNPs) A1, C1/C2, and D (Eversole and Maizels, 2000; Ford, 2000; LaBranche, 1998), molecular chaperones p23/hsp90 (Holt, 1999), double-stranded RNA binding protein hStau (Le, 2000), ribosomal protein L22, the La autoantigen (Aigner et al., 2000; Ford, 2001), and vault protein TEP1 (Harrington et al., 1997a). Despite the fact that within a given cell the telomere length shows some heterogeneity, the overall average in tumor cells is maintained within a narrow distribution and this is largely due to very controlled activity of human telomerase. Regulation of telomerase activity is exerted at multiple steps; the transcriptional activation of hTERT and hTR, post-translational modification of hTERT, recruitment of telomerase to the telomeres, assembly into an active enzyme, and subcellular localization (reviewed in Cong et al., 2002; Kelleher et al., 2002). In addition, another level of control acting *in cis* at the level of each telomere ends dictates the extension boundary of telomerase (for review(Smogorzewska and de Lange, 2004). The detailed mechanisms of telomere length homeostasis in human cells are not fully understood.

Telomerase regulation is part of a negative feedback loop, whereby certain telomere binding proteins act in *cis* as negative regulators for telomerase activity. TRF1, Tin2, Pot1, TPP1, Rap1 are the human telomere binding proteins that control telomere length homeostasis and additional factors, Rif1, and Rif2 act *in Saccharomyces cereviseae* (Smogorzewska and de Lange, 2004). The main regulator is TRF1 (van Steensel and de

Lange, 1997) since overexpressing it causes telomeres to gradually shorten while inhibiting it by a dominant negative form that removes endogenous TRF1 from the telomeres results in telomere elongation without affecting telomerase activity (Smogorzewska, 2000). TRF1 constitutes a protein counting model, whereby longer telomeres possess more TRF1 and have less chance of being elongated by telomerase than shorter telomeres with less TRF1. The direct proof for the *cis*-effect of TRF1 on telomere elongation comes from tethering experiments showing that loading lacI-TRF1 fusion protein to a subtelomeric array of lacO sites lead to telomere shortening (Ancelin et al., 2002). The TRF1 binding proteins Tin2 and tankyrase are also involved in regulating telomere length. Tin2 overexpression led to shortening of the telomeres while overexpressing a dominant-negative form of Tin2 elongated the telomeres (Kim et al., 1999). Tankyrase exerted its effect on telomere length via TRF1, whereby increased levels of tankyrase leads to TRF1 degradation and telomere elongation, while inhibiting tankyrase activity stabilized TRF1 and led to telomere shortening (Smith and de Lange, 2000). Rap1 also influences telomere length, such that overexpressing full-length hRap1 results in telomere shortening and overexpression of deletion mutants lacking the BRCT or the Myb domains lead to telomere elongation (Li and de Lange, 2003). POT1, the single-stranded telomeric DNA-binding protein, is suggested to be the terminal transducer of TRF1 telomere length control. Overexpressing POT1 delta OB, a mutant form lacking the DNA binding domain, generated excessive telomere elongation in telomerase positive cells (Loayza and De Lange, 2003). The mechanistic details of the POT1 effect on telomerase extension has been explained by *in vitro* assays and crystallography showing

that POT1 loading on the overhang can occur in such a way that hides the 3'end from telomerase, thereby blocking the ability of telomerase to add TTAGGG repeats. On the other hand, if POT1 loading does not hide the 3' end terminus, telomerase ability to extend that end is enhanced (Lei et al., 2003; Lei et al., 2004; Lei et al., 2005). Other telomere associated factors that affect telomere length homeostasis include DNA-PKcs (Espejel et al., 2002; Espejel et al., 2004), Rad54 (Jaco et al., 2003), Suv39h (Garcia-Cao et al., 2004), Nbs1 (Bai and Murnane, 2003a; Ranganathan et al., 2001), hEST1 (Reichenbach et al., 2003) and the retinoblastoma (RB) family of proteins (Gonzalo and Blasco, 2005). Many factors that affect telomere length homeostasis and regulate telomerase have been described, however what is yet to be determined is what aspect of telomerase regulation (recruitment, processivity or activity) do they impinge upon.

The telomere length in cancerous cells is at a steady state with an average length that is generally shorter that normal cells. This suggests that telomerase activity within a given tumor cell is just sufficient to maintain telomeres above the point where they would generate DNA damage signals. There is circumstantial evidence suggesting that telomerase gets preferentially recruited to the shortest telomeres. Evidence comes from studies done on human cells whereby telomerase hTERT was overexpressed in BJ (human foreskin fibroblasts) cells for a limited number of doubling using the cre-lox system. When the "transiently" telomerized cells reached senescence, their overall telomere length was significantly shorter than the average telomere length of the control (telomerase negative cells) senescent cells (Steinert et al., 2000). This implied that the telomerase activity, that was present for few doublings, preferentially elongated the shortest telomeres

and the cells reached senescence when a bigger fraction of ends had short telomeres. In another study after telomerase activity dropped to low levels in transfected normal cells (presumably due to promoter hypermethylation) the cells still grew indefinitely but had an average telomere length that is extremely short (Ouellette et al., 2000a). Further evidence comes from mouse models, whereby a 3rd generation telomerase hTR knockout mouse (hTR -/-) was crossed with a heterozygote hTR (+/-) mouse. Comparing the telomere length in the progeny showed that a fraction of very short telomere that was present in the null hTR mice was absent from the heterozygote mice with limited amount of telomerase (Hemann et al., 2001).

Telomere-length homeostasis has been deciphered in *Saccharomyces cerevisiae* (Diede and Gottschling, 1999; Marcand et al., 2000; Teixeira et al., 2004; Teixeira et al., 2002). A recent study measured telomere elongation in a single cell cycle at a nucleotide resolution level using genetics and telomere sequencing (Teixeira et al., 2004). Their results unequivocally show that in yeast, telomerase does not act on every telomere end in every cell cycle. Short telomeres are more frequently elongated, with the number of nucleotides added being random. Based on those experiments a model was drawn, showing that telomere exists in two states, short telomeres have an extendible state that is permissive for telomerase elongation, and long telomeres are in non-extendible state that blocks telomere elongation. The threshold between short and long is set by a protein counting model, in which telomere binding protein Rif1 relays the message to Tel1 which in turn controls telomerase recruitment or activity (Teixeira et al., 2004). In human cells, a precise understanding of detailed mechanism of telomerase action and its relationship to

telomere replication has been limited mainly due to the lack of assays that defines the dynamics of telomere extension events at the level of individual telomere molecules.

TELOMERE LENGTH DYNAMICS AND WAYS TO MEASURE IT

Telomere length dynamics reflect an intricate interplay between biological mechanisms that cause telomere shortening and others that lead to telomere elongation. Telomere shortening is mainly due to the end replication problem (Olovnikov, 1971; Watson, 1972) and nuclease resection (Makarov et al., 1997; Wellinger et al., 1996) as well as other stochastic influences like oxidative damage (Serra et al., 2000; von Zglinicki, 2000; von Zglinicki, 2002; von Zglinicki et al., 2000) and rapid deletion events (Baird et al., 2006; Baird and Kipling, 2004; Baird et al., 2003; Wang et al., 2004). Shortening is counteracted by telomerase extension in the germ line and 90% of tumor cell lines (Kim et al., 1994). A small fraction of tumor cells maintain their telomeres by engaging ALT (alternative lengthening of the telomere) that is based on recombination (Bryan et al., 1997).

Plenty of methods to assess telomere length have been developed, including southern blot (TRF: telomere restriction fragment analysis) (Allsopp et al., 1995; Harley et al., 1990), fluorescent *in situ* hybridization (FISH)(Lansdorp et al., 1996), Primed *in situ* (PRINS), flow cytometry and a PCR-based method to measure length of individual telomeric molecules on a given chromosome end (STELA: Single Telomere Length Analysis)(Baird et al., 2003). In a southern blot, genomic DNA is extracted and digested

with a four base cutter restriction enzyme, then run on an electrophoresis gel, blotted on membrane and probed with a radioactively labeled probe consisting of the telomeric TTAGGG or AATCCC sequence. The telomeres which consist of TTAGGG/AATCCC repeats that lack restriction sites will run as smear, such that one can calculate the TRF-length of a given sample or cell type by measuring the size and the intensity of the smear using densitometry (Harley et al., 1990; Oexle, 1998). With FISH one can use a fluoresecently labeled probe (PNA, phosphoamidate or oligonucleotide) and directly label the telomeres of an individual cell (Lansdorp et al., 1996). The cells are usually collected at metaphase and dropped on a slide for analysis. Throughout the years, both of these assays have been utilized immensely and allowed telomere length analysis to be performed with great accuracy. However, their major disadvantage is the inability to precisely analyze short telomeres. TRF and FISH are hybridization based and usually biased towards longer telomeres, masking the ability to carefully measure the length of the shortest telomere. Given the importance of the shortest telomere in signaling senescence in normal cells and recruiting telomerase in cancer cells, ways to assess their length were needed. In 2003, Baird *et al.,* developed the STELA (Single telomere length analysis) assay, a PCR-based technique that provided the full spectrum of telomere lengths on a given chromosome (Baird et al., 2003). The basic idea behind STELA is to ligate an oligonucleotide with a unique sequence to the last base of the telomeric C-rich strand. The telomere is PCR amplified using a forward primer within the subtelomeric region of a specific chromosome arm and a reverse primer that is complimentary to the unique sequence ligated to the telomere end. A southern blot is then performed and the whole

spectrum of telomere length can be analyzed without being biased for longer telomeres (fig. 1-6).

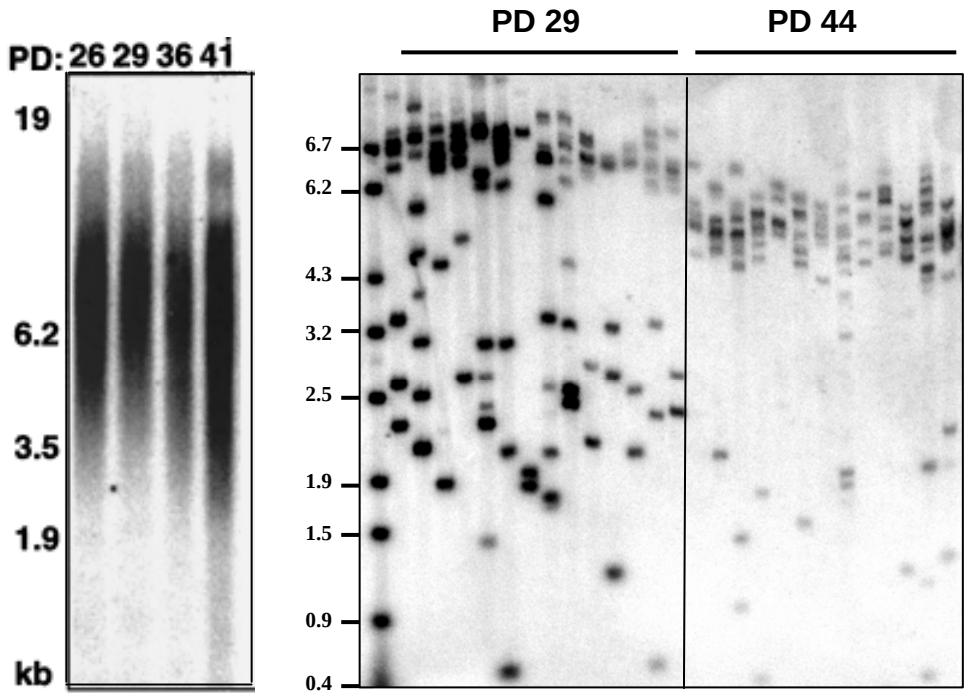


Figure 1.6- TRF vs. STELA. Telomere length analysis of WI-38 (lung fibroblasts) cells using TRF (left panel) (courtesy of (Forsyth et al., 2003) and STELA (right panel) assays. A set of short telomeres that is monitored by STELA, could not be distinguished on a TRF gel.

Telomere length is thought to be variable nevertheless, certain studies proposed that it is inheritable with a 78% heritability factor. The mechanism behind this inheritance is largely unknown (Bischoff et al., 2005; Slagboom et al., 1994). Different tissues of the same individual could have similar telomere length on a specific chromosome arm

(Graakjaer et al., 2006). Telomere lengths within a human somatic cell are heterogeneous (Lansdorp et al., 1996; Londono-Vallejo, 2004; Martens et al., 1998). This heterogeneity is marked by a non-random distribution of lengths on different chromosomes and many groups reported the existence of a chromosome-specific telomere length pattern (Graakjaer et al., 2003; Graakjaer et al., 2004). Certain studies suggested that longer chromosomes have longer telomeres (Martens et al., 1998) and accrocentric chromosomes possess the shortest telomeres. Studies by Londano et al., (Londono-Vallejo et al., 2001) showed that the telomere length difference is not just between different chromosomes but is also between two homologous telomeres of the same chromosome. Using the STELA technique, Baird et al., (2003) also showed that normal human fibroblasts have a bimodal distribution of telomere length with difference that can reach up to 6 kilobases. This difference was due inter-allelic variation whereby individual telomere length is presumably set in the germ line in a stochastic fashion. Monozygotic twins have similar telomere lengths on a given chromosome end while dizygotic twins have less similar telomere lengths. This shows that there are some epigenetic factors that might influence telomere length throughout one's life (Graakjaer, 2004).

CHAPTER TWO

Telomere-End Processing; the Terminal Nucleotides of Human Chromosomes

ABSTRACT

Mammalian telomeres end in single-stranded, G-rich 3'-overhangs resulting from both the "end-replication problem" (the inability of DNA polymerase to replicate the very end of the telomeres) and post replication processing. Telomeric G-rich overhangs are precisely defined in ciliates; the length and the terminal nucleotides are fixed. Human telomeres have very long overhangs that are heterogeneous in size (35-600nt), indicating that their processing must differ in some respects from model organisms. We developed telomere-end ligation protocols that allowed us to identify the terminal nucleotides of both the C-rich and G-rich telomere strands. Up to approximately 80 % of the C-rich strands terminate in CCAATC-5', suggesting that following replication a nuclease with high specificity or constrained action acts on the C-strand. In contrast, the G-terminal nucleotide was less precise than *Tetrahymena* and *Euplotes* but still had a bias that changed as a function of telomerase expression.

INTRODUCTION

Telomeres are the ends of linear chromosomes and their "end-capping" function helps maintain the integrity of the genome by preventing end-end fusions and degradation. Mammalian telomeric DNA contains TTAGGG repeats bound by specialized proteins (Smogorzewska and de Lange, 2004). Human telomeres end in a single stranded G-rich 3'-overhang (35-600nt in length) ((Huffman et al., 2000; Keys et al., 2004; Makarov et al., 1997; Stewart et al., 2003; Wright et al., 1997). Ultrastructural studies have shown that this overhang invades the preceding double-stranded region of the telomere to form the t-loop, a lariat like structure stabilized by base pairing and protein-protein interactions (Griffith et al., 1999). The t-loop is proposed to protect the end from being perceived as a double-stranded break (de Lange, 2002; Stansel et al., 2001). Telomeric overhangs result from both the "end-replication problem" (the inability of the DNA polymerase to replicate the very end of the telomere) and post replication processing events. Upon the completion of lagging strand synthesis, removal of the final primer should result in a short overhang (around 9-12 nt long) if it were placed at the extreme end of the template telomere. Alternatively, a longer and more variable overhang would result if the last priming event did not take place at the very end. Leading-strand synthesis, which is continuous, should produce a blunt end if it proceeds to the very end of the telomere, or might leave a 5'-overhang if the replication machinery falls off prematurely (Cimino-Reale et al., 2001). Since overhangs are present on both chromosome ends, a nuclease must act upon the

5'end of at least the leading strand to generate overhangs. It is not known if leading and lagging daughters are subject to the same regulatory processing.

Most of the evidence for overhang processing comes from yeast and ciliates that possess short overhangs (10-21 nt) of relatively uniform length. *Euplotes* overhang generation gives precise terminal nucleotides at both ends, GGTTTTG**G**-3' at the G-rich strand and AAAACC**C**-5' at the C-rich strand and the length of the overhang is always 14nt (in order to facilitate alignment, G-rich sequences are presented in the 5' to 3' orientation, C-rich sequences in the reversed 3' to 5' orientation, and the terminal nucleotides are shown bolded and underlined). Extensive studies characterizing mechanisms of overhang processing have been done in *Tetrahymena* (Fan and Price, 1997; Jacob et al., 2003; Jacob et al., 2001). Instead of ending in GGGTT**G**-3' as would be expected if the terminus is generated by dissociation of telomerase during the translocation step, most *Tetrahymena* telomeres end in TGGGG**T**-3'. *Tetrahymena* C-rich strands end with CAACC**C**-5' or CCAAC**C**-5'. This suggests that overhang generation is mediated by two separate processing steps; one cleaves the G-strand and the other resects the C-strand, and both steps are distinctively terminated at a specific base.

As a first step in characterizing overhang processing events in human cells, we established ligation-mediated methods to identify the last nucleotide of both the C-rich and the G-rich strands. The human pattern differs from that seen in *Tetrahymena* and *Euplotes*, suggesting a divergence in the underlying processing mechanisms. The terminal nucleotide of the C-strand was uniform, 80% of human C-strands beginning with the

sequence CCAAT**C**-5'. The G-strand terminal nucleotide was much more variable, with a bias that changed with telomerase expression. This definition of the terminal nucleotide should facilitate the identification of the factors involved in telomeric end-processing events.

RESULTS

C-strand processing of human telomeres is very tightly regulated

We used two independent assays identify the terminal nucleotide of the C-rich telomeric strand. In the primer ligation assay we tagged the C-strand with a known oligonucleotide. Six permutations of the telomeric sequence AATCCC were synthesized at the 3' end of a cassette containing two PCR primers, separated by a variable spacer so that each permutation could be identified by its spacer size (fig. 2-1A). Only the oligonucleotides (s) annealing immediately adjacent to the 5' terminus could be ligated to the C-strand. Following ligation of an equal mix of these six oligonucleotides, free oligonucleotides were removed and the telomere-bound oligonucleotides were PCR amplified. The amplification products were resolved on acrylamide gels, such that the size of the bands specified the oligonucleotide (s) that was ligated to the telomere, which in turn identified the terminal nucleotide for the C-strand. The intensity of each band determined the proportion of the oligonucleotide that was successfully ligated to the telomere. This was validated on an artificial telomeric overhang ending in one specific base, which gave the correct single band on the gel (fig. 2-1B top panel). A plasmid that

contained a mix of six terminal nucleotides generated six bands of equal intensities (fig. 2-1B bottom panel).

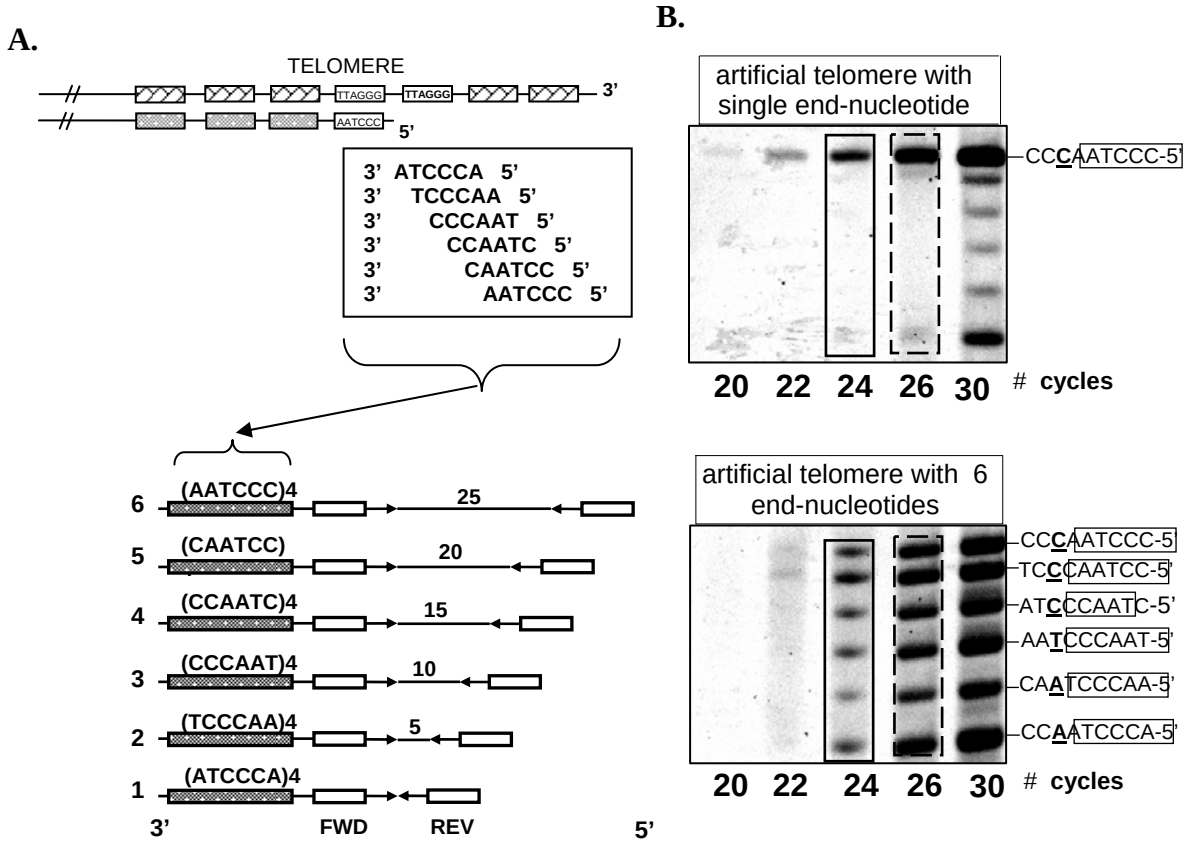
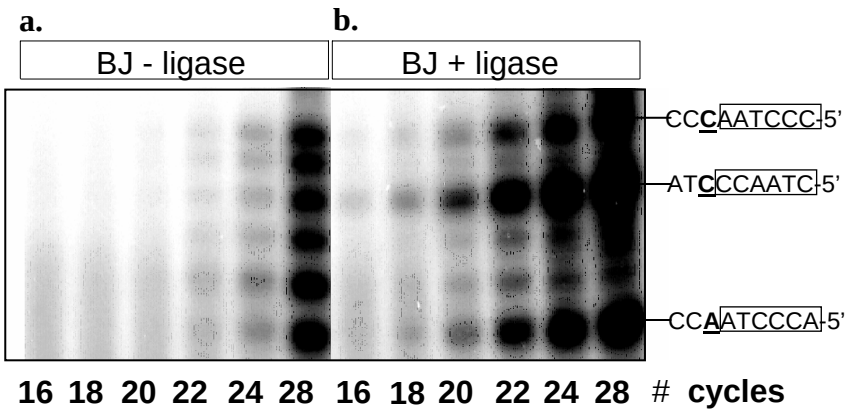


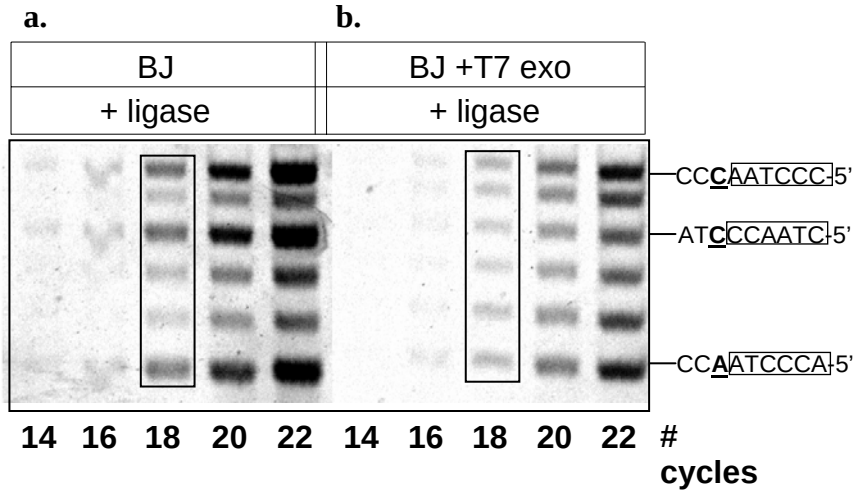
Figure 2.1- Strategy for Primer Ligation assay to determine the identity of the C-strand terminal nucleotide: A) Six oligonucleotides are annealed to the overhang at the chromosome end. Each contains a permutation of the telomeric sequence (AATCCC). Only the linker that anneals in correct register and adjacent to the last base of the C-strand terminal nucleotide can be ligated. The six oligonucleotides contain four AATCCC repeats followed by a tail containing two known sequences that are used for PCR amplification. Each permutation of the sequence AATCCC is paired with a different length spacer between the two PCR primer sequences. The first three nucleotides of the labels represent the terminal nucleotides of the G-rich strand with the last base in bold and underlined, while the boxed sequence represents the sequence of the ligated nucleotide. B) Two artificially generated telomeric overhangs possessing either one specific terminal nucleotide (top) or a mix of six nucleotides representing all permutations of the telomeric repeat (AATCCC) (bottom) showed the predicted patterns of band distribution and intensities on an acrylamide gel. The background appearing in the later cycles is due to the failure to remove all unligated primers**.**

Using DNA from cultured human BJ fibroblasts, most of the primer ligation products represented the oligonucleotide with 3'-**C**CAATC corresponding to a C-strand ending in CCAAT**C**-5' (fig. 2-2A). After randomization with the 5'→3' T7 exonuclease, the preference for the C-strand terminus CCAAT**C**-5' was completely lost (fig. 2-2B panel a and b), while the small enrichment for the top and bottom oligonucleotides (Fig. 2.2B panel a) was preserved. Moreover, treating with Exonuclease I to digest the overhang did not abolish the enrichment for the top and bottom oligonucleotides (fig. 2-2C), suggesting that the slight bias for theses oligonucleotides was not significant.

A.


a.
b.
BJ - ligase
BJ + ligase
CCCAATCCC-5'
ATCCCAATC-5'
CCAATCCCA-5'
16 18 20 22 24 28 16 18 20 22 24 28 # cycles

B.


a.
b.
BJ
+ ligase
BJ +T7 exo
+ ligase
CCCAATCCC-5'
ATCCCAATC-5'
CCAATCCCA-5'
14 16 18 20 22 14 16 18 20 22 # cycles

C.

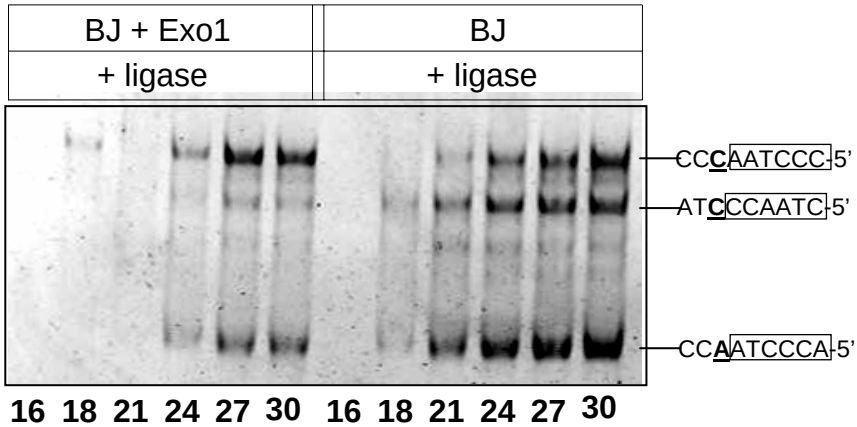

BJ + Exo1
+ ligase
BJ
+ ligase
CCCAATCCC-5'
ATCCCAATC-5'
CCAATCCCA-5'
16 18 21 24 27 30 16 18 21 24 27 30

Figure 2.2- The last base of the C-strand based on the Primer Ligation Assay. A) The Primer Ligation assay was applied to genomic DNA from BJ cells. Semi-quantitative PCR using ^{32}P labeled primers and increasing number of cycles was performed. Panel (a) is a negative control (-Ligase). Panel (b) shows major preference for the oligonucleotide possessing 3'-**C**CAATC end, indicating that the C-strand most frequently ends in CCAAT**C**-5'. B) Primer Ligation assay applied to DNA from BJ cells (panel a) or treated with T7 exonuclease to randomize the ends (panel b). A minor bias for the top and bottom oligonucleotides was observed in the ends randomized by T7 exonuclease treatment. C) Digestion of the overhangs with 5'- 3' nuclease Exo1 diminished the specific ligation to C-strands with AT**C**-5' termini without changing the artifactual PCR preference for telorettes ligating to CC**C**-5' and CC**A**-5'

Mammary epithelial cells (HMEC), breast tumor cells (MCF7), mouse cells (3T3) and human lung fibroblasts (WI38) all displayed the same pattern, with most ligation products corresponding to telomeric C-strands ending in CCAAT**C**-5' (fig. 2-3).

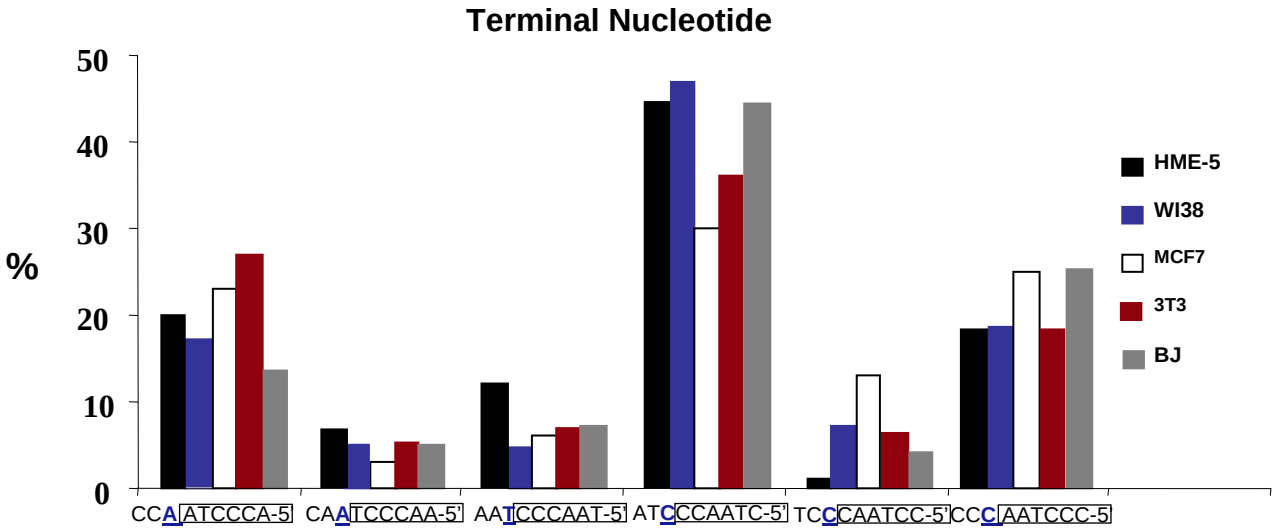


Figure 2.3 – The C-strand terminal nucleotide in different cell lines. All of the cell types tested including normal WI38 and BJ fibroblast, HME-5 mammary epithelial cells, telomerase expressing MCF-7 mammary tumor cells and telomerase expressing mouse 3T3 fibroblasts exhibited the same preference for C-strands terminating in AT**C**-5' using the end-ligation assay.

We confirmed the above results using a modification of STELA (single telomere length analysis) (Baird et al., 2003) to look at individual chromosomes and identify their terminal nucleotide (s). In this assay, "C-telorette" oligonucleotides, containing seven nucleotides of telomeric repeats followed by a unique sequence, were ligated to genomic DNA. The DNA was then diluted until only a few amplifiable molecules were present and PCR amplified using XpYp chromosome-specific subtelomeric primers and "teltail" primers complementary to the unique sequence of the telorette. A Southern blot with Xp/Yp subtelomeric probes revealed individual bands, representing individual telomeric molecules. We performed six ligation reactions, each containing a telorette with one specific base at the 3'end representing a permutation of the telomeric repeat (AATCCC) (fig. 2-4A). The number of bands amplified with each C-telorette reflected the proportion of telomeres that were ligated to the 3'-end of the specific telorette, which in turn defined the end-nucleotide of the C-strand.

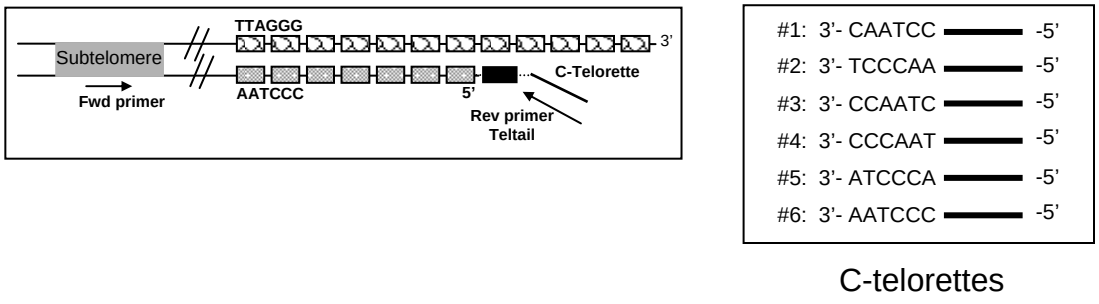


Figure 2.4- Strategy for C-STELA last base determination: Six individual C-telorettes are ligated in separate reactions to the same amount of DNA and then amplified using a forward Xp/Yp chromosome-specific subtelomeric primer and a reverse Teltail primer. Only the C-Telorette annealing adjacent to the last base of the C-strand will be ligated to the telomere end and can produce a PCR product.

Multiple amplification reactions were performed for each ligation reaction using BJ human foreskin fibroblast DNA. Non-specific ligations to all six telorettes were obtained using 0.9 μM of C-telorettes (as in Baird et al., 2003) (fog. 2-5A). This represents an approximately 4 x 10^7-fold excess of oligonucleotide to telomeres. Specific ligations were seen over a broad range of concentrations when the input telorettes were diluted 100-100,000 fold (fig. 2-5B).

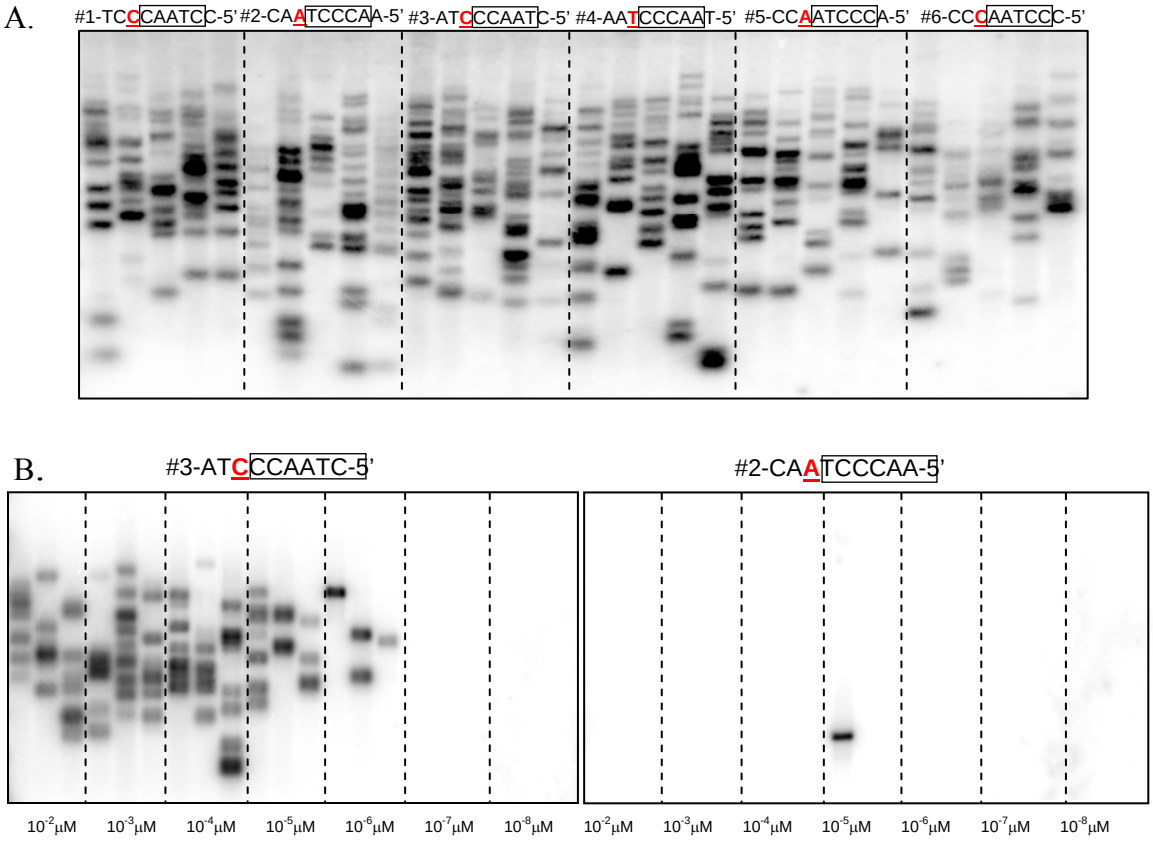


Figure 2.5- The identity of the C-strand terminal nucleotide: A) STELA adaptation performed with 6 different ligation reactions each with 0.9 μM telorettes. All six telorettes are able to ligate to the telomere end and generate similar number of amplification products. B) STELA adaptation was performed while diluting the concentration of telorettes # 3, 4 and 5 from 10^{-2} μM till 10^{-6} μM. For each ligation reaction multiple PCR reactions were performed. At lower concentrations (10^{-2} -10^{-6} μM) only telorette #3 was

significantly able to ligate to the telomere ends. STELA adaptation was performed while diluting the concentration of telorettes # 2 and 3 from 10^{-2} μM till 10^{-8} μM.

Approximately 80% of the amplification products represented the C-telorette that ends in 3'- CCAATC (fig. 2-6). This confirmed the primer-ligation assay results showing the terminus of most C-rich strands is CCAAT**C**-5'.

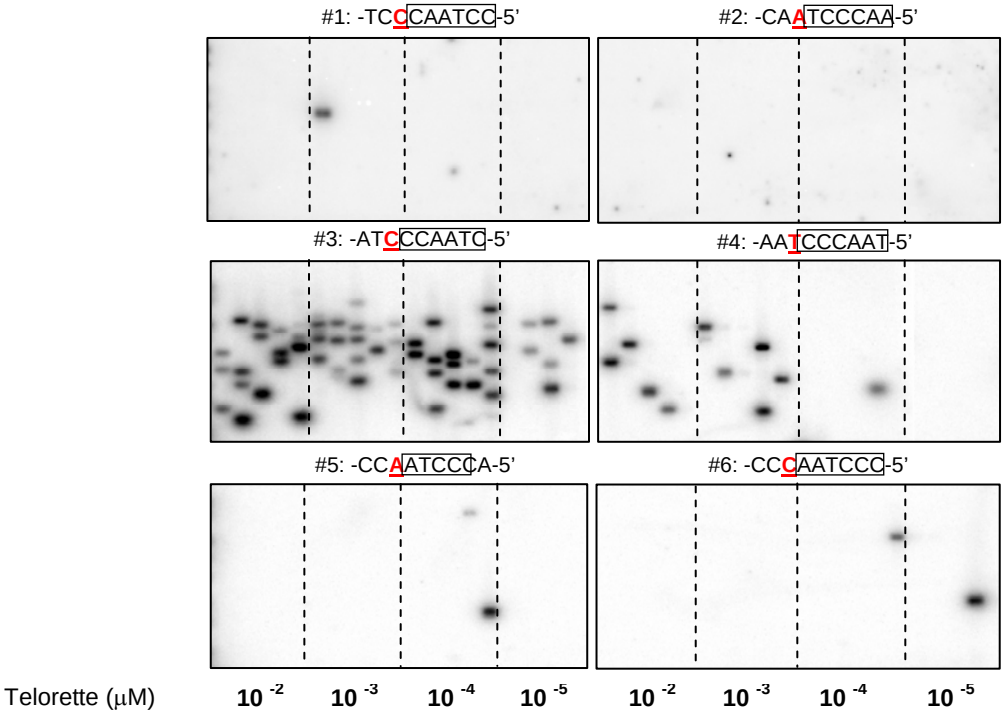


Figure 2.6- Identifying the C-strand terminal nucleotide by STELA on the Xp/Yp telomere. STELA adaptation with telorette concentration ranging from 10^{-2} -10^{-5}μM were individually ligated to DNA from BJ cells. Multiple amplifications using the XpYp E2 Fwd primer and the Teltail reverse primer show that 80% of the bands appear when using telorette #3 (ends in 3'-CCAATC). The first three nucleotides of the labels represent the terminal nucleotides of the C-rich telomeric strand with the last base in bold and underlined, while the boxed sequence represents the sequence of the ligated oligonucleotide.

Randomizing the terminal nucleotide by treating the DNA with the 5'-3' T7-exonuclease abolished the preference for C-telorette #3 (3'-CCAATC) (fig. 2-7).

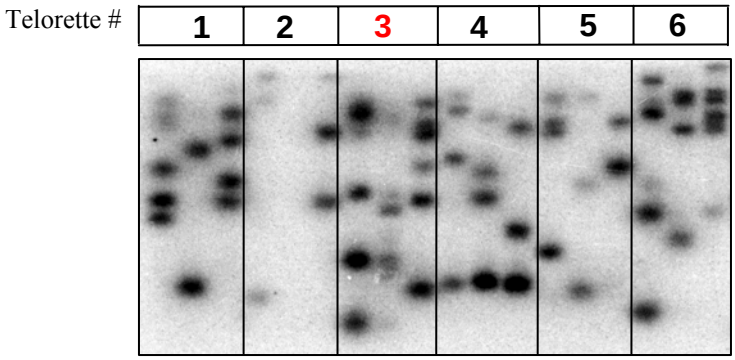


Figure 2.7- Last-base randomization using T7 exonuclease. STELA was performed on BJ DNA that was pre-treated with T7 exonuclease. This randomized the terminal nucleotide, and all telorettes (10^{-3} μM) gave similar numbers of amplification products. The panel numbers (1-6) match the numbers in Figure 2-4 with telorette #3 corresponding to AT**C**-5' as the last base.

Cells with telomerase activity exhibited the same end-nucleotide (fig 2-8), suggesting that telomerase did not alter the nucleotide specificity of C-strand processing. The terminal nucleotide of other chromosomes (7p and 10q) gave the same preference. Dephosphorylating and re-phosphorylating the telomeres prior to ligation did not alter the outcome, indicating the preference for CCAAT**C**-5' did not represent a bias for *in vivo* 5' phosphorylation of selected ends.

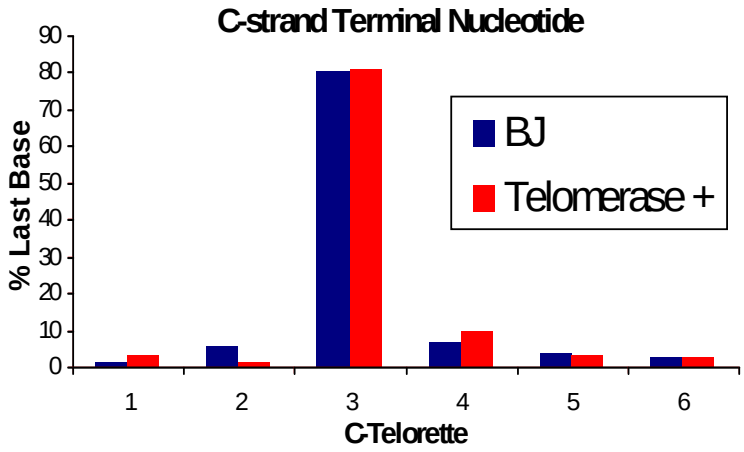


Figure 2.8- Telomerase does not alter the last base preference. Similar to BJ cells (telomerase negative), Hela cells (telomerase positive) show the same preference (~80%) for ATC-5' at the terminal nucleotide position.

The terminal nucleotide of the G-rich telomeric strand in human cells is less precisely regulated and is slightly altered by telomerase expression

In order to determine the terminal nucleotide of the G-rich strand, we first annealed an $(3'\text{-AATCCC-}5')_{10}$ oligonucleotide to the overhang. This "platform" produced a 5' overhang that guided the ligation of G-telorettes to the G-rich strand terminal nucleotide (fig. 2-9).

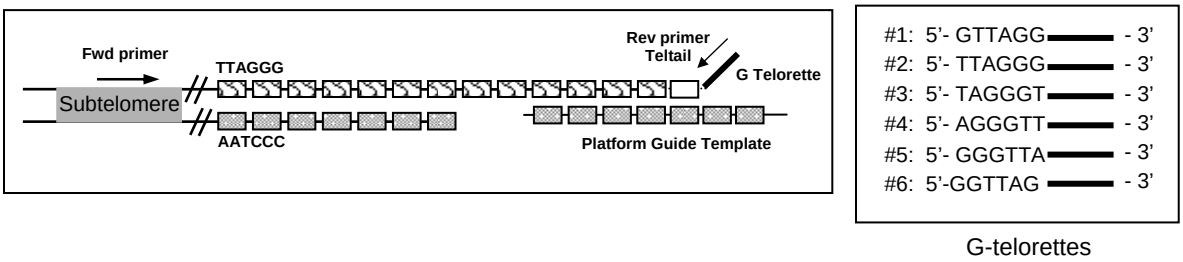


Figure 2.9- G-STELA strategy. Annealing a guide template to the overhang provides a platform for subsequently ligating G-telorettes to the 3' end of the G-rich strand. Six individual telorettes are ligated in separate reactions to the same amount of DNA and then PCR amplified using a forward chromosome-specific primer and a reverse Teltail primer.

After ligating six individual G-telorettes, each with a different permutation of the TTAGGG repeat, individual telorettes-tagged telomeric molecules were amplified and detected on a Southern blot. All six G-telorettes were able to ligate to some G-ends from BJ fibroblasts, suggesting that G-strand processing of mammalian telomeres is less precise than reported for *Tetrahymerna* and *Euplotes*. Nevertheless, G-strand processing was not totally random; ~70% of 990 telomeric molecules analyzed ended in GGTTA**G**-3', GGGTT**A**-3' or AGGGT**T**-3' (last base underlined and in bold) (fig. 2-10).

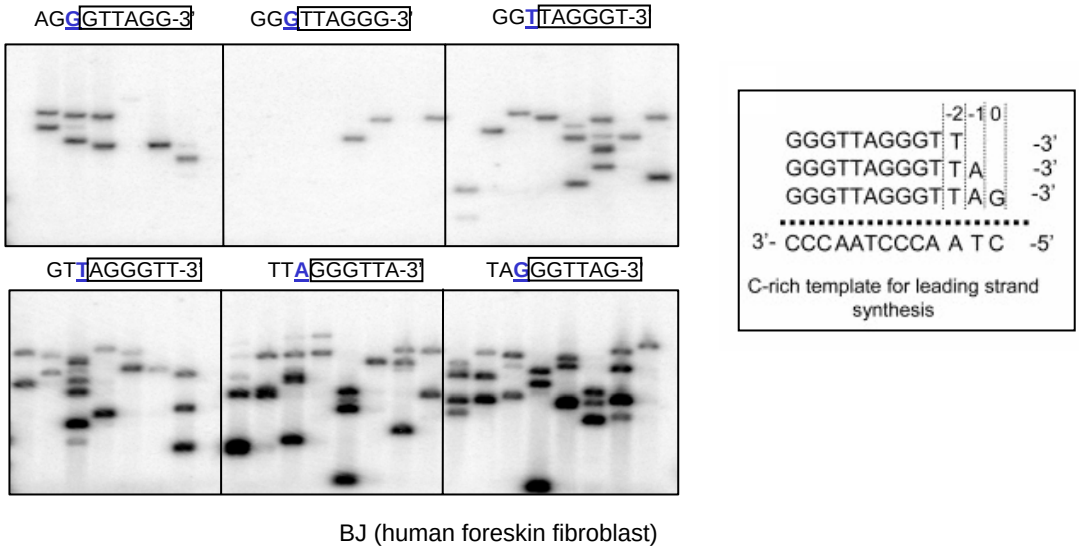


Figure 2.10- The identity of the G-strand terminal nucleotide on the Xp chromosomes of normal cells. STELA adaptation with G-telorettes individually ligated to DNA from BJ cells. Multiple amplifications show that G-telorettes that ligate in register to AGGGT**T**-3', GGGTT**A**-3' and GGTTA**G**-3' generate 70% of the total number of bands. The first three nucleotides of the labels represent the terminal nucleotides of the G-rich strand with the last base in bold and underlined, while the boxed sequence representing the sequence of the ligated nucleotide. (insert) The pattern of variability observed for the G-terminal nucleotide is consistent with the replication complex sometimes generating a blunt end (leaving a GGTTA**G**-3' end) or dissociating one or two nucleotides prior to the terminus.

We next determined whether the enzymatic activity of telomerase altered the G-terminus. A clear shift in the distribution pattern of G-terminal nucleotides was observed in telomerase positive Hela cells, resulting in a greater enrichment (~40% of total

telomeres) for GGTTA**G**-3' (fig. 2-11A and B). The G-terminal nucleotide GGTTA**G**-3' matches the last base of the hTR (telomerase RNA) template region and is the pause site following which telomerase translocates prior to the next cycle of repeat synthesis.

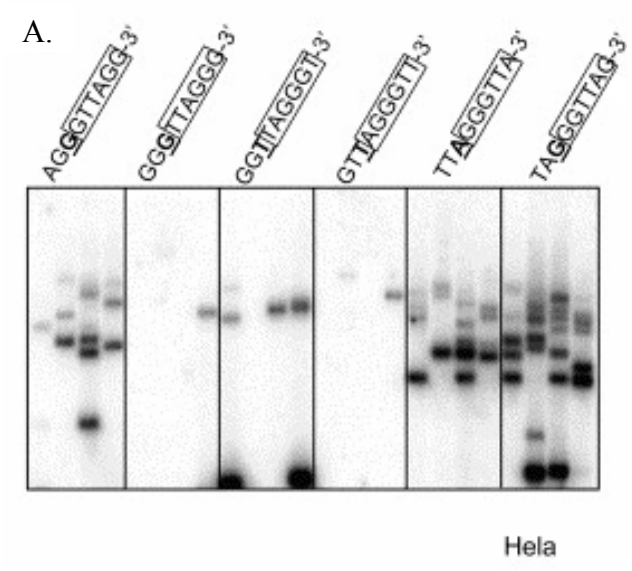


Figure 2.11- The G-terminal nucleotide in telomerase positive cells. A) STELA adaptation was applied to DNA from telomerase positive Hela cells. Results show further enrichment for the G-telorette ligation to overhangs ending in GGTTA**G**-3'. B) Percentage for each terminal nucleotide from 3B (BJ cells) and 3C (Hela cells). 990 telomeric molecules were analyzed for BJ cells and 330 telomeres for Hela cells. The

dotted line shows what would be expected for a random distribution. The difference in the frequency of the GGTTA**G**-3' ends in Hela *vs*. BJ cells is highly significant by the Student's t-test.

We next investigated whether leading and lagging daughter strands end in the same terminal nucleotide(s). Cells were fed the thymidine analogue BrdU for one doubling, during which leading strand synthesis (using AATCCC as the template) incorporated twice as much BrdU as lagging strand synthesis (using TTAGGG as the template). The different densities of the strands allowed their separation on a CsCl gradient (fig. 2-12). STELA ligations and PCR showed that both leading and lagging strands exhibited the same major preference for the sequence CCAAT**C**-5' at the 5'end of the C-rich strand (fig. 2-13). This suggests that the overhang on the lagging strand is not solely produced by removing the last Okazaki primer; most likely it is subject to the same final C-strand nuclease processing step as leading strand.

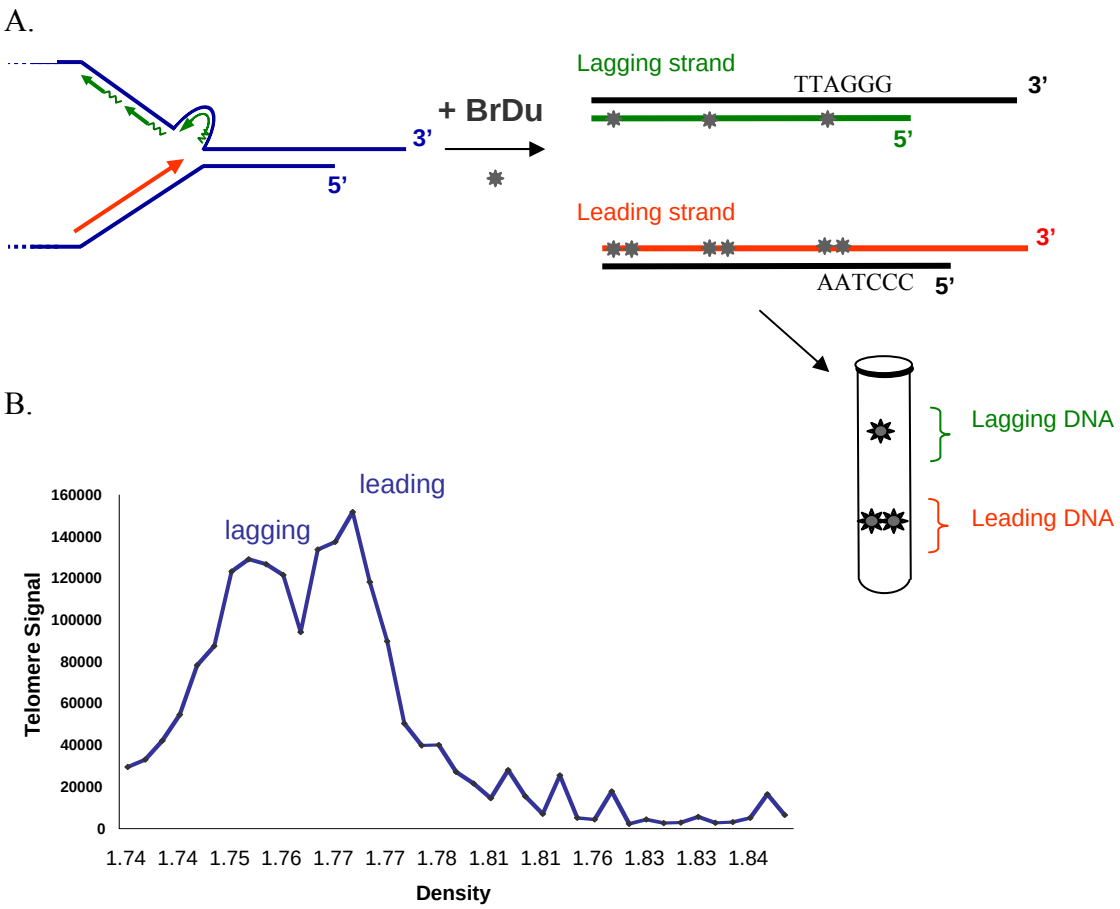


Figure 2.12- Scheme for the separation of the leading and lagging telomeres. A) Cells are grown in the presence of BrdU (thymidine analogue) for one division during which lagging daughter telomere replication will incorporate one BrdU molecule per telomeric repeat (six nucleotides) while replication of leading telomeres incorporates two BrdU molecules. Leading daughter telomeres will be heavier than the lagging telomeres allowing their separation in a CsCl gradient. B) Telomeric DNA from BJ cells labeled with BrdU for 1 PD was separated on CsCl density gradient. DNA with leading telomeres run at density of approximately 1.775 g/ml and the lagging strand daughter DNA runs at density of approximately 1.750 g/ml.

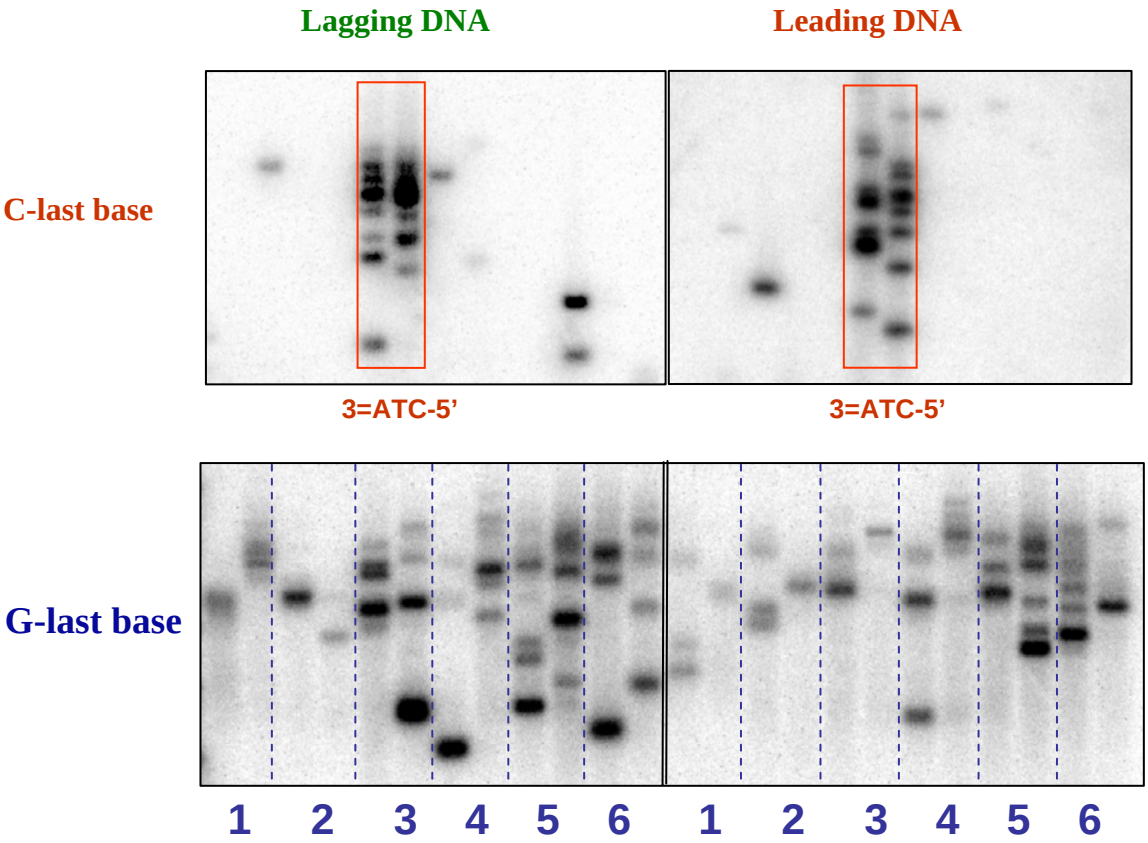


Figure 2.13- Terminal nucleotides of leading/lagging strands of DNA replication C-STELA was applied to leading and lagging DNA using six individual ligation reactions possessing C-telorettes at 10^{-3} μM concentration. Both daughter telomeres have a major preference for C-strands terminating in CCAATC-5' (top panel). Similarly, the lower panel shows the last base of the G-strand of leading and lagging DNA as determined by G-STELA. The same distribution was apparent for both strands. (The number of the G-telorettes corresponds to the number in Figure 2-9)

DISCUSSION

Overhangs are a critical component of the telomere-end structure. Their importance exceeds simple telomere capping functions since they establish telomere shortening rates and influence replicative senescence. Our observations define the products of the final steps of telomere end processing for both the C-rich and G-rich strands in human cells. Although the detailed mechanisms remain to be determined, the results have important implications for understanding the generation of telomeric overhangs. We show that the vast majority of telomeric C-strands end with the sequence CCAAT**C**-5', thus C-strand processing is very tightly specified. This is in agreement with results from ciliates (*Euplotes* and *Tetrahymena*) that possess a specific terminal nucleotide, suggesting that the mechanism of C-strand resection and the factors involved in the processing events are most likely conserved.

Leading-daughter overhangs are only about 60% as long as lagging-strand daughters (Chai et al., 2006a) indicating some difference in length control during overhang processing. Our present observations do not distinguish between extensive C-strand processing of both daughters when replication is completed versus the simple removal of the RNA primer from the last Okazaki fragment of the lagging-strand daughter with extensive resection of the C-strand on leading daughter. Nevertheless, our results imply that although there might be differences in the steps involved in producing these different-sized overhangs, the mechanism specifying the terminal nucleotide and the final step in C-strand processing is likely to be shared.

What determines the precision of the C-strand resection and the identity of the terminal nucleotide is still unknown; it could be due to a base-specific endonuclease/helicase that cleaves exclusively between two C residues, or to a telomere-binding protein that provides the nucleotide specific boundary for 5'→3' exonuclease trimming. Such a protein could be a single-stranded binding protein, or a protein that binds to double-stranded DNA and physically hinders further resection by the nuclease. A set of telomere binding proteins bind to the telomere-end with great specificity and cap the chromosome end. Their role in overhang processing is unknown. The identity of the C-strand nuclease is unknown is a fundamental gap in the understanding of telomere replication.

We found a non-random mixture of terminal G-strand nucleotides with marked preference for GGTTA**G**-3', GGGTT**A**-3', and AGGGTT-3'. This contrasts with the highly specified G-terminal nucleotides of model organisms. If the leading strand replication machinery is able to accurately synthesize DNA all the way to the final C-rich template nucleotide (CCAAT**C**-5'), then G-strands should end in GGTTA**G**-3'. Variability could occur if replication sometimes generated a blunt end (leaving a GGTTA**G**-3' end) but also dissociated one or two nucleotides prior to the terminus, leaving GGGTT**A**-3' or AGGGT**T**-3' ends. In this scenario no processing of the human G-strand would take place and the terminal nucleotide would simply represent the failure of the replication complex to always copy the final one or two nucleotides. The significance of an abundance of TTAGG**G**-3' termini greater than predicted by this model remains to be determined. The results are also consistent with some imprecise nuclease

processing of the G-strand that yields less nucleotide specificity than C-strand processing. Such processing would be regulated by a set of proteins that bind the overhangs with some flexibility to generate a less precise terminal nucleotide.

Interestingly, we found some change in the distribution of G-ends in the presence of telomerase. Although our results do not exclude telomerase participating in a processing complex (Oulton and Harrington, 2004), the increased preference for GGTTA**G**-3' termination in cells expressing telomerase is fully consistent with the hypothesis that the altered distribution is due to telomerase dissociation without further processing to generate the terminal nucleotide on some of the telomeres.

Table 1 compares current knowledge of the overhangs and terminal nucleotides of some model organisms and humans. *Saccharomyces cerevisiae* have overhangs that are 12-14 nucleotides in length (Larrivee et al., 2004). Their G-strand termini show no base specificity (Forestemann et al., 2000) while their C-strand termini have not been directly determined. *Euplotes* and *Tetrahymena* continuously express telomerase and have very short and uniform telomeric overhangs. The two ciliates are known to exhibit precise processing to generate specific G-rich and C-rich terminal nucleotides (Dionne and Wellinger, 1996; Fan and Price, 1997; Jacob et al., 2003; Jacob et al., 2001). Human telomeres have variable overhang lengths ranging from 35-600nt (Huffman et al., 2000; Makarov et al., 1997; Stewart et al., 2003; Wright et al., 1997). The present report demonstrates that end processing at human telomeres have some aspects that are common to model organisms and others that are different, probably reflecting these different telomeric dynamics. Understanding the mechanisms of end processing in human cells may

permit interventions to accelerate the loss of telomere length during telomerase-inhibition based cancer therapy or to reduce the rate of telomere shortening to retard replicative senescence.

	Telomere composition	Overhang length	Last base of C-strand	Last base of G-strand
Euplotes	TTTTGGGG	14	AAAACCC -5'	TTTTGG -3'
Tetrahymena	TTGGGG	14-15 20-21	AACCC -5' AACC -5'	GGGGT -3'
Sacchar. cerevisieae	TG $_{1-3}$	12-14	Unknown	Random
Homo sapiens	TTAGGG	35-200	AATC -5'	GGTTAG -3' GGTTA -3' GGTT -3'

Table 2.1- Comparison of the telomeric-end structures. The size of the G-rich single-stranded overhang and the terminal nucleotides for each strand are shown for two ciliates, yeast and humans.

MATERIALS AND METHODS

Cell Culture

Cells were grown in a 4:1 mixture of DMEM and Medium 199 containing 10% iron-supplemented calf serum (Hyclone) and gentamicin (25 μg/ml; Sigma) at 37°C in 5% CO_2.

Primer-ligation Assay

DNA extracted using DNeasy Tissue Kit (Qiagen), was ligated to 200 fmol of an equal mix of the six oligonucleotides shown below using Taq ligase (New England biolabs; 40U/μL) at 60^0C for 30 min, followed by melting at 85^0C for 3min. The ligation/melting steps were repeated for 15 cycles. Free oligonucleotides were removed by purifying high molecular weight DNA from denaturing agarose gels (1%) using the QIAquick Gel Extraction Kit (Qiagen). PCR amplification (95^0C for 15 sec, 60^0C for 20 sec and 72^0C for 30 sec for increasing number of cycles) used 2.6 U of polymerase from the High fidelity PCR system (Roche applied science) with 200μM dNTP, 300 nM forward and reverse primer, and 1X high fidelity PCR buffer with 1.5mM $MgCl_2$. Products were resolved on an 8% acrylamide gel in 1X TAE and stained with ethidium bromide. For semi-quantitative PCR, the Forward and Reverse primers were end-labeled with p32 γ-ATP and the gel exposed to a PhosphorImager screen.

Six different primers were used, each containing a spacer of different size (highlighted in grey) and a sequence complimentary to TTAGGG but ending in a different 3' nucleotide (underlined).

Oligonucleotides and primers:

#1- 5'-GCCAGTCACGAGGTTGTATTTGC ATGCA GCTGTGTGAAACTGTTATCCGCT G ACCCTAACCCTAACCCTACCCTA -3'

#2- 5'-GCCAGTCACGAGGTTGTATTTGC ACAGAAATGCA GCTGTGTGAAACTGTTATCCGCT G AACCCTAACCCTAACCCTAACCCT-3'

#3- 5'-GCCAGTCACGAGGTTGTATTTGC GCACTACAGAAATGCA GCTGTGTGAAACTGTTATCCGCT G TAACCCTAACCCTAACCCTAACCC-3'

#4- 5'-GCCAGTCACGAGGTTGTATTTGC TACCGGCACTACAGAAATGCA GCTGTGTGAAACTGTTATCCGCT G CTAACCCTAACCCTAACCCTAACC-3'

#5- 5'-GCCAGTCACGAGGTTGTATTTGC AACTCTACCGGCACTACAGAAATGCA GCTGTGTGAAACTGTTATCCGCT G CCTAACCCTAACCCTAACCCTAAC -3'

#6- 5'-GCCAGTCACGAGGTTGTATTTGC CGGTAACTCTACCGGCACTACAGAATGCA GCTGTGTGAAACTGTTATCCGCT G CCCTAACCCTAACCCTAACCCTAA -3'

Forward Primer: 5'- GCCAGTCACGAGGTTGTATTTGC -3'

Reverse Primer: 5'- AGCGGATAACAGTTTCACACAGC -3'

C-strand STELA

Multiple ligation reaction were performed with individual C-telorettes, whereby 10 ng EcoRI-digested DNA was incubated in 10μl reaction (1X ligase buffer, 0.5 U T4 ligase, $10^{-2} – 10^{-5}$ μM of individual telorettes), at 35^0C for 12 hrs. Multiple amplification reactions were performed (26 cycles, of 95^0C for 15 sec, 58^0C for 20 sec and 72^0C for 10 min) using 1U of Fail Safe enzyme mix (Epicenter), 12.5 μl Fail Safe buffer H (2X, provided by manufacturer) and 0.1μM primers (XpYp E2 forward primer and Teltail reverse primer) in a final volume of 25μL, containing 200 pg/μL DNA. The amplification products were resolved on a 0.5% agarose gel, denatured, transferred onto a positively charged nylon membrane (Zeta probe; Bio-Rad), fixed with UV and hybridized with a subtelomeric probe (generated by PCR using XpYpE2 and XpYpB2 and labeled by random priming). The membrane was exposed to a Phosphor Imager screen, and scanned.

Oligonucleotides and primers:

XpYpE2 (forward primer subtelomeric): 5'-TTGTCTCAGGGTCCTAGTG-3'

XpYpB2 (reverse primer subtelomeric): 5'-TCTGAAAGTGGACC(A/T)ATCAG-3'

C-telorette 1: 5'-TGCTCCGTGCATCTGGCATCCCCTAAC-3'

C-telorette 2: 5'-TGCTCCGTGCATCTGGCATCTAACCCT-3'

C-telorette 3: 5'-TGCTCCGTGCATCTGGCATCCCTAACC-3'

C-telorette 4: 5'-TGCTCCGTGCATCTGGCATCCTAACCC-3'

C-telorette 5: 5'-TGCTCCGTGCATCTGGCATCAACCCTA-3'

C-telorette 6: 5'-TGCTCCGTGCATCTGGCATCACCCTAA-3'

C-teltail (reverse primer): 5'-TGCTCCGTGCATCTGGCATC-3'

G-strand STELA

EcoRI digested DNA (60ng) was incubated with 10^{-4} μM of the oligonucleotide sequence $(AATCCC)_{10}$, the platform guide template, at 70^0C for 1hr. 10^{-3} μM individual G-telorettes were then ligated to 10ng aliquots of the DNA+guide template. The subsequent Ligation and PCR reactions followed the same condition and protocol as the C-strand STELA.

Oligonucleotides and primers:

Platform guide template: 5'-CCCTAACCCTAACCCTAACCCCTAACCCCTAACCCTAACCCTAACCCTAACCCTACCCTAA-3'

XpYpE2 (forward primer subtelomeric): 5'-TTGTCTCAGGGTCCTAGTG-3'

XpYpB2 (reverse primer subtelomeric): 5'-TCTGAAAGTGGACC(A/T)ATCAG-3'

G-telorette 1: 5'/5Phos/-GTTAGGGCTACGGTCTACGTGCCTCGT-3'

G-telorette 2: 5'/5Phos/-TTAGGGTCTACGGTCTACGTGCCTCGT-3'

G-telorette 3: 5'/5Phos/-TAGGGTTCTACGGTCTACGTGCCTCGT-3'

G-telorette 4: 5'/5Phos/-AGGGTTACTACGGTCTACGTGCCTCGT-3'

G-telorette 5: 5'/5Phos/-GGGTTAGCTACGGTCTACGTGCCTCGT-3'

G-telorette 6: 5'/5Phos/-GGTTAGGCTACGGTCTACGTGCCTCGT-3'

G-teltail (reverse primer): 5'-ACGAGGCACGTAGACCGTAG-3'

Generation of Artificial Telomeres

PUC19 plasmid was digested with AatII and Nde I (Promega 1U/μg), then dephopshorylated with alkaline phosphatase (Roche). In parallel, six G-rich oligonucleotides (sequence below) were individually annealed to a complimentary oligonucleotide (see below) such that they reconstituted an AatII site on the 5'end and a 3' telomeric overhang with specific terminal nucleotides. Using T4 ligase (Roche applied science 10U/μL), the vector was ligated to the double stranded oligonucleotides. Gel purification was applied to purify the overhang-possessing vectors from the free double stranded oligonucleotides.

Oligonucleotides:

#1: 5'Phos- CGTCAGCCAAAC $(TTAGGG)_5$ -3'

#2: 5'Phos- CGTCAGCCAAAC $(TAGGGT)_5$ -3'

#3: 5'Phos- CGTCAGCCAAAC $(AGGGTT)_5$ -3'

#4: 5'Phos- CGTCAGCCAAAC $(GGGTTA)_5$ -3'

#5: 5'Phos- CGTCAGCCAAAC $(GGTTAG)_5$ -3'

#6: 5'Phos- CGTCAGCCAAAC $(GTTAGG)_5$ -3'

Compl. Plasmid: 3'-TGCAGCAGTCGGTTTG-5'

Randomizing the Terminal Nucleotides:

To randomize the C-rich terminus, DNA was incubated with T7 Exonuclease (10 U/μl) (New England Biolabs) in 1X NEB buffer 4 at 25°C for 3min.

Separation of the leading and lagging telomeric DNA.

Cells were cultured in the presence of 30 μM of BrdU and 20 μM of dC (deoxycytidine) for one round of replication (Meuth and Green, 1974). Genomic DNA was digested with Rsa I and loaded onto CsCl solution with a density of ~1.73 g/ml. The tubes are sealed and the samples were ultracentrifuged at 55,000 rpm for 24 hours at 21 °C. Then, fractions with ~ 25 μL of solution were eluted from the tubes and aliquots of DNA from each fraction were diluted with water to 1:10 and denatured in 0.1M NaOH for 10 min at 37 °C. 6X SSC was then added to the samples for neutralization and samples were immediately loaded on a slot-blot. A telomeric $(TTAGGG)_3$ probe was used to determine which fractions contained the telomeric DNA. Pooled DNA from all aliquots that contain the corresponding strands were ethanol precipitated, and resuspended in TE.

CHAPTER THREE

In search of proteins that alter last base specificity and nucleases that resect the telomere end

INTRODUCTION

Determining the terminal nucleotide of the telomeres provided us with a tool to better understand the nature of the resecting nuclease(s) and the regulatory proteins that generate the overhangs. The specificity observed for the C-strand terminal nucleotide could stem from a sequence specific nuclease that preferentially clips following a particular nucleotide. There have been no reports of an exonuclease possessing base-specificity. If it were the nuclease by itself that defines the precision of the terminal nucleotide, then such nuclease would most likely be an endo-nuclease that preferentially cleaves the C-strand at the AATC^CC position. Possible end-processing nucleases include already identified endonucelases like FEN-1 (Liu et al., 2004b) and Dna2 (Kao et al., 2004; Tomita et al., 2004) or exonucleases such as Artemis (Rooney et al., 2003; Rooney et al., 2002), Werner (Bai and Murnane, 2003b; Crabbe et al., 2004), and MRE11 (D'Amours and Jackson, 2002; Verdun et al., 2005). Such nucleases are involved in normal cell functions like VDJ recombination (Artemis), Non-Homologous End Joining and Homologous Recombination (MRE-11), leading strand replication (Werner) and Okazaki fragment processing upon DNA synthesis (FEN-1 and Dna2). Some of these nucleases (Werner, Artemis and MRE-11) have been linked to telomere stability.

However, their direct involvement in telomeric overhang generation post-replication has not been determined. Alternatively, the end-nuclease could be an unidentified telomere-specific nuclease that is yet to be characterized.

The precision of the C-strand last base could also result from telomere binding proteins that direct the cleavage of a given nuclease to the specified terminal nucleotide, and many different models are possible. One model predicts that after replication is complete, a 5'-3' exonuclease starts resecting the 5'end of the C-rich strand. In parallel, telomere binding proteins (such as TRF1 and TRF2) are loaded onto the preceding double-stranded DNA. The last protein complex loaded close to the telomere end could create a barrier that blocks further nuclease resection. That protein should bind to the double stranded DNA with great specificity to dictate the identity of the last base (fig. 3-1A).

An alternative model predicts that single stranded DNA binding proteins such as Pot-1, RPA or hnRNPA are being loaded on a 3'-G-rich overhang that is created by partial exonuclease resection of the 5'-end of the C-rich strand. When a certain threshold protein level is achieved, only the ATC 5'-ends are protected from further resection by the 5'-3' exonuclease (fig. 3-1B). The docking space for telomeric single stranded DNA binding proteins might be created by helicase unwinding of the telomere end instead of partial exonuclease resection. Once sufficient proteins are loaded, they could recruit an endonuclease that would specifically cleave at the AATC^CC boundary (fig. 3-1C).

A. B.

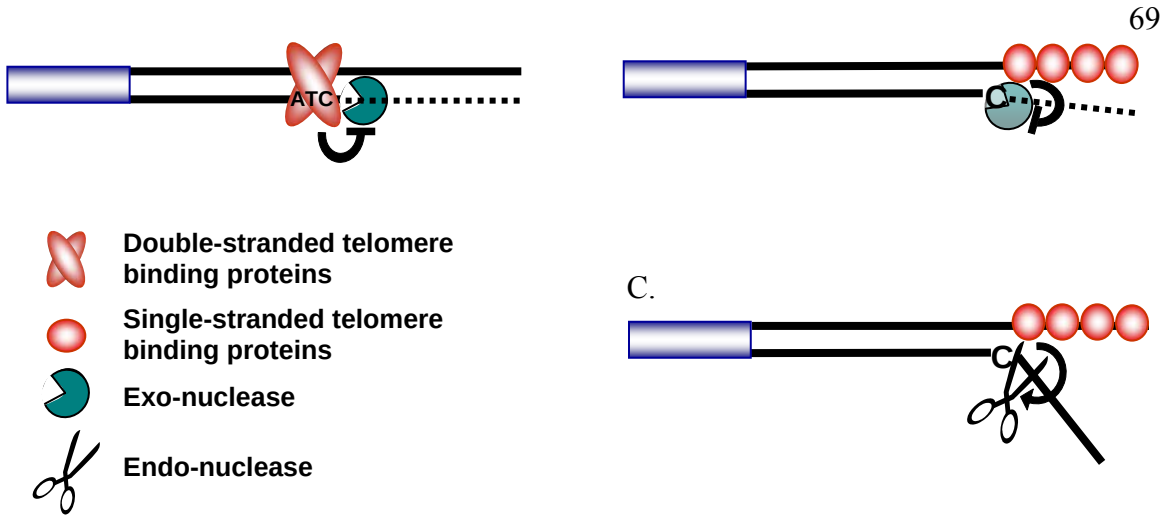


Figure 3.1-Potential end processing mechanisms: Telomeric Proteins binding to the double stranded DNA post-replication could create the boundaries for exonuclease resection (A). Alternatively, telomeric binding proteins that bind to the single-stranded overhang create the boundaries blocking further exonuclease resection beyond ATC-5' (B) or they could bring an endonuclease that would specifically cleave at the precise location ATC^CC (C). Helicases with or without interactions with the telomere-specific single stranded binding proteins, could also unwind the telomere end prior to endonuclease cleavage.

Substantial progress has been made in identifying the telomere-associated proteins and assessing their effect on telomere length, stability and structure. However the role of these proteins on overhang generation was not considered. A number of nucleases and helicases have been characterized in human cells. Determining the telomere-end nuclease and or helicase is the key to understand the molecular mechanism of end processing.

In order to assess their role in end processing, I targeted certain nuclease and telomere associated proteins using SiRNA/ShRNA, dominant negative forms or by using cells lacking certain proteins. Ultimately, I determined the end nucleotides using STELA, to figure out which protein is involved in last base determination during overhang processing.

RESULTS

Nucleases

Artemis

Artemis is a 5'→3' exo-nuclease with an endonuclease activity that is stimulated by DNA-PK. It is involved in Non Homologous End Joining and VDJ recombination. RS-SCID patients are characterized by Artemis deficiency and Artemis deficiency in marine ES cells resulted in increased telomere end-end fusion (Rooney et al., 2003; Rooney et al., 2002). Thus Artemis presented a good candidate for being a C-strand nuclease and we applied our C-STELA assay on fibroblasts from SCID patients that lack Artemis protein. We found the same preference for AT**C**-5' at the last base position in Artemis deficient cells (fig. 3-2). Our results do not rule out the role of Artemis in overhang generation, but show that Artemis resection is not required for the accuracy of the processing.

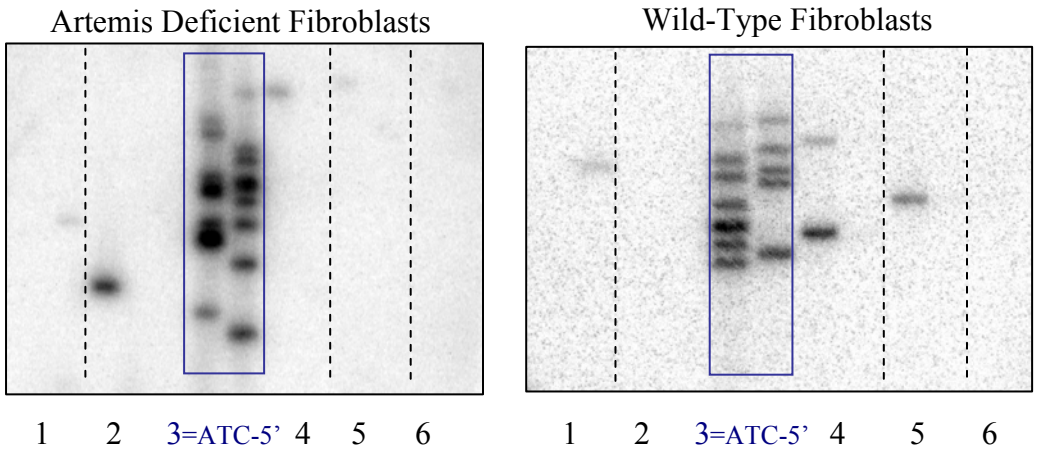


Fig 3.2- Artemis is not involved in specifying the last base of the telomeric C-strand. Artemis deficient cells (Guetel) (left panel) show the same last base specificity as the wild type cells (Otel) (right panel). In both cases, major preference for ATC-5' was noted. (The telorette numbers correspond to a last base of 1= TC**C**-5', 2=CA**A**-5', 3=AT**C**-5', 4=AA**T**-5', 5=CC**A**-5', 6=CC**C**-5').

Werner:

Werner Syndrome patients have a mutation in the Werner RecQ helicase and show a premature aging phenotype for review (Ozgenc and Loeb, 2005). Fibroblasts from WS patients have telomere dysfunction and undergo premature replicative-senescence, and the cells could be immortalized upon overexpressing ectopic telomerase (Ouellette et al., 2000b). Werner is 3'-5' exonuclease and a helicase. Its role in maintaining telomere integrity has been extensively studied. It is involved in telomere replication and in lagging strand DNA synthesis in particular (Crabbe et al., 2004). Werner protein interacts with TRF2 and it helicase activity is stimulated by POT1 that would unwind telomeric substrates (Machwe et al., 2004; Opresko et al., 2005; Opresko et al., 2002). Hence, the Werner protein assists in telomere replication and G-overhang protection by resolving G-

quartets that stall replication fork. We studied its potential role in overhang generation by examining the AGO4110 fibroblasts that were isolated from Werner patients. The absence of Werner did not alter the last base identity (ATC-5') suggesting that is not involved in end processing after replication (fig. 3-3).

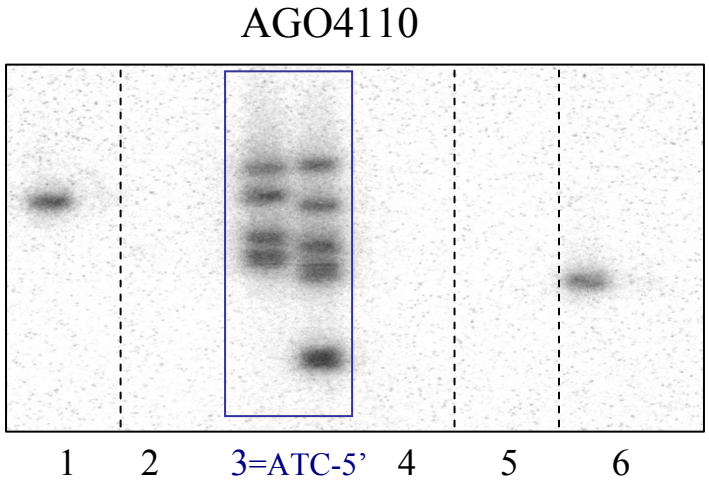


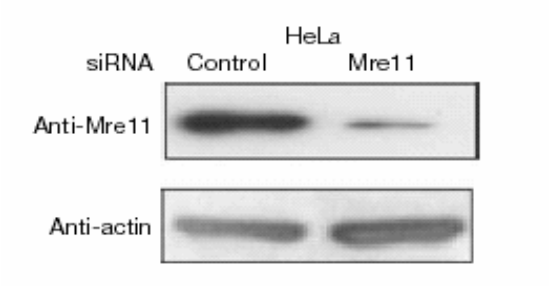
Figure 3.3- Werner nuclease/helicase is not important for the base-specificity during telomere end-processing. AGO4110 fibroblasts show a major preference for ATC-5' at the end, and this is consistent with normal cells. (The telorette numbers correspond to a last base of 1= TC**C**-5', 2=CA**A**-5', 3=AT**C**-5', 4=AA**T**-5', 5=CC**A**-5', 6=CC**C**-5').

MRE11:

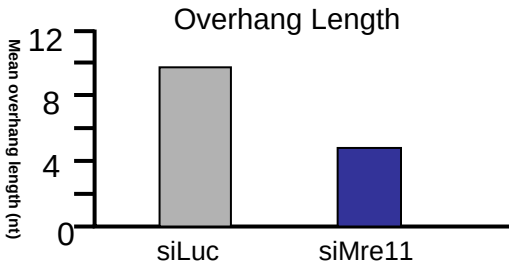
Mre11 is part of the MRN complex that is recruited to double stranded breaks to initiate DNA repair by HR or NHEJ machinery. Mre11 is a 3'-5' exonuclease with endonuclease activity as well and it is involved in processing the DNA structure at break sites to allow repair (Boulton and Jackson, 1998; Ciapponi et al., 2004; D'Amours and Jackson, 2002; Jackson, 2002; Paull and Gellert, 1998). The MRN complex compelx has

been shown to be required for telomere integrity in mammalian cells (Lombard and Guarente, 2000; Ranganathan et al., 2001; Zhu et al., 2000). Deleting Mre11 in budding yeast generated smaller overhangs and caused telomeres to shorten (Larrivee et al., 2004). Mre11 associates with human telomeres by interacting with TRF2 and its levels at the telomeres peak during the G2 phase of the cell cycle (Zhu et al., 2000). We investigated whether Mre11 is involved in human G-rich overhang generation by knocking it down in normal human cells (BJ) and cancer cells (Hela) (Chai et al., 2006b). Using SiRNA to target Mre11, its levels in Hela cells were reduced by ~90% (fig. 3-4A). Hela cells with Mre11 knockdown had markedly shorter average overhang length as measured by Telomere Overhang Protection Assay and in gel hybridization assay (fig. 3-4B). On the other hand, knocking down Mre11 in BJ cells that are telomerase negative had no effect on the overhang length. In both cell types the overall telomere length was not affected. To examine the identity of the terminal nucleotide, DNA from Hela cells treated with SiRNA against Mre11 and a control siRNA were subjected to C-STELA and G-STELA. The C-strands of Mre-11 diminished cells as well as control cells ended in ATC-5', the natural end of human telomeres (fig. 3-4C). Furthermore, the G-strand end showed similar pattern of distribution in Mre-11 knockdown cells as well as control cells (fig. 3-4D). Our results indicated that Mre11 reduction did not alter the specificity of the telomere last bases and as such Mre11 might not be involved in G-rich overhang processing.

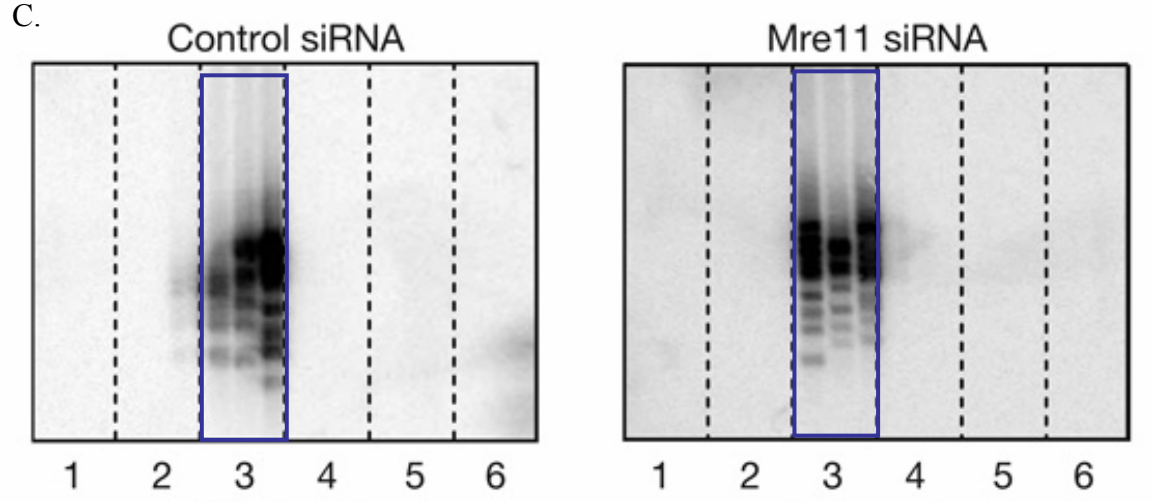
A.


HeLa
siRNA
Control
Mre11
Anti-Mre11
Anti-actin

B.


Overhang Length
Mean overhang length (nt)
12
8
4
0
siLuc
siMre11

C.


Control siRNA
Mre11 siRNA
1 2 3 4 5 6
1 2 3 4 5 6

D.

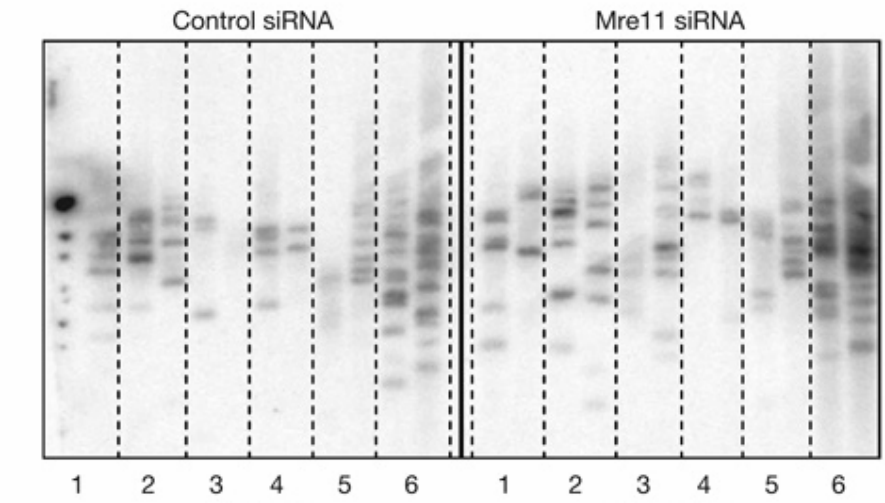

Control siRNA
Mre11 siRNA
1 2 3 4 5 6
1 2 3 4 5 6

Figure3.4- The role of Mre11 in G-overhang processing. A) Western blot for Hela cells treated with SiRNA targeting Mrell and a control SiRNA (Luc). Proteins were extracted a72 hours after treating the cells with the corresponding SiRNA. ~90% reduction in Mre11 could be noted. B) Average overhang size in Hela cells treated with SiRNA for Mre11 and Luciferase (negative control). C) C-STELA was run on DNA extracted from Control SiRNA and Mre11 SiRNA treated Hela cells. The results indicate the same preference for PCR products generated by ligation of telorette #3 to the end of the telomere. D) G-STELA was also run on Mre11 SiRNA treated cells and control cells. Similar distribution pattern was apparent in both cases. DNA used for C and G-STELA was the same as the one used to determine overhang length in (B).
(The C-telorette numbers correspond to a last base of 1= TC**C**-5', 2=CA**A**-5', 3=AT**C**-5', 4=AA**T**-5', 5=CC**A**-5', 6=CC**C**-5' and the G-telorettes corresponds to last bases of 1= AG**G** - 3', 2= GG**G** -3', 3= GG**T** -3', 4= GT**T** -3', 5= TT**A** -3' and 6= TA**G** -3').

Flap endonuclease 1 (FEN1):

FEN1 is a flap endonuclease that is involved in DNA replication, repair and recombination. It is important for Okazaki fragment maturation after DNA replication, whereby it cleaves the RNA primer from the DNA fragment (for review (Liu et al., 2004b). FEN1 interacts with Werner protein and resolves stalled replication forks (Brosh et al., 2001; Sharma et al., 2004; Sharma et al., 2003; Sharma et al., 2005). Given its importance for general cellular function, inhibiting FEN1 activity *in vivo* would be detrimental for the cells. Extensive studies were done to characterize the interaction of Fen1 with the DNA substrate and key residues that mediate the specific binding were identified. Based on those studies, an R47A mutant for of Fen1 was generated and while it maintained the endonuclease and exonuclease activity of FEN1, it altered the base specificity of nuclease resection. *In vitro,* the endonuclease cleavage specificity was shifted by two positions form the end of the flap (Qiu et al., 2002).

The end of the lagging strands is an Okazaki fragment that might require Fen1 to cleave its RNA primer, likewise the last portion of leading strands could resemble an Okazaki fragment in that it might be cleaved by Fen1 when the last portion of the double stranded DNA is unwinded by a helicase. To test this hypothesis and determine if Fen1 is the nuclease that acts on the ends and determines the last base specificity, we overexpressed the R47A mutant form of FEN1 in a tetracycline inducible manner (tet-off system). If Fen1 were the C-strand nuclease, overexpressing R47A should generate C-strands with CC**A**-5' as their last base. His-tagged mutant R47A FEN1 was induced upon the withdrawal of doxycycline from the media of Hela rtTA cells resulted in a significant overexpression. The proliferation rates of Hela cells overexpressing R47A mutant form was not altered. Furthermore, the last base identity was not altered and the cells still maintained AT**C**-5'as the preferential end of their chromosomes (fig. 3-5). Our results exclude a role for Fen1 in last base specificity after overhang processing.

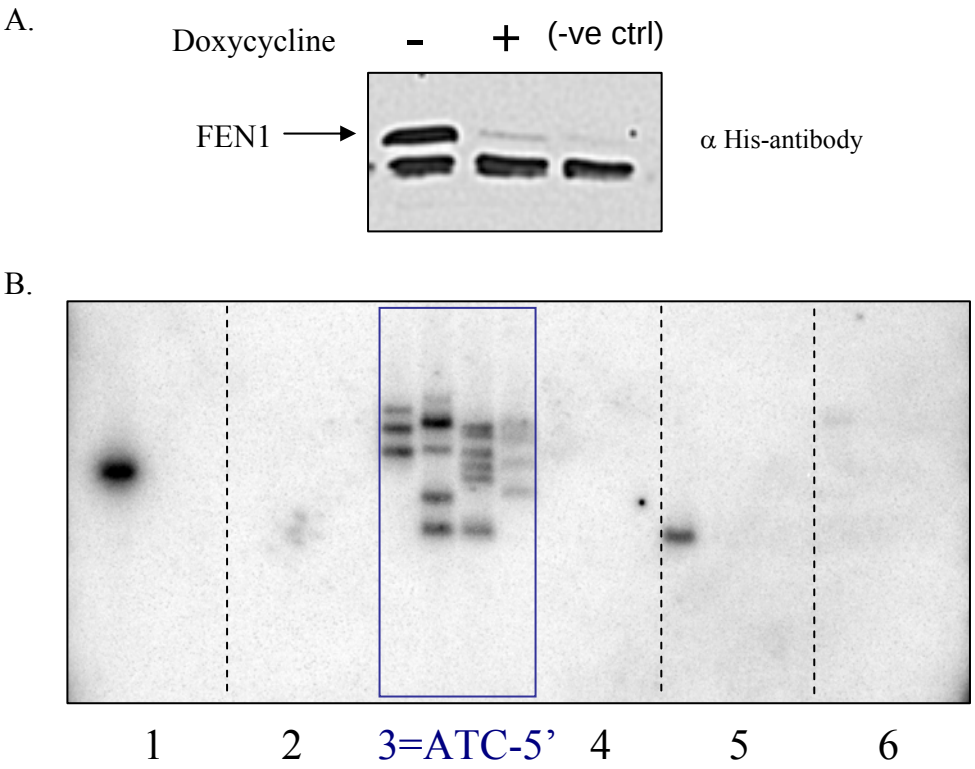


Figure 3.5- Overexpression of FEN1 mutant (R47A) and its effect on the identity of the terminal nucleotide. A) Western blot analysis for His-FEN1 levels in Hela rtTA cells infected with the His-R47A FEN1 72 hours after removing Doxycycline from the media (lane 1) compared to cells growing in the presence 2 μg/ml of Doxycycline (lane 2) and cells that do were not infected with the mutant plasmid (lane 3). B) DNA samples from cells overexpressing Fen-1 R47A were subject to C-STELA assay using 6 telorettes and multiple PCR amplification reaction for each telorette ligation reaction. (The telorette numbers correspond to a last base of 1= TC**C**-5', 2=CA**A**-5', 3=AT**C**-5', 4=AA**T**-5', 5=CC**A**-5', 6=CC**C**-5').

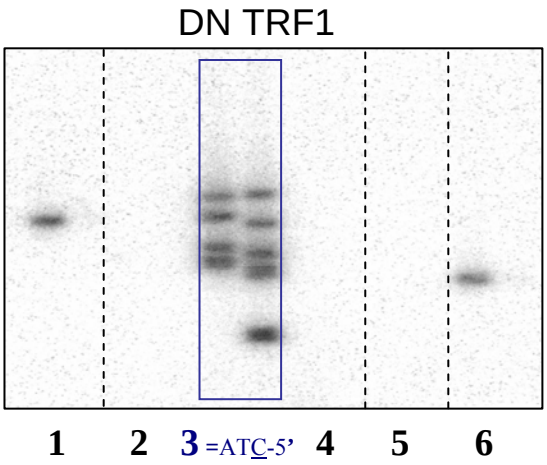
Telomere binding proteins:

TRF1, TRF2, and TPP1:

The proteins of six-member complex play an important role in telomere maintenance. To determine their exact role in end-processing individual members were

targeted and the last base was determined. TRF2 is important for G-rich overhang integrity since it protects it from nuclease clipping. Inhibiting TRF2 function using knockdowns or dominant negative forms lead to the complete loss of the 3'overhang on 50% of the telomere-end due to uncontrolled action of the ERCC1/XPF nuclease that clips the overhang at the juncture of single to double stranded region (Zhu et al., 2003). Knowing that an overhang of at least 7 bases is a key requirement for the C-STELA technique, it was technically impossible to alter TRF2 function and determine its effect on last base identity. TRF1 dominant negative mutant form was previously overexpressed in HT-1080 cells and showed a telomere–elongation phenotype (van Steensel and de Lange, 1997). DN TRF1 was overexpressed in Hela cells, and DNA from the cells was subject to C-STELA. Our results show that the identity of the last base showed the typical preference for AT**C**-5' (fig. 3-6A). TPP1 was identified based on its interaction with Tin2 and POT1 (Houghtaling et al., 2004; Liu et al., 2004a; Ye et al., 2004). It functions in anchoring POT1 to the six-member complex and recruiting it to telomere. Knocking down TPP1 using shRNA or altering its function overexpressing a mutant form, results in a telomere extension phenotype that is consistent with the loss of POT1 from the end. For that reason, we overexpressed in Hela cells the mutant form of TPP1 that lacks the POT1 binding site and has been shown to act as a dominant negative (Liu et al., 2004a). The last base of the C-strand of the Hela cells with altered TPP1 function had chromosomes that ended in AT**C**-5' consistent with normal cells (fig. 3-6 B).

A.



B.

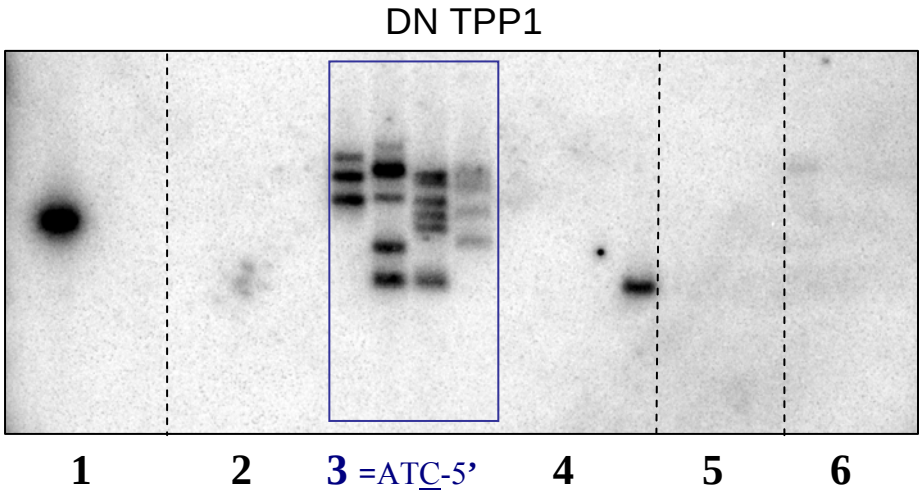


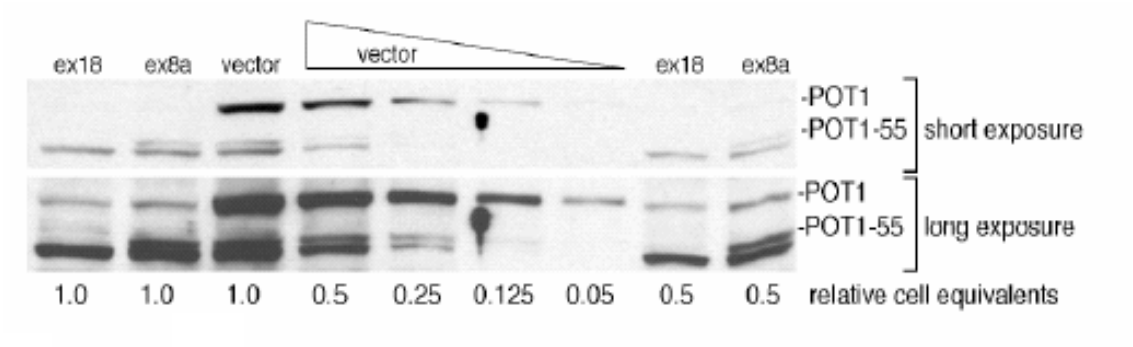
Figure 3.6- TRF1, TPP1 and the last base identity. A) Last base determination for Hela cells expressing a mutant form of TRF1. B) Hela cells overexpressing PTOP RD, a mutant form of TPP1 that displaces Pot1 from telomeres showed ATC-5' as the preferential last base

POT1:

POT1 (protection of telomere) is a telomeric single stranded DNA binding protein that binds to the 3'-overhang directly via two OB folds at its N terminus and indirectly by being part of the six-member telomeric complex (TRF1, TRF2, Tin2, PIP/PTOP/TINT (PPT1), hRAP1 and Pot1)(Liu et al., 2004a; Loayza and De Lange, 2003; Ye et al., 2004). Human POT1 has two isoforms, a 71kDa protein that constitutes 90% of the total POT1 and isoform that lacks an OB fold, termed POT1-55 (55kDa) and makes up 10% of the total protein. In vitro binding assays together with the solved crystal structure of its OB folds indicate that Pot1 binds very specifically to the 5'-TTAGGGTTAG-3' sequence (Kelleher et al., 2005; Lei et al., 2004; Loayza et al., 2004). Given this great specificity with which it binds the telomere, POT1 constitutes a good candidate for specifying the precise overhang end structure. Pot1 was targeted using shRNA to knockdown its levels by >90 % in Hela (fig. 3-7A) and to similar extent in BJ cells. The proliferation of the transformed Hela cells was not altered when Pot1 levels were diminished, however a transient DNA damage response was noted with a significant increase in the levels of TIF (telomere induced Foci) in G1 phase of the cell cycle (Hockemeyer et al., 2005). Knocking down Pot1 in normal BJ cells slowed down their proliferation tremendously and rapidly induced senescence in a subset of the cells. With respect to overhang phenotype, BJ cells with Pot1 inhibition showed some randomization in the identity of the last base of the C-strand and a slight decrease in overhang length (fig. 3-8). Hela cells with Pot1 ShRNA knocking down both forms showed total randomization of the C-strand last base such that a chromosome ended randomly in any of the 6 bases of the telomeric repeat (fig.

3-9). Hela cells expressing shRNA against ex8 that targets the OB fold present only in the full-length protein, showed diminished levels of POT1 while POT1-55 levels remained intact. The terminal nucleotide of the C-strand in those cells was equally randomized and the preference for AT**C**-5' was totally lost (fig. 3-9), emphasizing the importance of the full length POT1 and its OB fold in particular in overhang processing and last base determination.

A.



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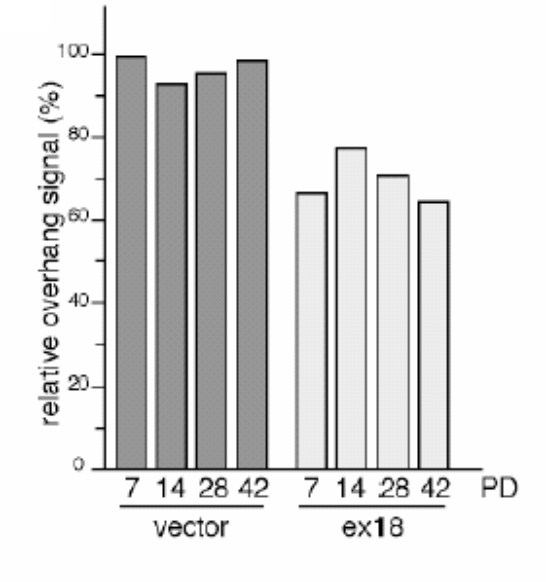


Figure 3.7- Treatment of Hela cells with ShRNA vectors targeting POT1. A) Western blot analysis to determine the level of POT1 knockdown in Hela cells expressing ex 18 (targeting POT1 and POT1-55) and ex 8a (targeting POT1 only). For quantitative purposes, serial dilution of a expressing vector was compared to levels of knockdowns. B) Bar graphs representing quantified overhang signals determined by in gel hybridization for Hela cells expressing ex18 ShRNA for up to 42 Pd. A loss in the overhang length was apparent as early as 7 PDs after POT1 inhibition and the reduction persisted up to PD 42.

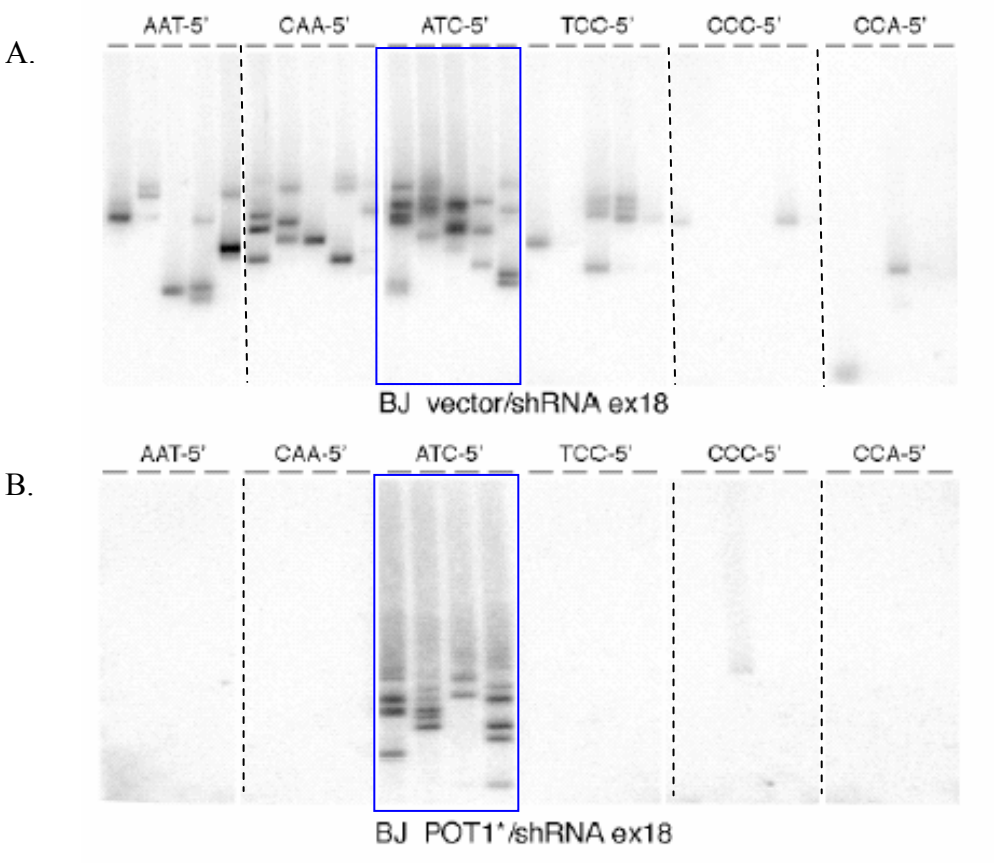


Figure 3.8- POT1 inhibition randomizes that last base in BJ cells.
BJ cells expressing a ShRNA that target ex18 of both forms of POT1, were infected with vectors expressing a non-degradable form of full length Pot1 (B) or a control empty vector (A). DNA samples were subject to C-STELA assay with multiple PCR amplification for each telorette ligation. The results show that knocking down Pot-1 to an extent randomizes the last base of BJ cells (a subset of these cells was in senescence as monitored by β-gal staining). B) Expressing a non-degradable Pot1 rescued the phenotype and respecified ATC-5' as the major end of the telomeres.

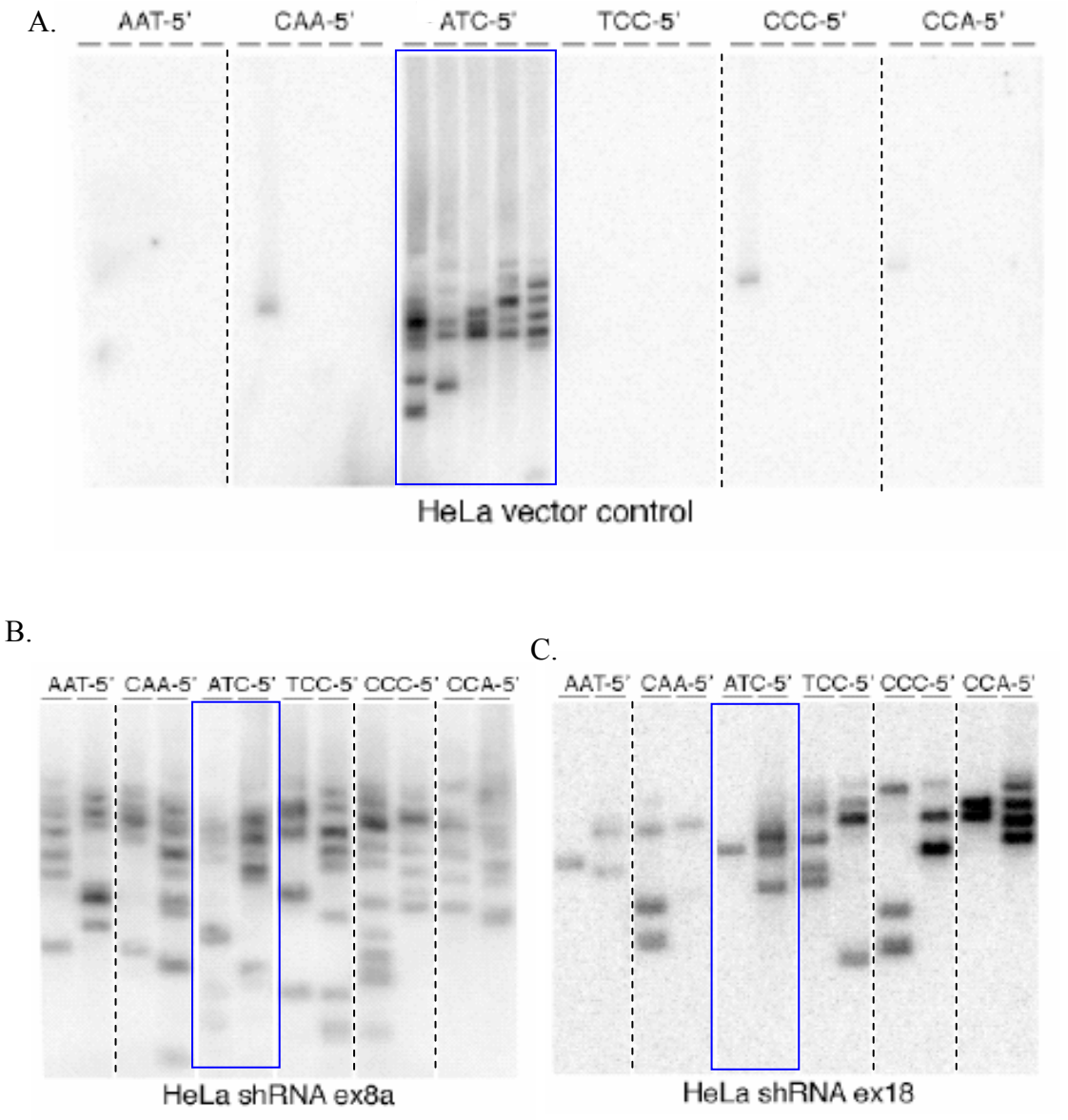


Figure 3.9- POT1 determines the terminal nucleotide of chromosome ends in Hela cells. A) Cells treated with a vector control show the usual preference for ATC-5' at the ends. However cells with diminished levels of POT1 (B) and (C) showed a complete randomization of the last base. Cells treated with shRNA against full length POT1 only and not POT1-55 (B) generated equivalent number of bands with all six telorettes. (C) Similarly, targeting both POT1 forms with shRNA ex18 results in complete randomization of the terminal nucleotide.

CONCLUSION

In this chapter a screen for proteins that alter the last base specificity was carried out. Certain nucleases with telomere-related effect and telomere binding proteins were targeted. The findings show that while many known nucleases affect telomere integrity, the end resecting nuclease is not identified yet. Furthermore, our findings indicate that overhang processing is a multistep process since many factors affected overhang length, but did not alter base specificity. The last base determination step is a very precise step involves the binding of POT1 to the overhang and specifically to the sequence 5'-TTAGGGTTAG-3' (Lei et al., 2004; Loayza et al., 2004) which starts two bases away from the normal telomere end . Thus POT1 could bring and endonuclease that specifically cleaves at the AATC/CC position, or it could physically hinder exonuclease resection beyond ATC, which would be close to its binding site. Is POT-1 determining the last base of the C-strand by the direct binding of its OB fold to the overhang or does it interact with other telomeric proteins to help create the proper ends? If the latter is the case, then what is the identity of the associated protein(s)? Furthermore, if POT-1 interacts with the telomere end nuclease, can this long sought-after nuclease be identified? The process of end-structure formation is important for our understanding of telomere replication, telomerase recruitment, T-loop function and telomere shortening. Answering these questions about the nuclease and mechanisms determining end-structure will provide much needed information for exploiting our knowledge of telomere biology for therapeutic purposes. The telomere-end nuclease remains the missing piece and the key factor in telomere biology.

MATERIALS AND METHODS

Cell Culture

All cell lines used in this study were grown in a 4:1 mixture of DMEM and Medium 199 containing 10% iron-supplemented calf serum (Hyclone) and gentamycin (25 μg/ml; Sigma) at 37^{o} C in 5% $CO_{2.}$. Cells were passaged by trypsinization and subsequent inactivation of the trypsin with serum-containing media, followed by replating. Guetel and Otel (artemis deficient cells) were from JP Villartay. HeLa rTA cells (Clontech, Palo Alto, CA) were grown in X media (4:1 DMEM: Medium 199) supplemented with 10% tetracycline-free serum (Donor Calf from Gibco, Gaithersburg, MD), and 2 ng/ml doxycycline. Doxycycline was removed upon induction.

Antibodies

The following primary antibodies were used: monoclonal anti-Mre11 (GeneTex, San Antonio, TX, USA), anti-His antibody (abcam), and antibodies for POT1 and POT1-55 was an anti-peptide antibody (# 978) as described previously (Loayza and De Lange 2003). Horseradish peroxidase-conjugated anti-mouse or anti-goat IgG were used as secondary antibodies.

Retroviral Infections and plasmids

Phoenix E cells were transiently transfected with 30 μg retroviral plasmid DNA using FuGENE6 (Roche). The retroviral plasmids used were PTOP RD (Songyang Z.), dominant negative TRF1 (de Lange T.), and His-FEN1 R47A mutant (Shen B). The supernatant from the cells was used to infect PA317 cells (amphotrophic retroviral packaging cell line) (Miller 1990). Following selection, supernatants were harvested from PA317 cells, and used to infect target cells. The cells were infected 2 or 3 times with 8 hour exposure time to the supernatant in the presence of 4 μg/mL (final) polybrene (Sigma, St. Louis, MO). Cells were then allowed to recover for 24 hours before adding the appropriate antibiotics for selection.

siRNA transfection and ShRNA infection.

siRNAs were designed and synthesized for the target sequence of Mre11 (ACAGGAGAAGAGATCAACT), The control siRNA targets luciferase and its sequence is available from BD Biosciences. HeLa cells were washed once with PBS, then transfected with siRNA and Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to Manufacturer's instructions. The cells were analyzed 72 hrs post transfection by extracting DNA for overhang measurement and STELA analysis and to isolate protein for immunoblotting. Knockdown in POT1 levels was maintained stably using shRNAs expressed from the pSUPERIOR retroviral vector (OligoEngine). The target sites in POT1 were Ex8a: 5'-GATATTGTTCGCTTTCACA-3' CAATACCGCATCCGAGCAA-3'; Ex18: 5'-GTACTAGAAGCCTATCTCA-3'.

Western-blot and immunofluorescence

The percentage of cells having their proteins knocked down using siRNA or shRNA was determined by Western blot detection using whole cell lysis. Cell pellets were lysed in 1X Western buffer (0.05M Tris, pH 7, 2% SDS, 5% sucrose) by vortexing for 30 min at 40C. Samples were diluted 1:2 in 2X Laemmli buffer (0.125M Tris, pH 6.8, 10% β-mercaptoethanol, 0.002% bromophenol blue, 4% SDS, and 20% glycerol for 2X) heated to 95º C for 2 minutes and run on an appropriate concentration SDS-PAGE gel. Gels were transferred to PVDF membrane (Millipore, Billerica, MA) for 75 minutes at 100 mA and blocked in PBS plus 0.05% Tween- 20 containing 5% milk. Primary antibodies were diluted in PBS plus 0.05% Tween-20 containing 0.5% milk and incubated for 2 hours at room temperature. Then the membrane is incubated with the corresponding secondary antibody for 1 hour. Blots were washed and the signal was detected using the ECL detection system (RPN2109 from Amersham, Piscataway, NJ) and X-ray film.

Overhang protection assay and in gel hybridization

Telomere overhang protection assay was carried out as described in Chai el al., 2005 and nondenaturing in-gel hybridization assay were carried out as described by (Chai et al., 2005; Dionne and Wellinger, 1996).

CHAPTER FOUR

Interplay between telomere length and telomerase action

INTRODUCTION

Telomere-length homeostasis in tumor cells is an intricate balance between shortening (due to the end-replication problem and nuclease resection) and telomerase-mediated extension. Although mammalian telomeres show some degree of heterogeneity (Lansdorp et al., 1996; Londono-Vallejo, 2004; Martens et al., 1998), their length is tightly controlled and displays a chromosome-specific length pattern (Graakjaer et al., 2003; Graakjaer et al., 2004) that might be inherited (Bischoff et al., 2005; Slagboom et al., 1994). In one series of experiments, the length of the donor end was maintained after translocating a telomere from one chromosome end to another, suggesting that cis-acting sequences in the subtelomere region might play a role in this regulation (Barnett et al., 1993; Hanish et al., 1994; Sprung et al., 1999). While the mechanism that specifies this non-random distribution of telomere length on different chromosomes is not quite understood, some factors influence the heterogeneity. For example, expressing deletion forms of Rap1 decreases the overall heterogeneity of the telomeres and the mechanism behind that is still unclear (Li and de Lange, 2003).

In *Saccharomyces cereviseae*, telomeres exist in two states, an open extendible state when their length is short and a closed non-extendible state when they are long. The corresponding state is controlled by a protein-counting model based on the number of

Rap1 and Rif1 molecules on a telomere (Teixeira et al., 2004). Certain studies showed that in human cells the telomere length is mediated by the *cis* acting factor TRF1 that binds to the duplex region of the telomere, forming a negative feedback loop that relays the message to POT1, which would in turn block further extension by telomerase (Loayza and De Lange, 2003; van Steensel and de Lange, 1997). POT1 is a negative regulator of telomerase, and structural studies have shown that POT1-bound telomeres are not optimal substrates for telomerase elongation (Lei et al., 2003; Lei et al., 2004; Lei et al., 2005).

Telomere length distribution in telomerase positive cells is maintained within a relatively narrow range. Telomere-length homeostasis might be regulated at many different levels; transcription of hTERT and hTR, hTERT post-transcriptional modification , assembly into a ribonucleoprotein enzyme, subcellular localization, recruitment to the telomere ends, and potentially processivity of the enzyme (reviewed in (Cong et al., 2002; Yi et al., 2001; Yi et al., 2000). Regulating telomerase levels in germ cells in important for telomere length homeostasis in such a way to allow replicative aging. In tumor cells the levels of hTERT and hTR are usually low and maintain telomeres at relatively short lengths. When both components are overexpressed in Hela cells, the telomere length constantly increases even after telomeres are 8 fold longer than physiological length (Cristofari and Lingner, 2006) .

The timing and mechanism of telomerase recruitment to the telomere end is widely studied in yeast. In one cell cycle 10% of the yeast telomeres are extended by telomerase (Teixeira et al., 2004). Telomerase activity at the end is tightly regulated such that telomeres get extended in late S phase when yeast telomeres are replicated (Fisher et al.,

2004; Marcand et al., 2000; Taggart et al., 2002). The action of telomerase has been shown to be coupled to the activity of lagging strand polymerases (DNA pol α and δ) and thus probably relies on lagging strand DNA replication machinery for C-strand, and the assembly/recruitment of telomerase for G-strand synthesis (Diede and Gottschling, 1999). Recruitment of telomerase components to the telomere is also cell cycle dependent whereby the localization of Cdc13, Ku and Est1 to the telomere end is restricted to S phase (Chandra et al., 2001; Fisher et al., 2004; Taggart et al., 2002).

There are no data on the temporal regulation of telomerase action in mammalian cells. Furthermore, there has been little information as to how mammalian telomerase is recruited to the telomere end. A mutation in the N-terminal DAT (dissociates activities of telomerase) domain of hTERT retains the catalytic activity of the enzyme but blocks its ability to extend telomeres and immortalize normal cells, suggesting that the DAT domain might be important for recruiting telomerase to the telomere end. Fusing the mutant DAT form of hTERT to hPOT1 rescues the phenotype such that telomerase-POT1 fusion protein is efficiently recruited the telomere end and is able to immortalize normal cells (Armbruster et al., 2004).

Human telomeres are technically more challenging to study, since they are much longer than in most model organisms and are not easily sequenced. As such, many key questions regarding the mechanism of telomere length homeostasis and telomerase action at the chromosome end are still unanswered. How many nucleotides can telomerase add per cycle? Does it add stretches of kilobases Kb to some of the telomeres per division or a few hundred bases to most of the telomeres in a relatively uniform manner? Does the

yeast model for extendible/non-extendible telomeres hold up in mammalian cells? Are short telomeres more frequently extended by telomerase, or do they get more repeats/ cell cycle? In this chapter, attempts to addresssome of these questions will be highlighted.

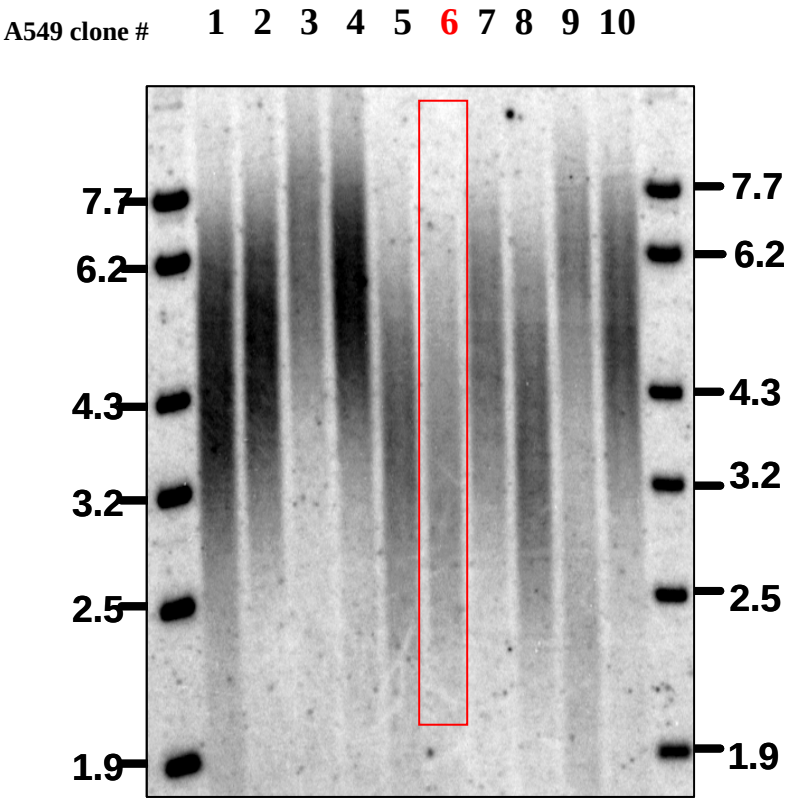
RESULTS

Developing a system to study the mechanism of telomerase action in human cells.

Cell culture system:

Human telomere length within a cell population is highly heterogeneous. In order to get a cell line with fairly homogeneous telomere length, MRC5 (telomerase negative fetal lung fibroblasts) and A549 (lung tumor) cells were plated at clonal density and individual cell colonies were isolated. The telomere length of the individual clones was determined by TRF and STELA. We selected the clones with shortest telomere length (A549-C6 and MRC5-C14). A549–C6 with an average telomere length of 3.5 Kb was treated with 1 μM GRN163L (telomerase hTR inhibitor) for ~20 PDs to drive shortening further and to get an average Xp telomere length of 1 Kb (fig. 4-1)

A.


A549 clone #
1 2 3 4 5 6 7 8 9 10
7.7
6.2
4.3
3.2
2.5
1.9
7.7
6.2
4.3
3.2
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1.9

B.

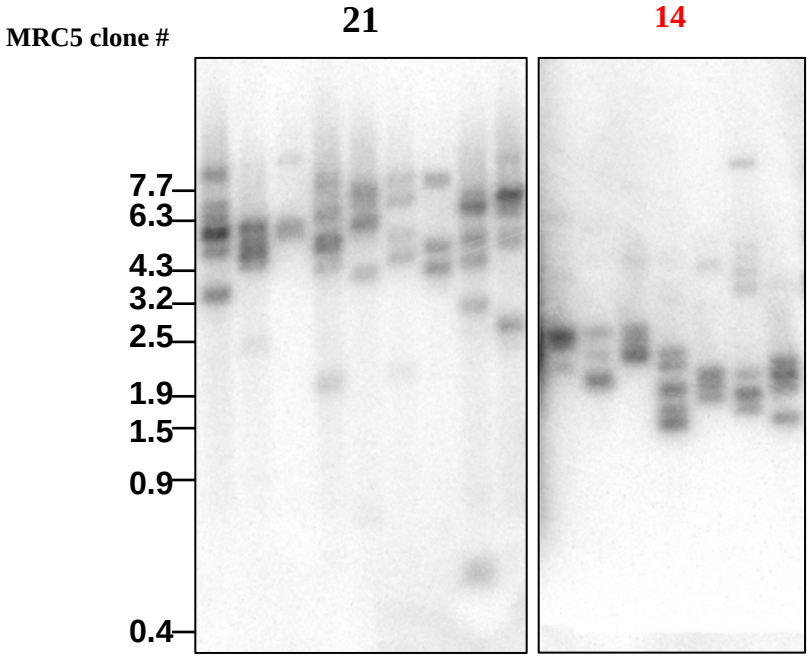
MRC5 clone #
21
14
7.7
6.3
4.3
3.2
2.5
1.9
1.5
0.9
0.4

Figure 4.1- Characterization of cell clones for telomere-length analysis. A) TRF (telomere restriction fragment analysis) was done on DNA samples of 10 A549 clones. Clone #6 was the one with shortest telomeres and will be used throughout the study. B) STELA was applied on DNA samples of 22 clones of MRC5 cells, showing 2 clones (#21 and #14). Clone 14 was used for telomere length analysis.

STELA:

We used the STELA technique that was developed by Baird et al., (2005) in order to monitor the telomere length of individual chromosomes with great accuracy and distinguish between the telomere lengths on different chromosome ends. STELA enabled us to measure the length of individual DNA molecules, and more importantly allowed us to examine very short telomeres, which could not be accurately monitored using hybridization-based assays. The initial report by Baird et al, analyzed the telomere length on the Xp /Yp telomere. Our first aim was to extend STELA to more chromosome ends. In order to get STELA products for the 92 chromosome the sequence of the subtelomeric region of the chromosomes is needed. These regions were among the last to be sequenced by the human genome project and in many instances have been mismapped. Today, sequence is available for only a few chromosome ends. In most cases the subtelomeres are highly variable, with some repetitive sequence, duplications and regions that are shared among different ends. On top of that, human subtelomeres are highly polymorphic, whereby some individuals possess hundreds of kilobases of subtelomeric DNA that is not present in others (Ciccodicola et al., 2000; Daniels et al., 2001; Heilig and Fonknechten, 2003; Riethman et al., 2004; Riethman et al., 2003; Riethman et al., 2005). Nevertheless, STELA primers could be designed for a subset of chromosomes ends, including 10p, 11q,

21q, 17p, 4p and 2p (fig 4-2). The sequence for the primers is included in table 4-1. The telomere length on these ends was not significantly different form the Xp/Yp telomere.

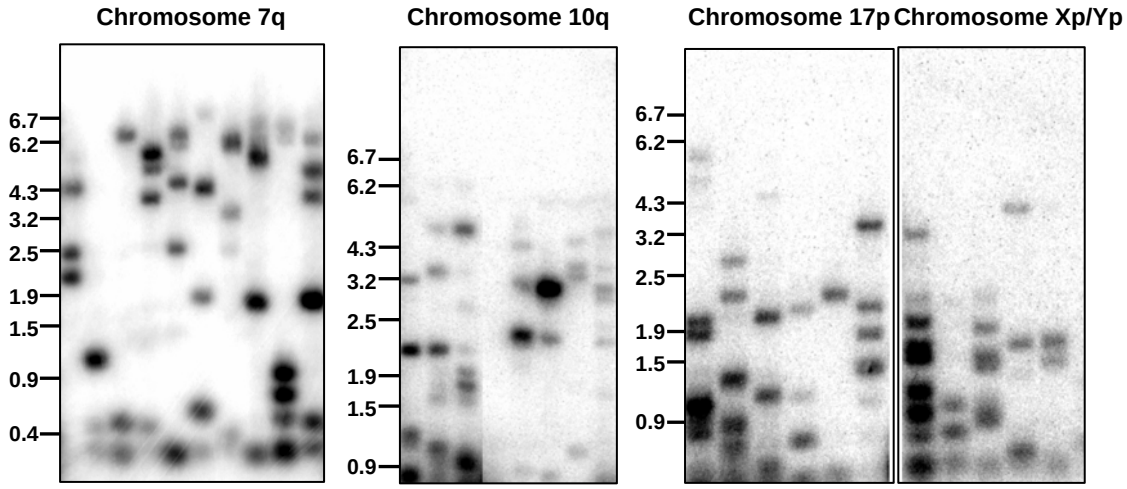

Figure 4.2- STELA on different chromosomes. STELA assay was extended to different chromosome ends and was applied on young BJ cells for chromosome 7q, and old BJ for 17p, 10q and Xp/Yp.

END	E2 PRIMER (forward)	B2 PRIMER (reverse)
17p	5'- GGCTGAACTATAGCCTCTGC -3'	5'- GAGTCAATGATTCCATTCCTAGC -3'
10q	5'- CTGCCTTTGGGATAACTCGGG -3'	5'- CTGAACCCTAACCCTCCATG -3'
4p	5'- GTGCATCTCTCCGCGTGACT -3'	5'- AGAATGCAGCTCCGTTATCG -3'
21q	5'- CTGCATGGCTTTGGGACAAC -3'	5'- GCGACGCTGCAGTTGAACCG -3'
2p	5'- TGCCCTAGTGGCCAGCAAGG -3'	5'- GGTCGTACTGCAGGTGCACAGC -3'
11q	5'- CTGTGTCCCAGCAGAACTCA -3'	5'- ACTTTCTGTGCTGGGTGAGC -3'

Table 4.1- STELA primer sequence for multiple chromosome ends. E2 is the Fwd primer. B2 is reverse primer used to generate a probe (E2-B2) for each chromosome end.

One major modification for the STELA technique was to use 1000-fold less telorette concentration than what was reported by Baird et al., 2003. Non-specific ligation was initially noted when C-STELA for last base determination (Chapter 2) was performed with 0.9 μM telorette concentration. Under those circumstances, all six telorettes ligated to the telomere ends in a non-specific fashion. We found that 1 μM of telorettes generated numerous small PCR amplification products that were were greatly reduced by using lower telorette concentrations (fig.4-3). This is attributed to the fact that when present in excess, the telorettes would ligate non-specifically to nicks along the duplex region of the telomeric DNA, generating smaller PCR products that do not correspond to full-length telomere molecules.

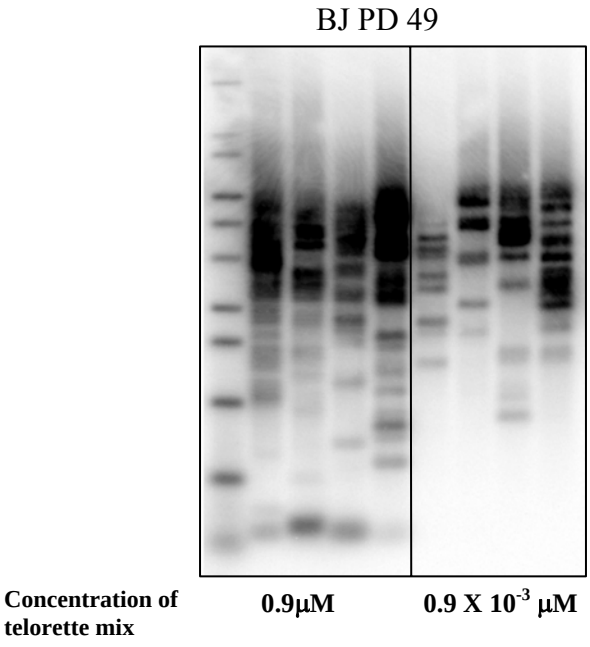

Figure 4.3- STELA modification for length determination purposes. A direct comparison of two STELA assays run on the same DNA sample using different telorette dilution.

How many nucleotides does telomerase add per division?

Currently, there are no data in mammalian cells to distinguish whether telomerase adds a kilobase of telomeric repeats to few telomere ends per division versus a relatively uniform addition of a few hundred bases to most of the telomeres. To determine this, MRC5 –C14 cells were grown to confluency and forced to enter the G_0 phase by contact inhibition for 10 days. hTERT was overexpressed by adenoviral infection for 12 hours, after which the cells were trypsinized and split into different plates. Immunostaining using hTERT antibody (1A4; Geron) was used to score for infection efficiency. 85-90% of the cells were hTERT positive (fig.4-4A). The TRAP assay verified that telomerase activity was produced. DNA was harvested at T_0 (24 hours post trypsinization) and at T_1 (after one doubling). The length of the Xp telomere at both time points was assessed by STELA and our result showed an increase in the average telomere length by ~260nt, suggesting that when overexpressed telomerase was able to add an average of 260 nt per telomere (fig. 4-4B).

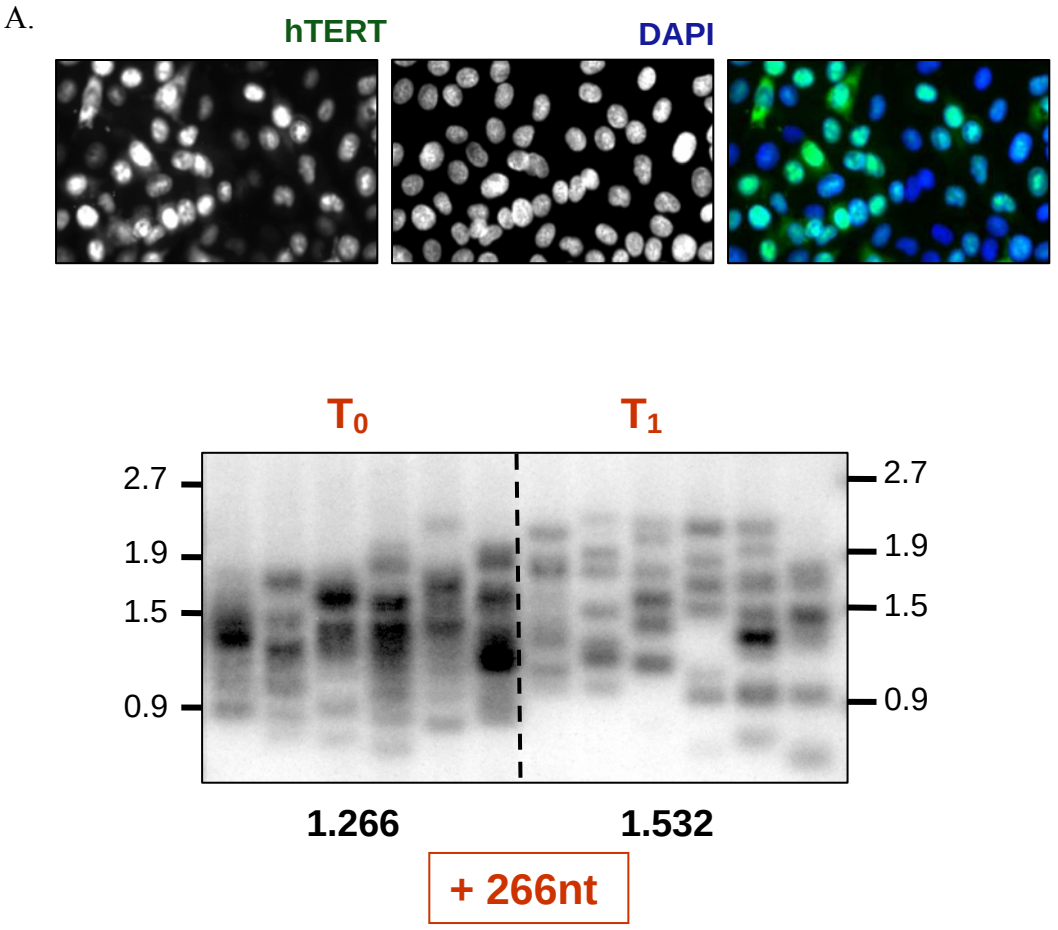

Figure 4.4- When overexpressed, telomerase can add an average of 260 nt/division. A) MRC5-C14 clone was stained with hTERT antibody (1A4; Geron) to determine the infection efficiency. B) STELA on the Xp/Yp telomere was applied on DNA from To and T1 (one replication doubling in the presence of hTERT. The length of approximately 300 telomeric molecules was analyzed.

Parallel experiments were done on A549-C6 cells with an average telomere length of ~1 Kb. To determine the average telomere lengthening that endogenous telomerase can add, STELA was performed on DNA samples harvested at T_0 and after one population doubling (T_1, 35 hours later). Additional plates of cells were treated with adenoviral hTERT for 12 hours to overexpress telomerase and then DNA was harvested at T_0 and T_1. The TRAP assay showed that when hTERT was overexpressed telomerase activity was 10-fold more than endogenous telomerase levels (fig. 4-6A). Our results indicate that the endogenous telomerase can add an average of 56 nucleotides/division (fig. 4-6B). When hTERT is ectopically overexpressed, the average telomere lengthening in the A549 clone was around 250 nt/division, similar to the average telomere-length increase in MRC5-C14 clone overexpressing hTERT. However, one major draw back of these experiments was the fact that the distribution of Xp/Yp telomere length was broad. Although individual clones of cells were picked, by the time the clones had 500,000 cells, telomere length was highly heterogeneous. As a result, our data could not definitively indicate whether all telomeres were extended by 250 bp, or a lesser fraction was extended by a greater amount (fig. 4-7).

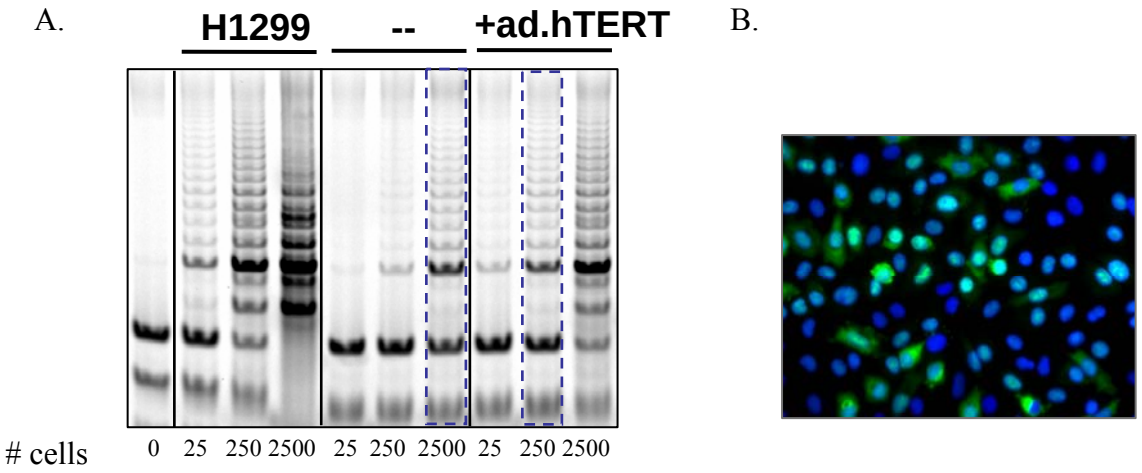

Figure 4.5- Telomerase levels in A549-C6 (+/- adenovirus hTERT). A) TRAP assay was performed to compare the telomerase activity in A549-C6 cells that were overexpressing hTERT by adenoviral infection (last 3 lanes) to endogenous levels of telomerase activity in A549-C6 control cells. 250 A549-C6 +hTERT cells had almost 10 fold more activity than endogenous telomerase. (the activity of 250 overexpressing cells is equivalent to that in 2500 uninfected cells) B) To determine the efficiency of Adenoviral infection, immunostaining with anti-hTERT antibody that can not detect endogenous levels of telomerase was performed. hTERT: green. DAPI: nuclei.

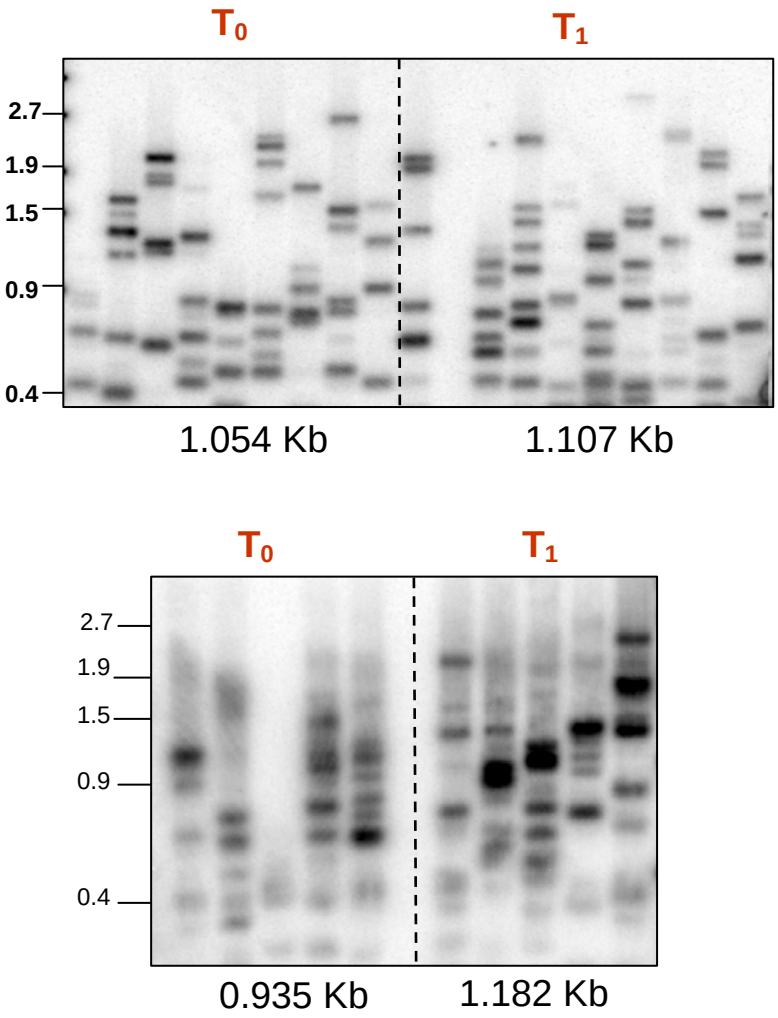

Figure 4.6- Endogenous *vs.* exogenous telomerase with respect to the number of repeats added. (Top panel) STELA was applied on DNA from T_o and T_1 of A549-C6 cells (after removing the telomerase inhibitor GRN163L). The assay enabled us to determine that endogenous telomerase extends telomeres by an average of ~56 nucleotides / population doubling. (Bottom panel) STELA assay was applied to DNA from T_0 (control cells), and T_1 (one PD post TERT expression) of A549-C6 cells (after removing the telomerase inhibitor GRN163L) overexpressing adenoviral hTERT. When overexpressed telomerase adds 240nt/division

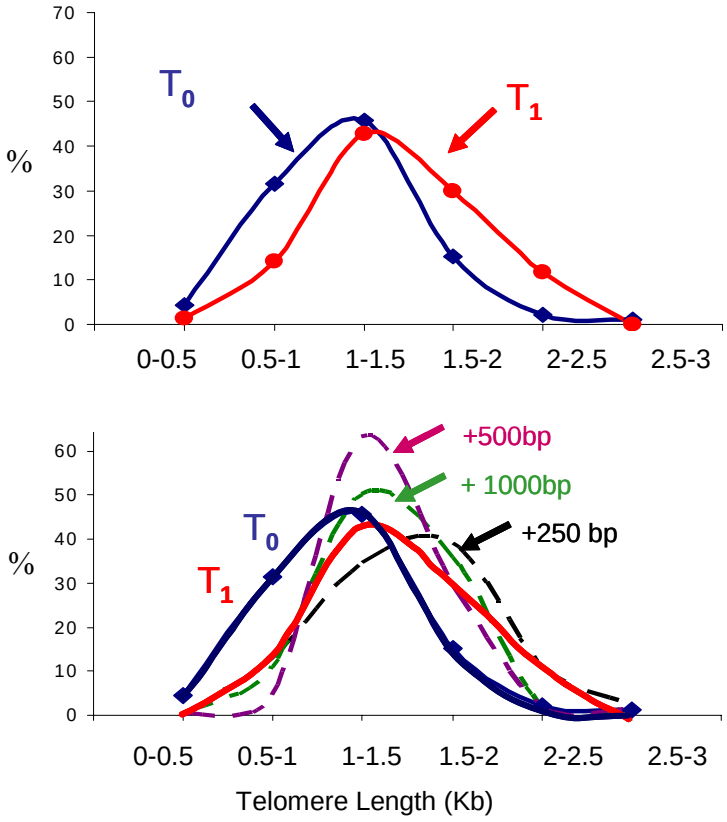

Figure 4.7- Modeling of telomere length distribution upon elongation by telomerase. The distribution of Xp telomere length of A549-C6 cells at T0 (blue line and T1 (red line) where telomerase acted for one division and added an average of ~ 250 nucleotides. Theoretical result from adding 250 to all T_0 telomeres, 500 to the shortest 50% or 1000 bp the shortest 25%.

When does telomerase act?

Human telomeres replicate throughout S phase (Ten Hagen et al., 1990; Wright et al., 1999). Each telomere on a particular chromosome end has a distinctive replication-timing within S phase (Zou, manuscript under preparation). When does telomerase extend each telomere end? Knowing that telomerase preferentially acts on the shortest telomeres, two possibilities can describe its temporal regulation. Based on the data in yeast, in which telomerase action is temporally coincident with replication, the most likely possibility was that telomerase action in human cells would be coupled to the replication of individual ends. Under such circumstances, the *cis*-factors acting at the level of each individual telomere would determine whether that precise end is short and to what extent it should be elongated by telomerase. Another possibility was that telomerase preferentially acts at the end of S-phase following the replication of all telomeres, and this might allow it to identify and selectively extend the shortest ends.

To understand the temporal regulation of telomerase action, A549-C6 cells were synchronized at the G1/S interphase using double thymidine block (Fig. 4-8). During the second thymidine treatment and 12 hours before the release from the block, hTERT was overexpressed ectopically by an adenovirus. Upon release, the replication timing of the Xp telomere was determined using the newly developed ReDFISH (Zou et al., 2004a) technique and the dynamics of the ~250-nt extension by telomerase was monitored by STELA. Cell cycle synchronization followed by propidium iodine staining / flow

cytometry showed that A549-C6 are consistently synchronized at G1/S and require 7 hours to exit S-phase and go into G2 phase (fig. 4-8).

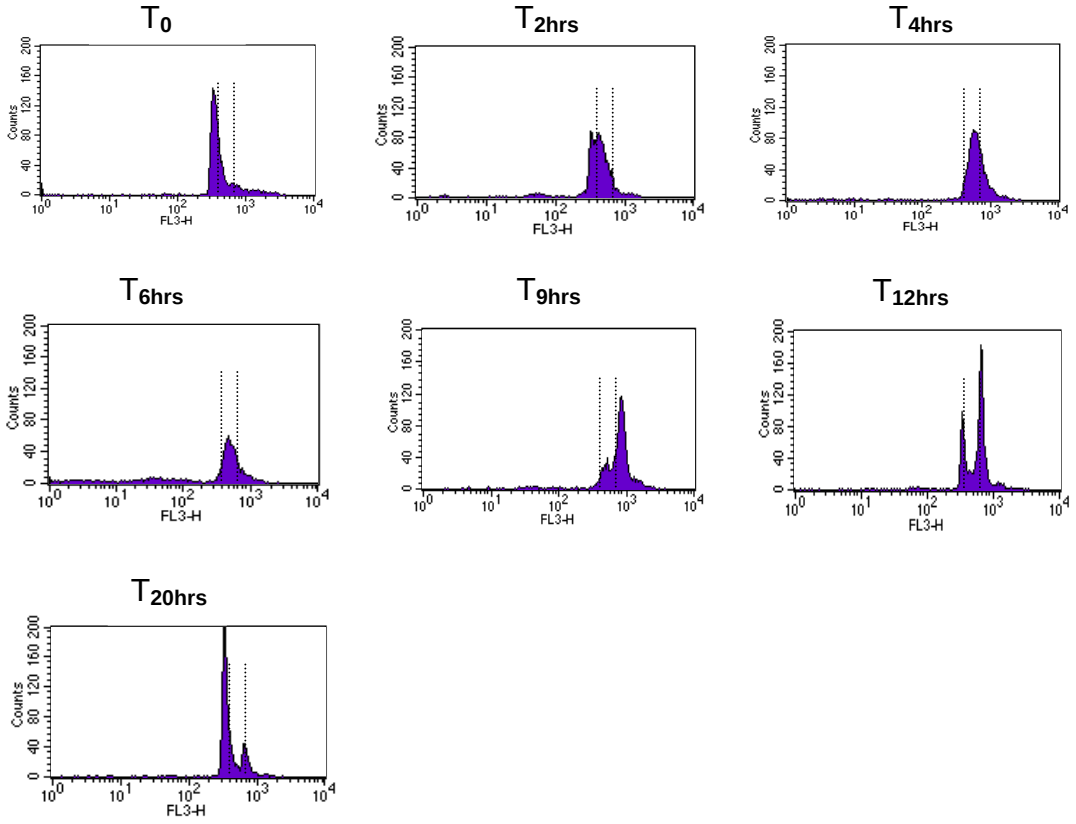

Figure 4.8- FACS analysis of synchronized HeLa cells. *y* axis, cell numbers; *x* axis, relative DNA content on the basis of staining with propidium iodide.

The replication timing of each individual human telomere in BJ cells has been determined in our lab (Zou, manuscript under preparation). In that study, the telomere of each chromosome arm had a unique timing of replication. Certain chromosomes replicated in early S-phase, some in mid-S and others, especially accrocentric chromosomes, replicated in late S phase. The significance and the reason behind the temporal regulation of telomere replication are yet to be determined. We applied the ReDFISH technique to determine when is the Xp telomere of A549-C6 cells replicating. The A549-C6 cells from the 2nd thymidine block were pulse-labeled with BrdU/BrdC (5'-bromo-2'-deoxyuridine and 5'-bromo-2'-cytidine, the analogues of thymidine and cytidine) for one-hour intervals during S phase. In effect, 7 plates were treated sequentially for one hour throughout S phase, one plate was treated continuously for 7 hours, and as a negative control one plate was not treated with BrdU/BrdC. All plates were provided with colcemid for 4 hours before mitosis and harvested at metaphase (fig. 4-9A). The cells were then dropped on a slide, treated with Hoechst dye and exposed to UV irradiation. This would preferentially create nicks in the BrdU/BrdC substituted daughter strands, which are subsequently digested with Exonuclease III. This treatment would leave the parental strands lacking BrdU/BrdC largely intact. Two probes (a Cy-3-labeled C-rich probe to label the TTAGGG strand and a FITC labeled G-rich probe to label the AATCCC strand) were sequentially used to tag the telomeres. When a telomere replicates in an interval where no BrdU/BrdC were present, both the parental and daughter strands would resist digestion and they would be labeled with both probes (FITC-G rich and Cy3-C rich) ultimately giving a yellow color (fig 4-9B). If a telomere replicates when

BrdU/BrdC is present, its daughter strands are digested while the parental strands remain. Chromatid arms produced by lagging strand synthesis would only retain the G-rich strand and hence would anneal to the Cy-3 labeled C-rich probe, generating a red signal. Chromtaid arms produced by leading strand syntheis which copies the C-rich telomeric strand and would only anneal to a FITC-conjugated G-rich probe giving a green signal.

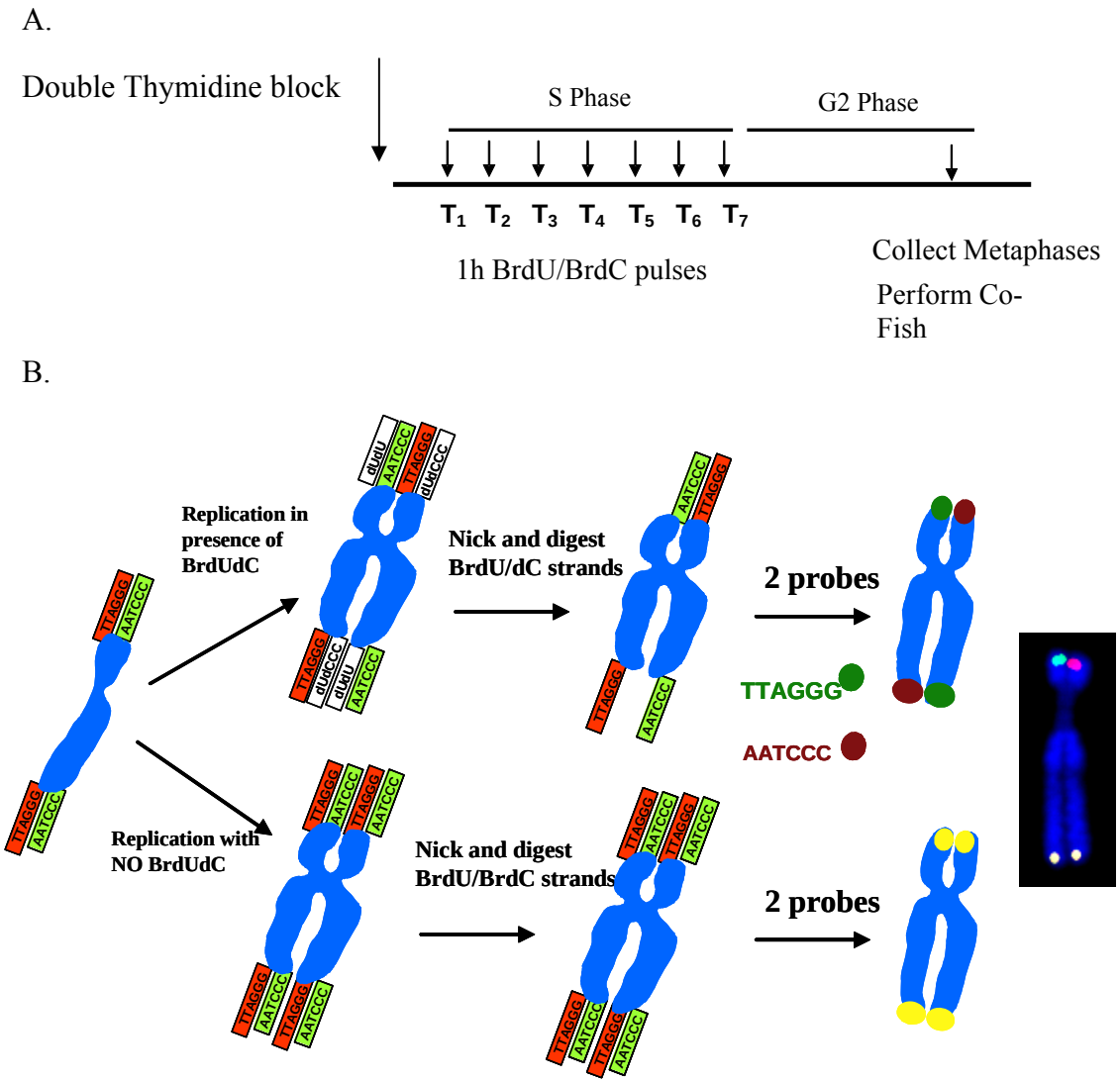

Figure 4.9- Schema for ReDFISH technique. A) Experimental design for ReDFISH whereby cells were pulse-labeled with BrdU/dC for one hour. The cells were treated with Colcemid for four hours prior to metaphase harvest. B) ReDFISH of a chromosome with its p arm replicating fully while, and its q arm replicating when no BrdU/dC is present. Newly synthesized telomeric strand of the p-arm incorporates BrdU/dC. The BrdU/dC containing strand is digested totally after nicking the DNA with Hoechst 33258 then UV irradiating and treating with Exonuclease III. As a result, this treatment leaves the parental strand intact and available for hybridization. On the other hand, both strands on the parm lack BrdU/BrdC and as such resist digestion and hybridize to both probes generating a yellow signal.

Scoring the red/green ends vs. yellow ends on the p arm of the X telomere (marked with a Cy-3 labeled centromeric PNA probe; a gift from Aviv, A) for each S-phase hourly interval indicated what fraction of the telomeres replicated during that interval. An end that did not replicate within a given interval would generate a yellow signal and this is exemplified in fig. 4-10A where the A549-C6 cells were treated with BrdU/BrdC during the 4th hourly interval. Cells treated with BrdU/dC continuously for 7 hours generated red/green signals on all ends including telomeres of the X-chromosome (figure 4-10B). Our results clearly showed that the highest fraction of telomeres on the p arm of the X chromosome replicated during the 2nd hour of S phase (figure 4-10C). Telomeres on the q-arm of the X-chromosome replicated during the 5th hour interval (Figure 4-9 D). This confirmed Zou et al.,(Zou et al., 2004a) results that the replication of telomeres on p and q arms were not coupled. By counting replicating vs. non-replicating ends, our results indicated that almost 90% of the Xp telomere have replicated by the end of the 3rd hour of S-phase (Figure 4-11).

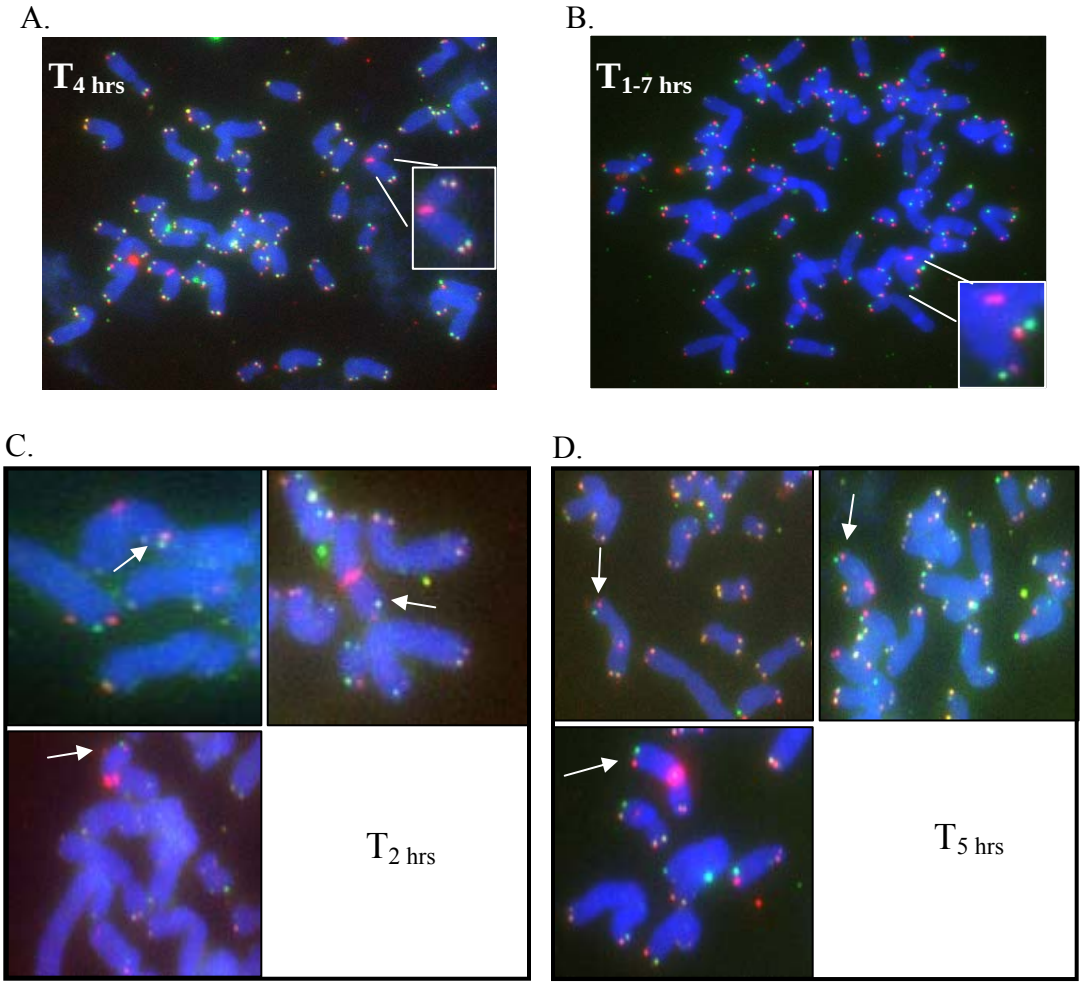

Figure 4.10- Characteristic replication timing of the telomere on the X-chromosome in A549-C6 cells. A) Representative metaphase spread of A549-C6 cells treated with BrdU/dC during the 4th hour interval of S phase. The insert shows an x-chromosome with its centromere labeled with a Cy3 labeled PNA probe and both arms generating yellow signals. This illustrates that the ends were not replicating in that specific interval. B) Positive control; a representative spread for cells treated with BrdU/dC throughout S phase (1-7 hours). The insert shows an X chromosome with both ends replicating in the presence of BrdU/dC and giving red/green signal. C) 3 spread from second interval treatment illustrating that most of the Xp telomeres replicated during 2nd hour of S phase D) Telomeres on the q arm mostly replicated during 5th times point, as illustrated by three representative spreads. Arrows point to the corresponding ends.

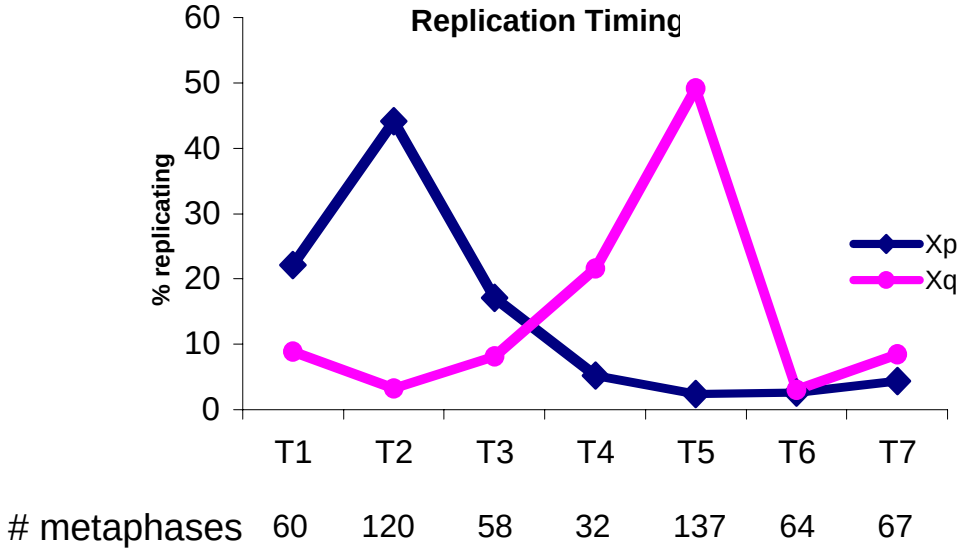

Figure 4.11- Timing pattern of telomere replication on the p and q arm of the X chromosome. Different pattern of telomere replication was observed for each arm. The number of metaphases analyzed for each time point in indicated below the graph.

Having established that Xp telomeres replicate during early S-phase, the next step was to study the correlation between telomere replication and its elongation by telomerase. To address that, upon releasing A549-C6 cells from the second thymidine block, DNA samples were extracted at different intervals throughout the cell cycle. The length of the Xp telomere in each interval was measured by STELA (fig. 4-12A). Surprisingly, our results clearly marked an elongation of ~250 nucleotides on the Xp telomere at the very end of S or early G2 phase, despite the fact that the Xp telomere mostly replicated during early S phase (fig. 4-12B). This clearly demonstrates that mammalian telomerase action is independent of telomere replication and telomerase acts at the end of S-phase/early G2 phase when all telomeres have replicated.

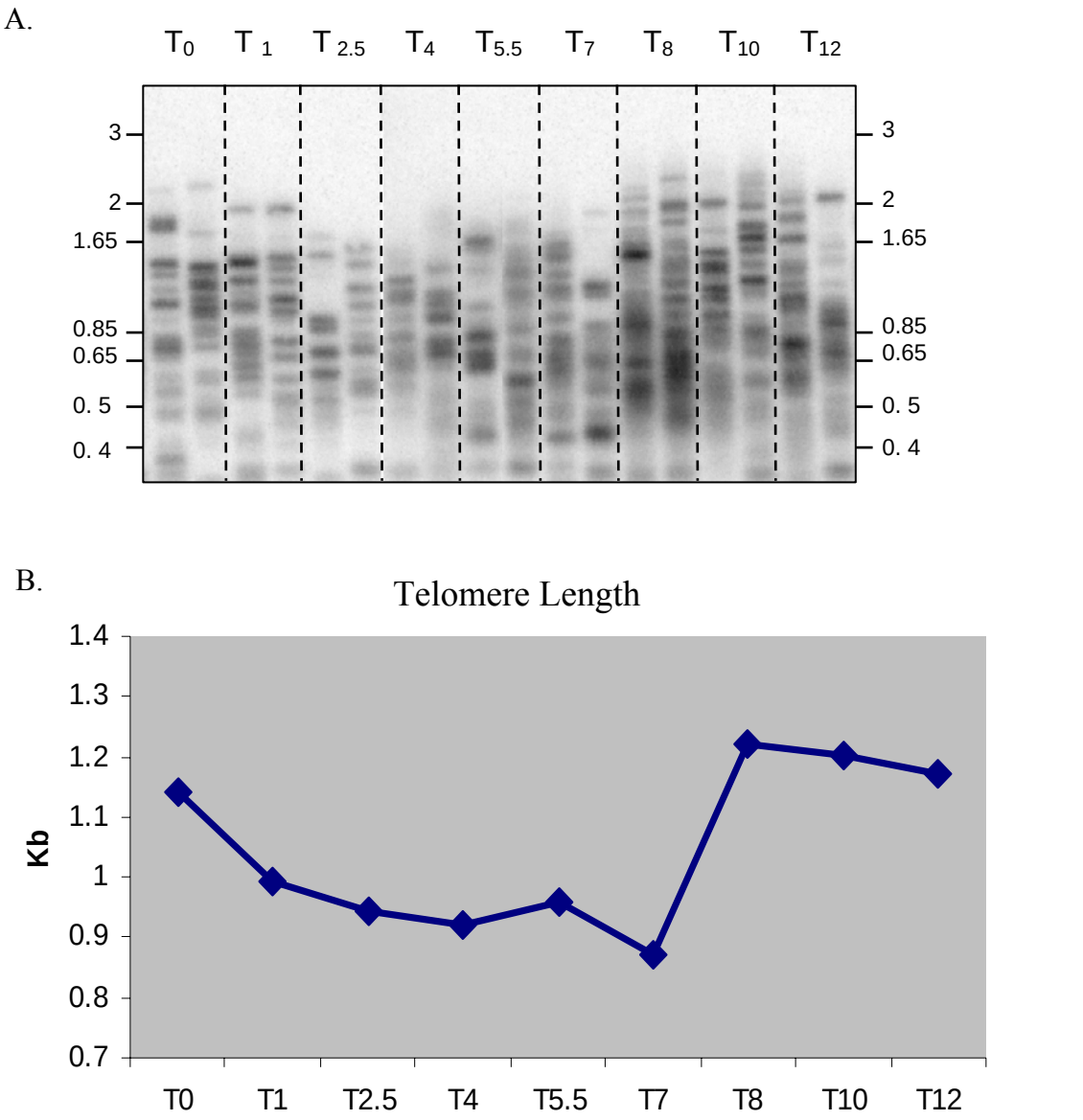

Figure 4.12- Mammalian telomerase action is not coupled to replication. A) Xp telomere length at different intervals during the cell cycle was determined using STELA. A representative graph is shown and the total number of telomeres analyzed was 603 molecules. B) A graph showing the average telomere length for each time point. This demonstrates that the 250-nucleotide extension is added by telomerase (adenoviral expressed hTERT) 8 hours after releasing the cells from the second thymidine block.

DISCUSSION AND FUTURE DIRECTIONS

Telomerase recruitment to the telomere is an area of telomerase regulation that is not fully understood. In this chapter we developed a system to study the dynamics of this recruitment and identify its key regulators. We were able to show that endogenous telomerase adds an average of 50 nucleotides per division, while overexpressed hTERT adds an average of 250 nucleotides. The pattern of size increase we observed when hTERT was overexpressed can best be explained by an average addition of about 250 nucleotides; however our results do not yet exclude other possibilities. The broadness of Xp telomere prohibited us from determining how the 250 nt extension was distributed among different telomeres. We could not determine whether all telomeres were extended in a uniform fashion or whether the short telomeres were preferentially elongated. With endogenous level of hTERT giving a 50 nt average increase it was similarly impossible to draw statistically significant models to explain the mode of extension. We will create a " homogeneously short telomere" by a chromosome-healing event (explained further in future directions section) in order to address this question.

One important finding was the fact that telomere elongation in mammalian cells was not coupled to replication. The significance of those findings and the key factors responsible for this tight temporal regulation are yet to be determined. Recent studies suggest that leading and lagging telomeric strands might be generated differently. Furthermore, the overhang length of lagging telomeres are significantly longer than that of

leading telomeres, and this difference is greatly reduced upon the expression of telomerase (Chai et al., 2006a). Are they recognized differently by telomerase? So far, there have been no reports as to whether telomerase act differently on lagging vs. leading telomeres. Using our cesium chloride system, one can separate leading from lagging telomeres that have replicated for one doubling in the presence of telomerase. Their elongation within that doubling will be monitored by STELA to determine if they both are equally lengthened by telomerase or ifone strand is preferentially elongated by 250nt.

Chromosome healing event to create a "short telomere"

In order to analyze the dynamics of telomere lengthening by telomerase with accuracy, one has to generate short telomeres with a very tight distribution. This could be achieved by seeding a telomere that could be manipulated to acquire a certain length. The strategy involves designing two plasmids that contain an I-Sce1 restriction site flanked by telomeric repeats (fig. 4-13). The length of the telomeric fragment preceding the I-Sce site would be 0.8 Kb or 1.6 Kb. A CMV-driven reporter gene (dsRED) and a drug selection gene (blasticidine) are placed internal to the telomere repeats. To carry out a chromosome-seeding event we would apply the method provided by Hanish et al., 1994, (Hanish et al., 1994) in which the plasmids containing the telomeric repeats will be linearlized and transfected into cells. When the plasmid gets integrated, it often leads to the truncation of a chromosome to create a newly seeded telomere in which the telomeric repeats on the plasmids get elongated by telomerase (Barnett et al., 1993; Sprung et al.,

1999). Clones could be isolated and characterized by *in situ* hybridization to determine the exact position of the seeded telomeres. As such, two cell clones will be considered, one containing the 0.8 Kb-telomere integrated plasmid and the other with the 1.6 Kb-telomere plasmid. The I-Sce 1 enzyme will then be overexpressed in the two clones and allowed to cut its restriction site that is within the telomere. As a result, an instantly created telomere with exactly 0.8 kb or 1.6 kb telomeres would be generated. One would compare the frequency as well as the extent of elongation of the 0.8 Kb telomere *vs*. the 1.6 Kb telomere and unequivocally monitor the dynamics of telomerase elongation.

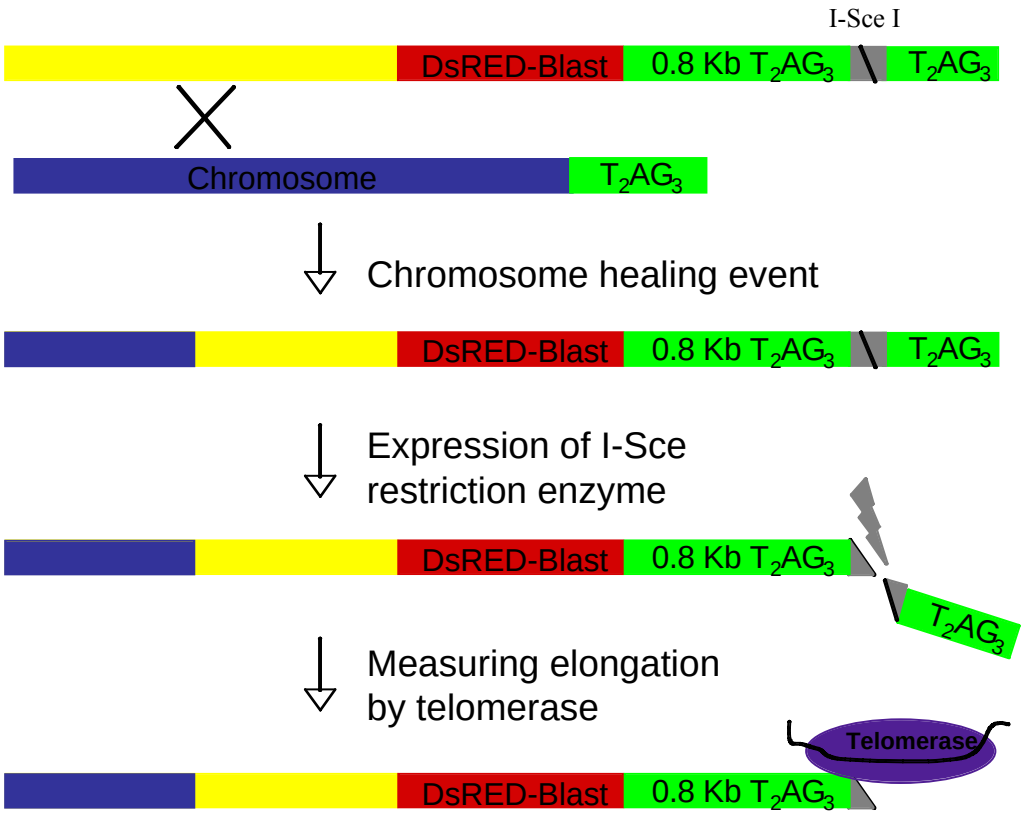

Figure 4.13- Strategy for a controlled chromosome healing event. This schema represents strategy for creating a 0.8 Kb telomere and the same procedure was followed to generate a 1.6 kb telomere. After transfecting the linearized plasmid (containing dsRED, Blast and telomeric repeats) into cells, the plasmid would recombine with an endogenous chromosome, resulting in the formation of a new telomere. I-SceI enzyme is expressed in the cells, allowing it to digest the I-SceI restriction site present in the plasmids following the 0.8 Kb or the 1.6 kb telomeric fragments. This creates a "short telomere" which would be elongated by telomerase.

MATERIALS AND METHODS

Cell culture and cell cloning.

MRC5 cells (ATCCC) and A549 cells were grown in a 4:1 mixture of Dulbecco's modified Eagle's medium: Medium 199 (Life Technologies, Inc) plus 20 % cosmic calf serum (HyClone Laboratories) at 37^{o} C in 5% CO_2/95% air with split ratios of 1:4. To pick individual cell clones, the cells were plated at clonal density (~200-300 cells on a 10 cm dish) and allowed to grow until clones reached a size that was easily visible (~1.5-2 weeks or several hundred cells). Individual clones were then isolated by placing a plastic ring over them (sealed with vacuum grease) and transferred to separate dishes by standard trypsinization methods. MRC5 clones were grown in low oxygen (0.2%) to protect he cells from oxidative damage.

Cell Synchronization and cell cycle analysis

1 million A549-C6 cells were plated in a 10 cm dish and treated with 2 mM thymidine 24 hours later. After 16 hrs, the cells were washed 3 times with pre-warmed wash solution and provided with fresh medium for 10 hours before adding 2 mM final concentration of thymidine. After 14 hrs, cells were again washed three times and provided with fresh medium to be released from the cell cycle block. At different time points, the cells were fixed in cold 70% ethanol overnight. The cells were then spun down by centrifugation and the pellet washed once with 10 ml PBS. After recovering the cells

by centrifugation, the cells were passed through a mesh and incubated with propidium iodide (Sigma) for 30 minutes at room temperature. DNA content was then analyzed using a FacscaliburII flow-cytometer and the Cellquest program.

hTERT adenovirus infection

Cells were infected with 30 moi/cell of hTERT adenovirus 10 hours before the cells were released from the cell cycle block.

Metaphase spread preparation

Cultured cells were treated with 10 μg/ml colcemid (Invitrogen) for 4 hours after which the media was collected form the cells and saved. The cells were trypsinized and resuspended in their corresponding media. Cells were then collected by centrifugation and incubated for 30 min in hypotonic solution (0.075 MKCL) at 37^{o} C. The cells were then fixed overnight with methanol/acetic acid (3:1). The next day cells were washed three times with methanol/acetic acid and finally resuspended in a smaller volume methanol/acetic acid (3:1) before being dropped onto pre-wetted and cold glass slides.

Terminal restriction fragment (TRF) analysis

Average telomere length was evaluated as previously described (Ouellette, 2000). After trypsinizing the cells and collecting the cell pellets by centrifugation, the pellets were suspended in lysis buffer (100 mM NaCl, 100 mM EDTA pH 8.0, and 10 mM Tris PH8.0, 1% Triton X-100 and 2mg/ml proteinase K). Cells were lysed and proteins were digested at 55^{o} C for 12h, then the proteinase K was inactivated at 70^{o} C for 30 min. The

samples were dialyzed overnight against 10 mM Tris, pH 8.0 and 0.1 mM EDTA and when required concentrated with a Centriprep-10 column (Amicon). Subsequently the DNA was digested with six 4 base cutter restriction enzymes (Alu I, Cfo I, Hae III, Hinf I, Msp I, and Rsa I) and run on a 0.7% agarose gel 70 V overnight. The following day, the gel was denatured for 20 min in 0.5 M NaOH and 1.5 M NaCl and dried for 2h at 50^{o}C using a gel dryer. Prior to labeling, the gel was neutralized in 1.5 M NaCl and 0.5 M Tris, pH 8.0. The gel was probed with 5'-^{32}P-labeled (TTAGGG)4 telomeric probe at 42 ^{o}C at least for 5 hrs. After that, the gel was washed once with 2 x SSC (1 X = 0.15 M sodium chloride and 0.015 M sodium citrate, PH 7.0) for 15 min at room temperature and twice (10 min each time) in 0.1x SSC/ 0.1% SDS, the gel was exposed to a PhosphoImager screen and scanned using a Storm 860 PhosphorImager (Molecular Dynamics/Amersham).

Measurement of telomerase activity

Telomerase activity was measured with the telomere repeat amplification protocol (TRAP) by using the TRAPeze kit (Intergen) with some modifications (Holt, 1996). The PCR cycle consisted of 3 cycles; (94^{o}C for 30s, 52^{o}C for 30s, 72^{o}C for 30s) repeated 29 times and using a Cy5-labeled TS primer (Integrated DNA Technologies, Inc.). The products are run on a 10% polyacrylamide gel. The gel is scanner on a STORM 860 scanner system (Molecular Dynamics) using a 650-nm filter. Telomerase activity is quantitated by calculating the ratio of the sum intensity of telomerase ladder to the intensity of the 36-bp internal standard.

STELA:

Multiple ligation reaction were performed with C-telorette #3 (5'-TGCTCCGTGCATCTGGCATCCCTAACC-3') , whereby 10 ng EcoRI-digested DNA was incubated in 10µl reaction (1X ligase buffer, 0.5 U T4 ligase, 10^{-3} µM of telorette #3), at 35^0C for 12 hrs. Multiple amplification reactions were performed (26 cycles, of 95^0C for 15 sec, 58^0C for 20 sec and 72^0C for 10 min) using 1U of Fail Safe enzyme mix (Epicenter), 12.5 µl Fail Safe buffer H (2X, provided by manufacturer) and 0.1µM primers (E2 forward primers that are chromosomes specific and Teltail reverse primer) in a final volume of 25µL, containing 200 pg/µL DNA. The amplification products were resolved on a agarose gel (0.9 % gel for measuring telomeres that are less that are less than 2 Kb in length and 0.5% for all other telomere length analysis) denatured, transferred onto a positively charged nylon membrane (Zeta probe; Bio-Rad), fixed with UV and hybridized with a subtelomeric probe (generated by PCR using E2 and B2 and labeled by random priming). The membrane was exposed to a Phosphor Imager screen, and scanned.

Red-FISH technique:

The ReDFISH technique followed which was described by Zou et al., and based on the CO-FISH technique that was developed by Bailey and Cornforth (Bailey et al., 2001; Goodwin and Meyne, 1993). Metaphase spreads from cells growing in BrdU/dC were prepared and dropped on glass slides. The slides were treated with ribonuclease A (0.5 mg/ml, Roche) for 10 min at 37°C, stained with Hoechst 33258 (0.5 µg/ml; Sigma)

for 15 min at room temperature, then mounted with McIlvaine's buffer at pH 8.0, and exposed to 365-nm ultraviolet light (Stratalinker 1800 UV irradiator). Following that, the slides were treated with Exonuclease III (3U/ul; Promega) for 10 min at room temperature. The C-rich strands (templates for leading strand synthesis) were stained by hybridizing them to Cy3- conjugated $(TTAGGG)_3$ 2'-deoxyoligonucleotide $N_{3'}$-$P_{5'}$ phosphoramidate probe (Egli and Gryaznov, 2000; Herbert et al., 2002) in 70% formamide, and 5% $MgCl_2$ in 10 mM Tris, pH 7.2 for 2 hours at room temperature. Then, the slides were washed twice with 70% formamide, 0.1% bovine serum albumin, 10 mM Tris, pH 7.2 (7 minutes each) and washed once with 0.15 M NaCl, 0.05% Tween 20, 0.05 M Tris (10 minutes). The slides were dehydrated through an ethanol series (70%, 85%, and 100%), and air-dried. Subsequently, the G-rich strands were hybridized by incubating them with 3'-FITC-conjugated $(CCCTAA)_3$ 2'-deoxyoligonucleotide $N_{3'}$-$P_{5'}$ phosphoramidate probe (same hybridization buffer as above) and a centromeric probe for the X chromosome (PNA probe a gift from Aviv A) for 2 hr at room temperature. Upon washing the slides and airdrying them, the chromosomes were counterstained with DAPI (Vector Laboratories). The slides were digitally imaged on a Zeiss Axioplan 2 microscope (63X; 1.4 NA; Plan-Apochromatic oil immersion objective) with precision Cy3/FITC/DAPI Cell bandpass filter sets. Cy3, FITC, and DAPI images were captured separately with a CCD (Hamamatsu) camera and merged using Openlab software.

CHAPTER FIVE

Discussion

Aging in model organisms such as nematodes (Kenyon, 2005), flies (Helfand and Rogina, 2003) and yeast (Bitterman et al., 2003; Kenyon, 2005) is strongly influence by genetics. For example, gene mutations that extend organismal lifespan have been reported. Mammalian aging is more complex and involves many inputs including the accumulation of reactive oxygen species (and their harmful effect on DNA, protein and lipid), protein cross-linking, immune system failure, mitochondrial dysfunction, and cellular senescence driven by short telomeres and DNA repair (Holliday, 1998; Wright and Shay, 2002). The interplay of all of these processes will lead to the decline in organismal function. The theory that telomere/telomerase is one important aspect of human longevity is controversial and the only way to ascertain the validity of this hypothesis would be to show that in elderly people telomere shortening hinders proliferation of certain cells whose expansion is absolutely needed to maintain physiological function. While the telomere hypothesis of aging still awaits direct evidence, the theory that telomeres constitute the mitotic clock of cells and are the major determinant of in vitro cellular aging is well established (Allsopp et al., 1995; Allsopp and Harley, 1995; Allsopp et al., 1992; Harley et al., 1990). Furthermore, the effect of inherited short telomeres on age related diseases such as Dyskeratosis Congenita is evident (Mitchell et al., 1999) and one very important disease of aging is cancer. Almost

universally telomerase mediated telomere maintenance is a required step for the formation of cancer (Kim et al., 1994).

Telomere metabolism is an intricate balance between telomere shortening and telomere elongation. The coordination between these two processes keeps the average telomere length within a given cell type in a state of equilibrium and this is fundamental for chromosome maintenance and stability. By tipping that equilibrium state, one can modify telomere function and accordingly alter the behavior of cells. In the previous chapters, I presented studies related to both telomere-shortening and telomere-elongation. Telomere shortening was tackled by deciphering the mechanism underlying telomere end processing, a key step that impinges upon shortening rates. In contrast, telomere elongation was studied by analyzing the dynamics of telomerase action. The following discussion will underline the significance of those studies on telomere biology and its link to disease. Furthermore, areas that require further investigation or consideration will be presented.

In normal cells that lack telomerase activity, telomere dynamics rely only on telomere shortening that eventually forces the cells into replicative aging. Shortening is mainly due to the end-replication problem and end processing (nuclease degradation) of the telomere ends. Altering end processing in such a way to reduce telomere-shortening rates in normal cells might delay replicative senescence.

In tumor cells, telomere shortening is counteracted mainly by telomerase-driven telomere elongation. Since telomerase is the obligate means for cancer cells to grow indefinitely, it constitutes a very promising target for cancer therapy. Hence,

understanding the mechanistic details of its action is the key that would allow telomerase to be further explored in cancer therapy. In fact, most current telomerase-directed cancer therapies are based on inhibiting the enzymatic activity of telomerase and relying on telomere shortening to drive tumor cell death (Shay and Wright, 2002). Based on the inherent telomere length of the tumor cells, it might take up to 60 divisions before the telomeres get too short and block the proliferative capacity of the tumor. Accordingly, one would like to decrease this lag phase and limit the number of population doublings cancerous cells undergo before they growth arrest or undergo apoptosis. By increasing overhang processing one could increase the rate of telomere shortening during telomerase inhibitor treatment, thereby dramatically reducing the lifespan of tumor cells. In parallel, understanding the pathway and identifying the key players that control the recruitment of telomerase to the telomere ends provides an alternative way for telomerase-targeted anti-cancer therapy. Blocking the ability of telomerase to elongate the shortest and most critical telomeres within a cancer cell might be a faster and a more efficient way to drive tumor cells into crisis.

FINE-TUNING THE CHROMOSOME END

The result of telomere-end processing is a well-defined terminal structure that is crucial for optimal function. In fact, proper telomere end structures give telomeres their unique identity as ends that do not require repair. Telomere end structure is critical for capping telomeres, forming T-loops, blocking NHEJ, and mediating telomerase-based

elongation. The structure of the end has been carefully analyzed in model organisms and consists of G-rich single stranded overhangs with very precise lengths (Wellinger, Wolf et al. 1993; Wellinger, Wolf et al. 1993; Fan and Price 1997; Jacob, Skopp et al. 2001; Jacob, Kirk et al. 2003) and bounded by unique last bases (Fan and Price, 1997; Jacob et al., 2003; Jacob et al., 2001). The only previously known mammalian overhang characteristic was the fact that they are highly variable with length ranging from 35-300 nucleotides (Chai et al., 2006a; Chai et al., 2005; Cimino-Reale et al., 2001; Huffman et al., 2000; Wright et al., 1997). In order to better understand how the ends were processed and what factors are involved in overhang generation, there was a need to define a very distinctive telomere feature such as the identity of their last bases. Having established that the last base of the C-strand was in fact very precise is an important finding, not just because it allowed us to understand the structure better, but also because it establishes basic information needed to discover factors that regulate the processing mechanism.

Analyzing leading and lagging strands of DNA replication showed that they both ended in the same last base, despite the fact that they possess different overhang lengths (Chai et al., 2006a). This clearly suggests that overhang processing is a mutli-step process, and the last fine-tuning step is to specify the ends. This is further supported by the fact that when certain proteins such Mre11 were targeted overhang length was altered, yet the identity of the last base remained intact. So far, POT1 was the only protein that was linked to last base specificity and thus is responsible for executing at least part of the fine-tuning steps. None of the identified nucleases that were linked to telomeres are in

fact the nuclease (s) that resects the 5'end of the telomere and the question remains, what is identity of this nuclease?

One significant question to be addressed is when are mammalian telomere ends processed to generate proper overhangs. Studies in yeast have shown that long overhangs are generated immediately after replication and are then processed to form the 14 nucleotide overhangs that are present throughout the cell cycle (Dionne and Wellinger, 1996; Wellinger et al., 1993a; Wellinger et al., 1993b). In contrast to yeast that replicate their telomeres in late S phase (Wellinger et al., 1993c), mammalian telomeres replicate throughout S phase (Wright et al., 1999; Zou et al., 2004a). Is their end processing coupled to DNA replication and does it occur independently on each telomere end? If this is correct does it implicate that the DNA replication machinery has a role in telomere-end processing? An alternative model would predict that end-processing occurs in a synchronized fashion following the replication of all telomeres. This implies that the complex involved is independent of replication machinery and most likely based on telomere-associated factors (telomere binding proteins; POT1, TRF1, TRF2, Tin2, TRPP1, RAP1, NHEJ proteins, HR proteins and others). Mammalian telomeres are being recognized as transient double stranded breaks during G2 phase of every cell cycle. They engage the NHEJ pathway whereby NBS1 and Mre11 are actively recruited to the telomere during G2 (Verdun et al., 2005). While the significance of this phenomenon is undetermined, it might be interesting to discover whether this transient DNA damage response is involved in telomere-end processing. Under such circumstances, NHEJ factors do perceive the telomere end as a double stranded DNA break but their function at

the telomere is tweaked by telomere binding factors in such a way as to generate overhangs with defined lengths and precise ends. One approach that would allow the differentiation between these two models and unravel the temporal regulation of end-processing would be to synchronize cells with POT1 knockdown resulting in random C-terminal nucleotides at the G1/S interphase. The cells must also contain an inducible form of POT1 that is resistant to shRNA degradation that is induced before the cells go into S phase. Following the release from the second thymidine block, last base determination assays on the Xp end would be initiated at different time intervals to determine when during the cell cycle the last base of the Xp end is specified to end in ATC-5'. This timing of end-processing could then be correlated to replication timing of the Xp telomere.

What is the identity of the nuclease that resects telomeric C-strands to generate mammalian overhangs? This constitutes one of the key questions that the telomere field has yet to unravel. Identification of this nuclease would improve the molecular understanding of overhang-processing and telomere biology in general. Many groups are trying to characterize the nuclease, yet until the present time it remains a mystery. Pull down experiments with the six telomere binding proteins has failed to uncover any nuclease (s). Most of the known nucleases that affect telomere function have been tested and are not involved in C-strand resection. So it is fair to ask where do we go from here? Our results indicate that the nuclease resection is regulated up to the base-specificity level and that the OB fold of POT1 is the key factor in defining that specificity. This suggests that the OB fold of POT1 must interact at least transiently with the end-nuclease. This could be an exonuclease and POT1 could hinder its ability to resect beyond ATC-5' or an

endonuclease that is recruited by POT1 to clip the C-strand at AATC∧CC. At this point we generated two key pieces of information to direct our future search for the nuclease. For that purpose, a screen for POT1 OB binding proteins or pull down assays with POT1 in the presence of a DNA substrate mimicking the telomere end might be one approach to uncover the nuclease.

A final yet very significant question to consider is what is the significance of having very precise ends? Why do our chromosomes end in AT**C**-5'? Why do mammalian cells favor end processing machinery that is tightly regulated to the base-specificity level? If one had to speculate, having a defined last base would be advantageous for creating the right structure that would help anchor the six member complex of the telomere binding proteins onto the DNA and create the proper DNA-protein interactions that is required for optimal telomere function. One can envision that POT1 and TRF1, which bind independently to the DNA portion of the telomere, can only form a complex when there is appropriate spacing between them. In order to test this idea, one should randomize the last base of the C-strand without altering POT1 function on the telomere and study the effect of such randomization on telomere stability and cell survival. POT1 binds through its OB folds to the G-overhang with great sequence specificity and specifically to 5'- TTAGGGTTAG-3' (Lei et al., 2004; Loayza et al., 2004). It may be informative to test whether certain mutations could be generated such that they alter the specificity with which POT1 binds to the overhang without affecting its binding affinity to DNA. As such, POT1 would still bind to the single stranded overhang and interact with other telomere binding proteins but would randomize the last base.

TELOMERASE REGULATION

Telomerase is the required feature that confers on most tumor cells their ability to divide indefinitely (Kim et al., 1994). Telomerase activation in tumor cells is primarily due to the transcriptional activation of the hTERT gene. However, optimal telomerase function relies on hTERT and hTR transcription, their ability to fold properly and assemble into the telomerase holoenzyme, the efficient recruitment of telomerase to the telomere end and its ability to extend the end (Aisner et al., 2002; Cong et al., 2002). Targeting telomerase for anti-cancer therapies relies on the ability to target its transcription, assembly, enzymatic activity as well as recruitment to the telomere. hTERT transcriptional activation, hTERT-hTR interaction and telomerase assembly have been the subject of careful investigations and we are just starting to understand the mechanisms underlying telomerase recruitment to the telomere. Presently, it is widely accepted that mammalian telomerase is preferentially recruited to the shortest telomeres (Ouellette et al., 2000a; Steinert et al., 2000) yet the mechanism dictating that is unknown. We designed an assay system that would allow us to decipher the mode of recruitment of telomerase to the shortest telomeres in tumor cells and identify its key regulators.

So far, there have been no studies to determine how telomerase acts during one cell division. Does it act immediately after telomere replication or does it act independently? Our results were surprising in that they showed, for the first time that in mammalian cells

telomerase action is uncoupled from telomere replication. While this is an interesting finding it raises many questions as to how telomerase recruitment is temporally regulated especially when its activity is the same throughout the cell cycle. Are the telomere ends capped by telomere binding proteins until all telomeres replicate and then are freed to be extended by telomerase to act? The regulation could also be at the level of telomerase sub-cellular localization; such that it is transported to the telomere immediately after replication of all chromosome DNA is complete. It would be very important to understand the dynamics of telomerase recruitment to the telomeres and to identify the key factors involved in that elegant mechanism. Lastly, knowing that each telomere has its own inherited length, how does telomerase elongate the shortest, yet maintain the telomere length distribution?

Even though this is the "end" of one aspect of these central questions, the telomere "end" still has many hidden secrets. Shedding additional light on these diverse areas of telomere and telomerase regulation will hopefully allow us in the future to use this knowledge for therapeutic purposes.

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VITAE

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