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A Critical Review of Telomerase Biology and Model Systems for the Study of Telomerase

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A Critical Review of Telomerase biology and model systems for the study of Telomerase.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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ABSTRACT

A CRITICAL REVIEW OF TELOMERASE BIOLOGY AND MODEL SYSTEMS FOR THE STUDY OF TELOMERASE

By Jeremy Charles Aisenberg

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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The study of telomere and telomerase biology holds substantial promise in uncovering the molecular process of aging and the treatment of cancers. Studies have shown that telomere shortening is directly linked to cellular aging and that telomerase expression is found in over 85% of human cancers, including 95% of all advanced malignancies. Development of effective model systems to elucidate the molecular mechanisms underlying the role of telomeres and telomerase in the processes aging and cancer is of particular importance. While inbred strains of mice have provided a wealth of information for a variety of pathways and diseases, the mouse model poses a challenge for the study of telomere biology due to their extremely large telomere sizes. The telomerase knockout mouse suffers no adverse effect for at least 6 generations, making studies of telomere shortening-associated pathology in single generations of animal very difficult. For this reason, the identification of new vertebrate models for the study of telomeres and telomerase biology is critical. The chicken, *Gallus gallus*, has been an important model system for the study of embryology and development for decades. The chicken has three classes of telomeres, characterized and differentiated by their size.

Class I ranging from 0.5 to 10 kb in size, Class II ranging between 10 to 40 kb in size, and Class III ranging between 40 kb to 2 Mb in size. The class I and II chicken telomeres are close enough in size to those of humans, which range between 4 and 15 kb, to allow for chicken studies to elucidate information valuable to the understanding of the molecular process in humans. The chicken telomerase genes have been cloned and characterized, providing a foundation for unlimited study of telomere biology in the chicken. Additional models, including many different fish species, will also hold promise as telomere models for aging and cancer. The development of an additional model of telomere biogenesis and telomerase regulation should provide important insights into the molecular processes surrounding both the development of cancer and organismal aging.

Chapter 1

The Telomere and Cellular Aging

Telomeres are defined as the repetitive DNA sequences at the ends of chromosomes and therefore do not contain protein-coding genes. Instead, telomeres have been shown to protect the ends of chromosomes from damage and end-to-end fusions (Sandell and Zakian, 1993). In vertebrates, the telomere is composed of tandem repeats of the sequence TTAGGG (Blackburn, 1999). The telomeres play a critical role in the localization of chromosomes within the nucleus as well as proper pairing and alignment during meiosis. Most notably, telomeres appear to play a central role in cellular aging, senescence, and cell cycle control.

The cell cycle has been the focus of intense research for more than 50 years. The control of timing of cellular events including signaling, development, division and other crucial pathways are directly influenced by the cell cycle. The eukaryotic cell cycle (Figure 1) includes an M phase, G1 phase, S phase, and G2 phase. Interphase includes G1, S and G2 phases, while M phase represents Mitosis, during which time the cell evenly distributes chromosomes into daughter cells. The S phase is the synthesizing phase during which the DNA content of the cell is duplicated. The G phases represent the gaps between the end of M phase and S phase (G1) and the end of S phase and the beginning of M phase (G2). In addition, many cell types enter the G0 phase, or resting phase, also known as quiescence, following mitosis. Cells can survive in quiescence for a significant amount of time, from days to weeks to months.

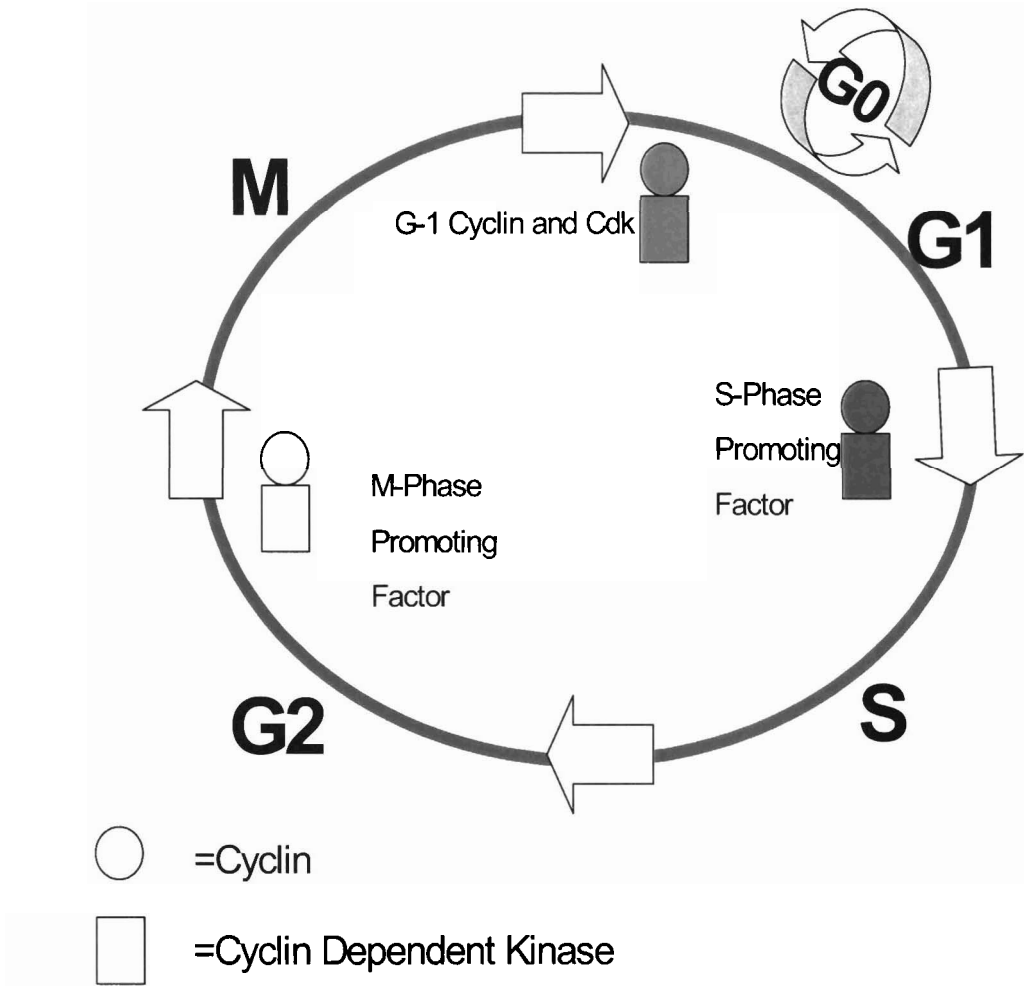


Figure 1. The Cell Cycle. The cell cycle, including G1, S, G2, and M phase as well as the growth arrest phase, G0, is represented as a clock and thought to be loosely based on a 24-hour period for many cell types. The cyclin and cyclin dependent kinase factors that drive transitions between these phases are also illustrated.

Normal eukaryotic cells were shown by Hayflick to have a finite number of population doublings before they reach a stage known as replicative senescence (Reviewed by Wright and Shay, 1992). Hayflick found that cells in culture will continue to replicate for a given number of population doublings before growth slows and eventually reaches the "Hayflick Limit" where no further cell division is observed. This cessation of cell growth was later termed cellular senescence. This phenomena is not observed in immortal cells such as germ cells, embryonic stem cells, and cell lines derived from tumor cells as telomere maintenance is achieved through the expression and activity of the telomerase protein, described in significant detail hereunder.

Senescence is marked by growth arrest during the G1 phase of the cell cycle, is irreversible, and is associated with an accumulation of cell cycle regulators p53, p21, and p16 (Reviewed in Bringold and Serrano, 2000). These cells are in an irreversible arrest phase whereby they are unable to undergo further cell divisions. Senescent cells can continue metabolic activities and are therefore not considered dead. Senescent cells in culture generally have a larger appearance, and populations of senescent cells generally have a heterogeneous size distribution. In culture, senescent cells are identifiable after staining for β-galactosidase at pH 6, which was a fortuitous observation and one of the clearest molecular and/or histochemical markers of cellular senescence (Dimri et al., 1995). Telomere length and shortening in successive replications appears to play a pivotal role in triggering the onset of cellular senescence.

Human telomeres range from 4 to 15 kilobases in length. There is generally a 100-150 base 3' overhang of single stranded TTAGGG repeats. The single stranded overhang has been shown to establish a tertiary structure known as T-loops, with associated proteins, which shall be discussed in further detail later (Griffith et al., 1999). T-loops have been identified in numerous organisms other including humans, and these T-loops help form protective end structures at the end of chromosomes to prevent chromosome ends from being recognized as damaged DNA. One theory for telomere-induced senescence is that erosion of telomeres, hence shortening, causes a conformational problem with the formation of the T-loops, thereby preventing the loop from forming and allowing telomeres to be bound by DNA repair enzymes.

Telomeres are believed to serve several critical functions. By capping the chromosome ends, they protect critical gene coding regions from loss due to the end replication problem described below. In addition, the telomeres protect the free ends of the chromosomes from being recognized as damaged DNA and the loss of the telomere is believed to trigger a DNA damage pathway leading to cell cycle arrest or apoptosis. Telomeric structures help cells to identify the chromosome ends. Without these complex protein/DNA structures, cells would not be able to differentiate from interstitial double stranded chromosomal breaks. When telomeres shorten or are uncapped, chromosome ends can be subject to degradation, recombination, or end-to-end fusions through cellular DNA repair systems (Chan and Blackburn, 2003).

When telomeres fuse, dicentric chromosomes are created. These abnormal structures are not correctly sorted during meiosis and lead to a variety of aberrant outcomes generally causing genomic rearrangements in the surviving cells (Gisselsson, 2003). Dysfunctional chromosome ends and genomic instability, either through DNA loss or protein dysfunction, can lead to a premature decrease in telomere length, which in turn can lead to early senescence. Conversely, dysfunctional telomeric elongation can lead to a delayed senescent response and a variety of aberrant outcomes. The mechanisms for such cellular activities are discussed below. The absence of functional telomeres clearly leads to genomic changes, which can lead to cancer and aging as discussed below (Gray and Collins, 2000).

Telomeres shorten in human somatic cells due to the end replication problem (Figure 2). While human telomeres range in size from 4 to 15 kb, gradual loss of anywhere from 50-200 base pairs after every cell division, takes place due to this problem. It has been shown that various cell types can lose from 20 bases (fibroblasts) up to 200 bases (epithelial cells). The end replication problem arises due to the absence of a primer for the lagging strand of DNA. During cell division, DNA dependent DNA polymerase can synthesize DNA on the leading strand to the most terminal point of the chromosome on the leading strand in a 5' to 3' direction. Because replication must proceed 5' to 3', the lagging strand is discontinuous in that as the replication fork opens, priming takes place followed by replication to the next RNA primer. As replication continues and RNA primers are replaced with DNA, the lagging strand is made up of Okazaki fragments that are eventually ligated together. However, at the very end of the linear chromosome,

there is either: 1) not enough DNA for RNA priming to take place, which leaves an unreplicated end piece, or 2) the priming event does occur at the very end, and when the RNA is removed, there is no Okazaki fragment to attach to, leaving an unreplicated fragment. It is likely that both mechanisms contribute to this end replication problem and

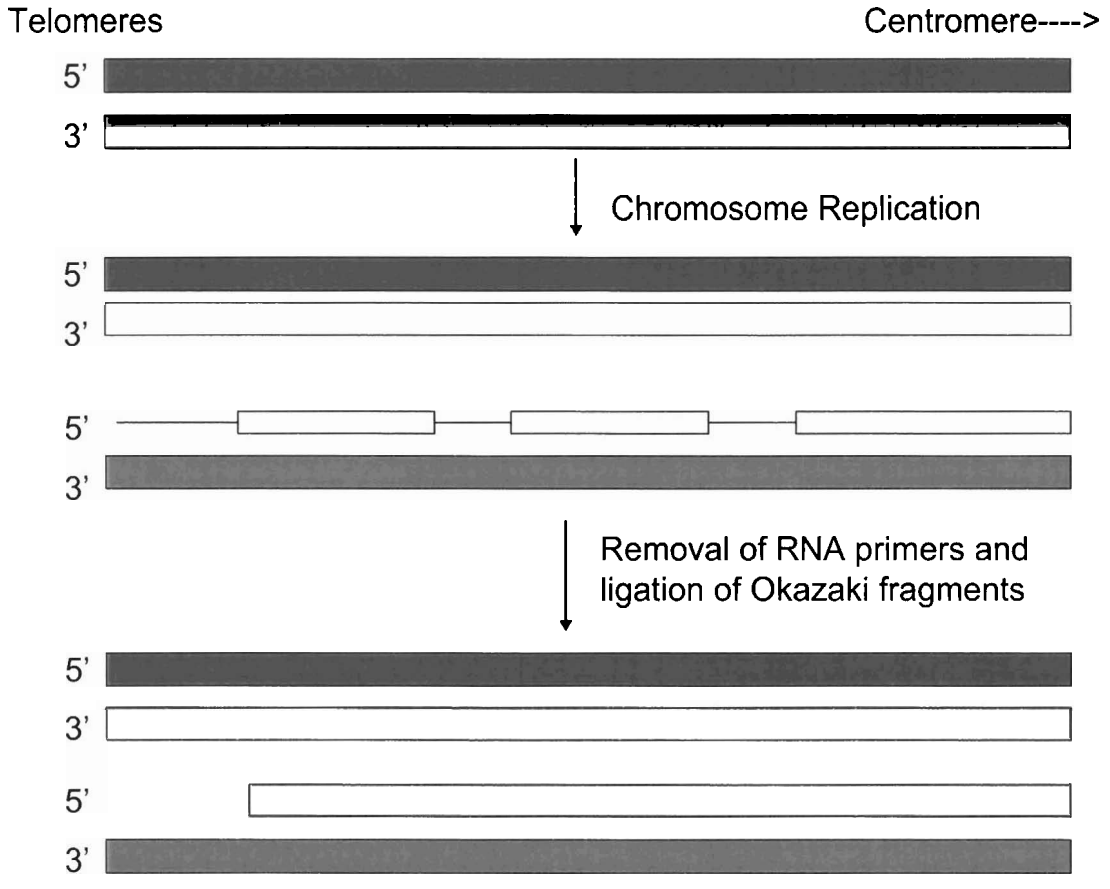


Figure 2. End Replication Problem. The end replication problem arises during cell division when the lagging strand, which uses Okazaki fragments as part of the replication process, does not have a site available to prime DNA synthesis at the terminal end of the lagging strand. Through repeated replication and cell divisions, telomeres become progressively shorter, which is a key mechanism in the onset of cellular senescence or apoptotic pathways.

telomere shortening, all of which was first proposed by a Russian scientist, Alexy Olovnikov (1973). Through repeated replication and cell divisions, telomeres become progressively shorter. This shortening of telomeres is likely the trigger to cellular senescence or apoptotic pathways.

Some cell types are not affected by the end replication problem. These include germ cells, stem cells, and tumor cells. In these cell types, telomerase has been found to be expressed and active. In a small proportion of cancers, telomere lengths are maintained or even lengthened in the absence of telomerase activation (discussed in more detail in Chapter 4). This pathway is referred to as the ALT pathway (alternative lengthening of telomeres) and appears to occur by a recombination process similar to processes seen in budding yeast lacking telomerase (Lundblad and Blackburn, 1993). This is a relatively minor pathway and is not the focus of the current thesis on telomeres and telomerase, although will be discussed in some detail below.

Chapter 2

Cellular Immortalization

Regulation of the cell cycle controls cellular proliferation through a variety of complex mechanisms, many of which are still not well understood. The control of cellular initiation of M phase is well studied. Mitosis Promoting Factor (MPF) is one transacting factor that promotes cellular entry into M phase. MPF consists of two subunits, a cyclin dependent kinase and a mitotic cyclin protein.

Cells are continuously exposed to a variety of stresses that affect function and proliferation. Changes that cause genetic mutations are deleterious to the cell's fate as when these changes are passed to subsequent generations of daughters, a number of various mutant phenotypes can occur, including aberrant and uncontrolled cell division leading to human cancers. Cells have evolved a series of complex mechanisms to monitor, prevent, or eliminate cells that develop such changes.

The transcription factor and tumor suppressor gene p53, identified initially as being essential in identifying DNA damage, has subsequently been found to have a broader role after cellular stresses (Reviewed in Yee and Vousden, 2006). The p53 protein functions as a tetrameric protein. It is found constitutively active at low levels in normal cells. Many different forms of cellular stress causes activation of a variety of pathways, which can cause post-translational changes to p53, affecting its function and increasing

its stability. Increased p53 stability leads to transcriptional activation of cell cycle inhibitory genes and genes involved in apoptosis, such as p21 and GADD45. Genes activated or repressed by p53 expression lead to outcomes such as stimulation of DNA repair, transient or permanent cell cycle arrest, and apoptosis.

The retinoblastoma protein, pRB, is another critical protein involved in the regulation of cell cycle control. Its level of phosphorylation determines whether or not a cell will continue normally through the cell cycle or enter into one of several pathways. High levels of phosphorylation of pRB allow the cell cycle to proceed, while low phosphorylation levels prevent cell cycle progression. Hypophosphorylated, or under phosphorylated, pRB is believed to inactivate the E2F transcription factors, while hyperphosphorylation of pRB causes release of E2F and promotion of cell growth. The E2F family of transcription factors controls a series of genes necessary for transition into the S phase of the cell cycle.

The phosphorylation level of pRB is dependent upon a variety of cyclin dependent kinases (CDK's). A family of proteins, known as cyclin dependent kinase inhibitors (CDKI's) has been shown to interrupt the activity of CDK's. Disruption of CDK activity during G1 of the cell cycle interferes with the phosphorylation of pRB, which leads to the prevention of progression of the cell cycle. One such CDKI is p16, which acts on CDK4 and CDK6. Previous studies show that introduction of p16 induces replicative senescence in fibroblasts and immortal cells without p53 activity (McConnell et al., 1998). Further, pRB is required for growth suppression driven by p16 expression. In

certain cell types, including tumor-derived cells, p16 can be inactivated by methylation of its promoter, which renders the pRB pathway inactive as it is continuously phosphorylated.

pRB phosphorylation can also be inhibited by p21, another cyclin dependent kinase inhibitor, which has also been shown to be induced during senescence. Several groups have found that p21 levels increase prior to p16 levels, leading to the conclusion that p21 possibly triggers senescence prior to p16 (Dulic et al., 2000). It has also been shown that p16 levels remain high over time in senescent cells, while p21 levels decrease after a short period of time (Stein and Dulic, 1998). Even so, p21 appears necessary for the induction of cellular senescence, while p16 may be required to maintain the senescent phenotype.

Taken together, p53 appears to recognize and be activated by DNA damage, including shortened telomeres (depicted in Figure 3). Following this activation, p53 is able to activate the cyclin dependent kinase inhibitors p16 and p21, which then inhibit cdk2, cdk4, and ckd6. This inhibition of kinase function ultimately prevents the phosphorylation of pRB, which is then unable to activate the E2F transcription factors, leading to either a pause in the progression of the cell cycle (quiescence) or a complete cessation of growth (senescence). While senescence experimentally may be reversible, it is generally thought that senescence, whether in tissue culture or in an organism, is an irreversible process that is distinct from cell death pathways, including apoptosis.

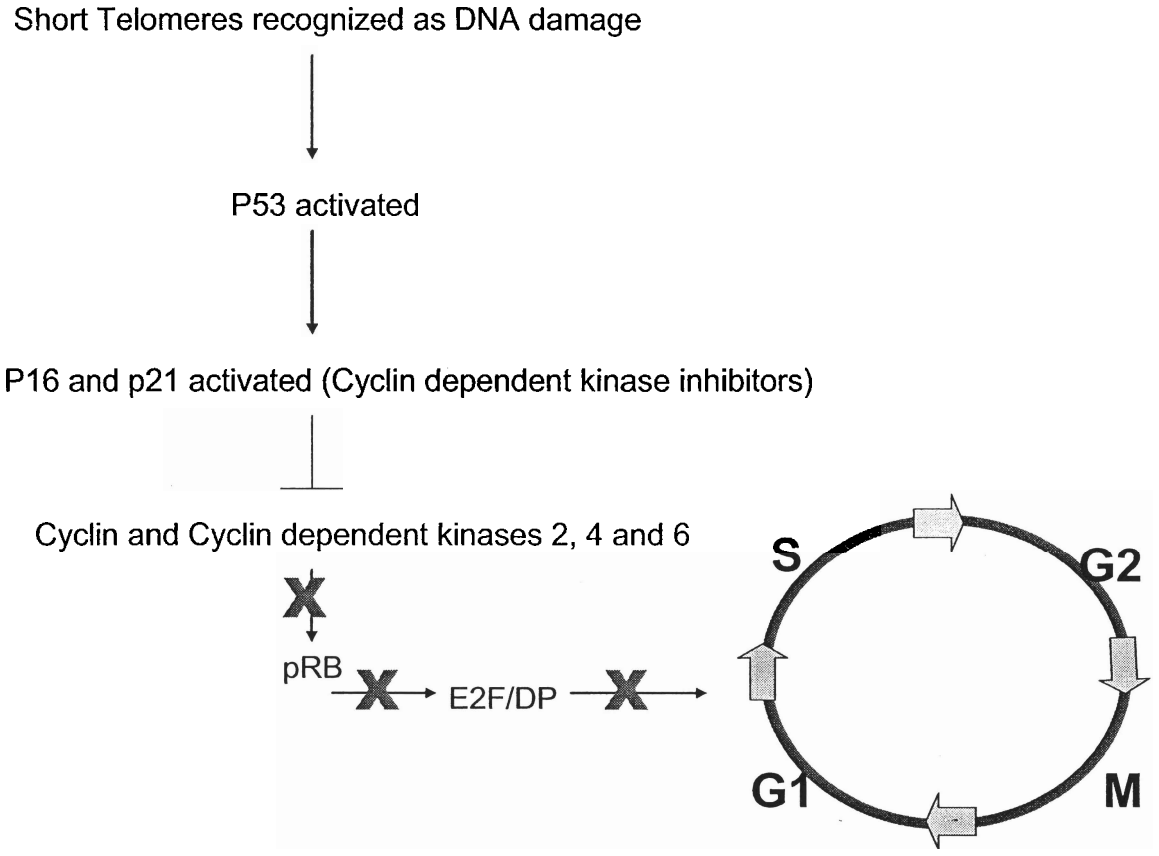


Figure 3. Shortened Telomere Response. Shortened telomeres may be recognized as DNA damage, activating p53, which either directly or indirectly activates the p16 and p21 CDK inhibitors. These CDKI's block cyclins and cyclin dependent kinases, which are then unable to phosphorylate pRB, thereby blocking E2F/DP's function in promoting the transition from G1 to S phase of the cell cycle.

The process of cellular immortalization has been the subject of intense study in elucidating insight into the development of neoplasia. Cell lines have been transfected or infected with viral oncogenes and studied for their ability to bypass or circumnavigate the senescence phenotype. These cells generally live longer, have an extended lifespan, and eventually enter a stage referred to as crisis. Cells that enter the crisis stage following rapid proliferation are generally marked by genomic instability, aneuploidy, and significant telomere shortening or dysfunction. During this crisis stage, some cells may enter apoptosis, and in fact, the vast majority of the cells undergo some sort of cell death pathway (Reviewed by Holt and Shay, 1999). The rare cell or cells that escape crisis are considered immortal and generally have activated some type of telomere maintenance pathway, which in the vast majority of cells, is the reactivation of telomerase enzyme to maintain telomere structure and function (Reviewed by Holt and Shay, 1999). Several groups have demonstrated that telomere maintenance is a critical step in immortalization and cancer development. These immortal cells also generally are found to express one or more oncogenes, as well as require the continuous inactivion of tumor suppressor genes such as p53 and pRB (Wright and Shay, 1992).

Chapter 3

Telomere Model Systems

A number of eukaryotic model systems have been used for the study of telomere function, aging and senescence. These models systems include tissue culture, such as human primary fibroblasts and epithelial cells, complex vertebrate models, and single celled organisms such as yeast.

The telomeres of most organisms' chromosomes consist of short asymmetric repeated sequences with lengths varying greatly across different species. Vertebrates tend to share the common hexameric telomeric sequence, TTAGGG, while other organisms have different telomeric repeats as shown in Figure 4. Humans have as many as thousands of TTAGGG repeats per telomere, while holotrichous ciliates such as Tetrahymena have approximately 50 repeats per telomere. Arabidopsis (plants) telomeres have less than 350 repeats, and *Saccharomyces cerevisiae* telomeres have repeats that stretch between 300 to 500 base pairs in length (Lundblad and Blackburn, 19993). *Drosophila melanogaster* chromosomes have transposable elements at the end of one of its chromosomes, making the study of the regulation of repeated telomere-like elements, especially related to aging, difficult in this organism, not to mention the fact that fruit fly and worm (*Caenorhabditis elegans*) cells are generally postmitotic in the adult organism (Holt and Shay, 1999; McChesney et al., 2005).

Gallus gallus (chicken)	TTAGGG
Tetrahymena, Paramecium (ciliates)	TTGGGG
Oxytrichia, Euplotes (ciliates)	TTTTGGGG
Trypanosoma, Leishmania (parasites)	TAGGG
Physarum (fungus)	TAGGG
Saccharomyces cerevisiae (yeast)	$TG_{1-6}TG_{2-3}$
Arabidopsis (plants)	TTAGGG
Danio rerio, Orizyas latipes (fish)	TTAGGG
Homo sapiens (humans)	TTAGGG
Caenorhabditis elegans (worms)	TTAGGG
Drosophila melanogaster (fruit fly)	transposable element
Kluyveromyces lactis (yeast strain)	ACGGATTTGATTAGGTATGTGGTGT

Figure 4. A comparison of Telomere sequences. Varying telomere sequences among different species, illustrating significant conservation of telomere species among very divergent organisms. Note that all vertebrates have the same hexmeric repeated sequence: TTAGGG.

Chicken telomeres, on the other hand, are of the TTAGGG variety, and they can be classified in three classes, with repeat lengths varying greatly between classes. Many studies comparing human and mouse telomeres have been performed. Mouse telomeres are tens of kilobases long; whereas humans have been shown to be much shorter, in the 10-20 kilobase pair range. A mouse line deleted for telomerase can survive for nearly 6 generations without exhibiting any serious genetic or phenotypic problems. Failures in meiosis and other problems emerge in the 6th generation when telomeres have gotten very short, and the major issue is that the mice are sterile (Blasco et al., 1997; Lee et al., 1998).

In Tetrahymena, the sequence is TTGGGG. Tetrahymena telomeres have approximately 50 repeats of this sequence compared to the many thousands of repeats in humans and other organisms (Reviewed by Blackburn, 1999). In Sacchaormyces, it is a more variable $TG_{1\text{-}3}$ repeat and a much smaller length (300-400 basepairs), nearly 200-fold less than inbred strains of mice and 50-fold less than humans.

Yeast cells, deleted for telomerase, slowly lose telomere sequences at a rate of about 10 base pairs per generation. After 50 generations, yeast cells begin to die as their telomeres become too short and cells begin to senesce (Lundblad and Blackburn, 1993). Another yeast, *Kluyveromyces lactis*, has a long, 25 basepair repeat sequence (Blackburn, 1999). Drosophila does not have repetitive telomeric elements, but instead their chromosome ends typically have a transposable element, such as HeT-A, at the ends of their chromosome. HeT-A serves as buffer between the chromosome ends and

active euchromatic regions, but does not provide a similar mechanistic model for studying telomere structure and function that would translate to eukaryotic systems. Yeast cells, when lacking telomerase, are able to survive by a similar strategy to the Drosophila system of retrotransposons: the addition of Y' elements (which may once have been transposable elements) to chromosome ends via homologous recombination events.

The telomeres of most mammals contain anywhere from less than 1 to over 50 kilobase pairs of telomeric DNA. Human telomeres, as stated above, range between 4 and 20 kilobase pairs, depending on the cell of origin. Telomeres from germline cells are usually 10-20 kilobase pairs, but telomeres are found to be shorter in somatic cells because of the inability of somatic cells to maintain telomere lengths (end replication problem). Germ- and stem cells have a telomere maintenance mechanism: telomerase, which will be discussed in the next chapter (Chapter 4). Interestingly, many forms of cancer exhibit cells with significantly shorter telomeres, and the reason for this is the significant cell divisions during the extended lifespan phase. Somatic cells lacking telomerase continue to shorten their telomeres while progressing past the senescence state and to the crisis stage, and in some cases, chromosomes will have severely shortened or undetectable telomere lengths. It is these cells that immortalize and stabilize their telomere lengths using telomerase. Importantly, there is not a rapid and significant elongation of telomere lengths but rather a stabilization of the length, which is the reason that most human cancers have shorter telomeres than surrounding normal cells (Reviewed in Holt and Shay, 1999).

Inbred strains of laboratory mice have telomeres that are quite often more than 50 kilobase pairs long, are heterogeneous in size, and exhibit differences from cell to cell and from strain to strain (Reviewed by Rodier et al., 2005). Two different species of mice, the inbred strain of *Mus musculus* and the outbred or wild strain of *Mus spretus*, have as much as a three-fold difference in telomere length (Rodier et al., 2005). Even with such significant differences in size, the lifespans of these two species of mouse are comparable, which suggests that telomere length, especially in the laboratory inbred strains, does not significantly contribute to aging in the mouse. Because of these long telomeres and their inability to relate to the mouse lifespan, inbred mice make a poor model for the study of telomere shortening as it relates to the aging process.

The study of telomere structure has revealed a variety of interesting findings. Human and mouse telomeres both "exhibit chromatin structures with arrays of nucleosomes that have a shorter repeat size than standard heterochromatic nucleosomes" (Tommerup et al., 2004). This observation would further strengthen the hypothesis that genes near the telomeres are subject to transcriptional silencing from position effects and progressive telomere shortening. Telomere specific proteins are also found associated with chromosome ends and are discussed in more detail below. The structure of chromatin at telomeres is unusual. There are several telomere sequence-specific telomere binding proteins (in mammals, TRF1 and TRF2, among others which are further discussed below) that help protect the DNA ends from degradation and presumably contribute to the specific heterochromatin associated with the telomere.

Specific understanding of the interaction between the telomeric DNA, bound proteins and tumor suppressor genes (hence, telomere structure) is interesting for many reasons. Telomeres have a 3', G-rich overhang, which has been shown to be critically important to formation of structures called T-loops. These T-loops are characterized by insertion of the G-rich overhang into the double stranded telomere structure (Figure 5), although it is not completely understood how the T-loop forms. It is hypothesized that the 3' overhang binds to the complementary C-rich strand at the point of displacement. The telomere binding protein TRF2 (telomere repeat binding factor 2) has been shown to modulate the formation of the T-loop structure, while the POT1 protein helps to maintain the structure and also to protect the 3' overhang from being degraded (Figure 5). There is substantial evidence that many organisms form T-loops to protect the exposed 3' end of the chromosome. The 'T-loop' complex is believed to loop back on the double stranded telomeric DNA, which was initially observed with electron microscopy by Griffith et al. (1999). The looping back of the single stranded overhang possibly explains how the single stranded sequence is not recognized by DNA damage sensory proteins and subjected to repair machinery. Another possible explanation for the lack of DNA repair in response to single stranded DNA ends is the association of the other telomere binding proteins, such as POT1 (protection of telomeres 1). In either case, it is believed that the associated proteins are vital to this protection, either facilitating the formation of the t-loop of simply protecting the single stranded DNA.

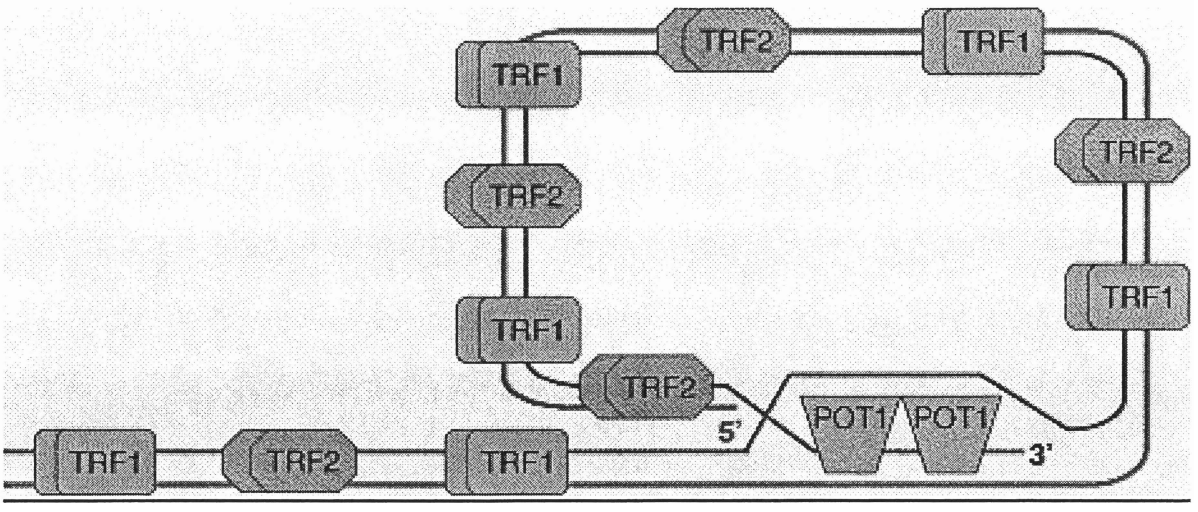


Figure 5. Hypothesized telomere associated protein binding and T-loop structure. (From Rodier et al., 2005). It is hypothesized that the telomere ends are stabilized by a variety of factors including TRF1 and TRF2, and that TRF2 is able to mediate the invasion of the 3' single stranded overhang into the duplex telomeric DNA to form the T-loop. It further thought that the invaded single stranded 3' overhang is stabilized by the POT1 protein. This structure prevents telomeres from being recognized as damaged DNA, provided the proper stability to the chromosome ends.

Additional proteins are thought to be associated with the telomere as illustrated in Figure 5. TRF1 is believed to inhibit telomerase access to the telomere (Karlseder, 2003), while also serving to preserve telomere integrity and structure. TRF2 has been shown to be essential for the formation of the T-loop and also for preservation of telomere structure and function. Disruption of TRF2 binding has been demonstrated to cause a loss of the G-strand overhang causing telomeric fusions and anaphase bridges (Karlseder et al., 1999). Loss of TRF2 in human primary fibroblasts was shown to elicit a senescent phenotype, which is p53 or pRB dependent (Smogorzewska and de Lange, 2002). Thus, maintenance of telomere structure and function in normal human cells is critical for prevention of cellular senescence and disruption of the delicate telomere balance can lead to either a gradual induction of senescence if not overtly severe or an immediate premature senescence phenotype.

Chapter 4

Telomerase

The first telomerase to be discovered was that of the *Tetrahymena thermophila* species in 1985 by a very driven graduate student in the laboratory of Dr. Elizabeth Blackburn at the University of California at San Francisco (Greider and Blackburn, 1985). Telomerase is a telomere specific DNA polymerase that is capable of maintaining telomeres. It is a ribonucleoprotein that adds telomeric repeats to chromosome ends. Telomerase is a protein RNA complex consisting of two subunits, TR and TERT. The protein catalytic subunit, or TERT, component was first identified in *Euplotes aediculatus* and contains typical reverse transcriptase motifs (Lingner et al., 1997). TR, the RNA component, provides the template for repeat synthesis by the TERT polymerase subunit. It has been shown that by mutating the template sequence of the RNA component, the sequence of the telomere can be modified (Yu et al., 1990) and in some cases, cause rapid elongation or shortening. The TERT (telomerase reverse transcriptase) subunit provides the catalytic activity of the enzyme.

The telomerase RNA subunit varies in size among different organisms. The smallest, only 150 base pairs, is found in ciliated protozoan, while mammals have longer TR subunits, with the human TR being 451 bases. Curiously, lower eukaryotes like *K. lactis* and *S. cerevisiae* have TR subunits of approximately 1.3 kilobases for reasons that are not completely understood.

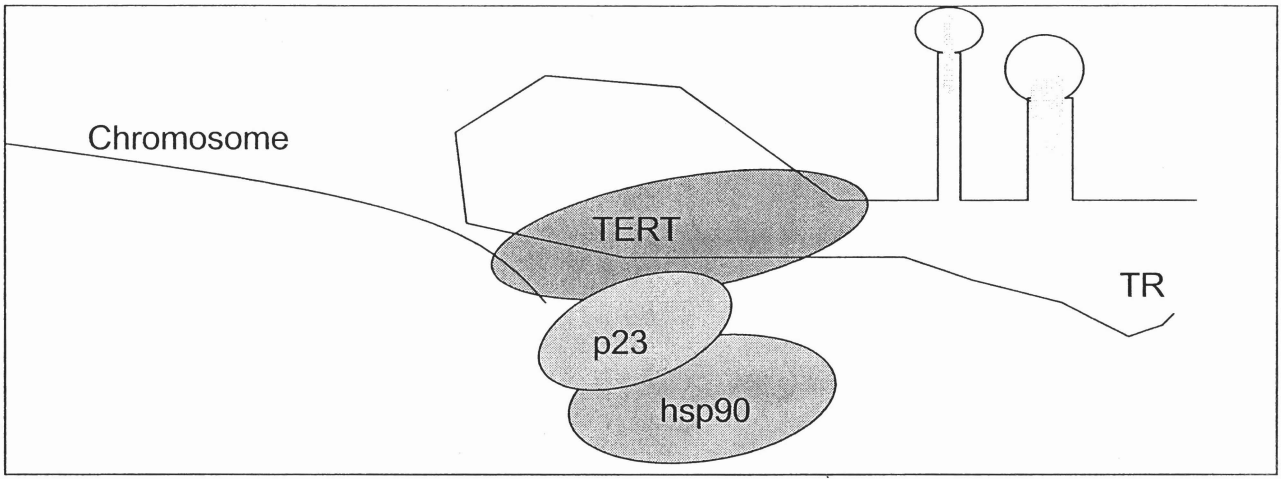


Figure 6. Hypothesized telomerase enzyme interaction with telomere. The telomerase enzyme is composed of TR (template RNA, shown with significant secondary structure) and TERT (telomerase reverse transcriptase, shown in green) and 2 proteins that serve to facilitate the folding of the enzyme complex, p23 and hsp90, One hypothesis thought is that not only do the hsp90 chaperones serve to stabilize the telomerase holoenzyme, they also are critical for its interaction with the telomere.

Like other reverse transcriptases, telomerase is a ribonucleoprotein consisting of an RNA template and a protein catalytic subunit. Across species, the protein subunit is highly conserved, while the RNA subunit is much more variable. In humans, the RNA component, hTR (human Telomerase RNA), is ubiquitously expressed, while the protein component, hTERT (human Telomerase Reverse Transcriptase), is only expressed in cells that activate telomerase activity such as stem cells, germ cells, and activated lymphocytes. The role of telomerase is to catalyze the sequential addition of telomeric repeats to the lagging strand of DNA in a dividing cell, by reverse transcribing DNA complementary to the hTR template. Telomerase works in a processive manner, meaning that it synthesizes 6 bases at a time. Once complete, the telomerase complex must translocate and synthesize 6 new bases. This process of elongation followed by translocation is repeated until the native telomere length is maintained. What makes telomerase a processive enzyme is the ability for 1 holoenzyme complex to continuously replicate telomere ends, rather than having another complex displace the enzyme already bound to the telomere (Figure 7).

Telomerase activity can be reconstituted *in vitro* by combining hTERT and hTR (Weinrich et al., 1997). These reconstitution assays have been used to study the activity of telomerase biochemically. In addition, many studies have identified numerous proteins that associate with telomerase, including hsp90 and p23 (Holt et al., 1999; Forsythe et al., 2001). The collective telomerase complex is referred to as the holoenzyme. This chaperone complex was found to interact directly with hTERT (Holt et al., 1999), but unlike other chaperone targets where chaperones were only

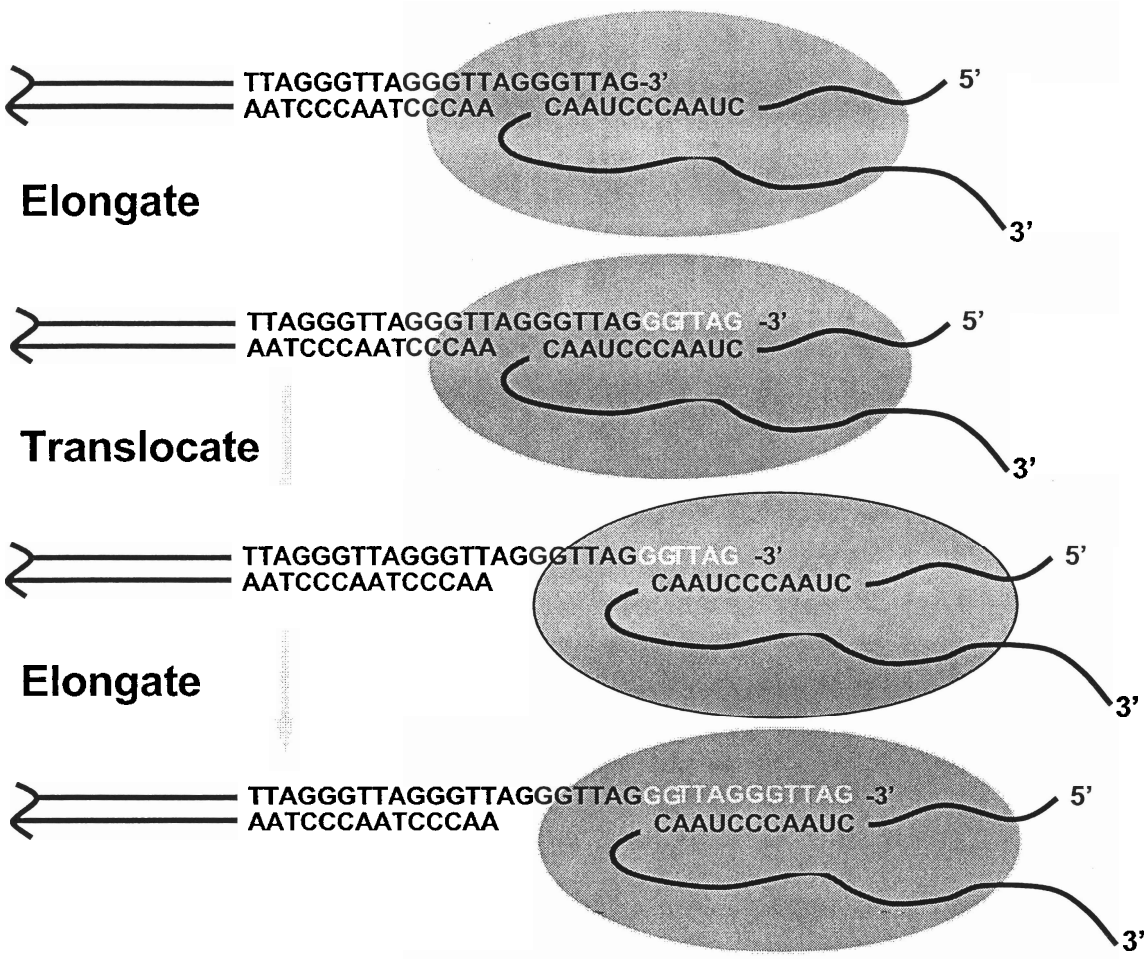


Figure 7. Telomerase Priming and Telomere Elongation. Telomeres are elongated by telomerase in a processive elongation, translocation, elongation pattern extending the lagging strand of the telomere from 5' to 3'. Taken from Harley and Villeponteau, 1995.

associated transiently, hsp90 and p23 remain bound to the active telomerase enzyme (Forsythe et al., 2001). The reason for this continued association of chaperones may have something to do with the telomerase enzyme's processive characteristics, given that it polymerizes telomere addition and translocates to the next available position to synthesize again. There may be some additional tweaking of enzyme structure or conformation that is required for effective and processive replication of telomere repeats. Alternatively, a recent hypothesis is that hsp90 and p23, while not specifically required for assembly of the complex initially, are necessary for telomerase to bind to telomeres (a.k.a. hsp90 and p23 are required for substrate recognition) (Dr. M. Jarsterfer, University of North Carolina, Chapel Hill, NC, personal communication). Additional required components for a functional telomerase holoenzyme include hsp70, HOP/p60 (hsp organizing protein) and hsp40 (Forsythe et al., 2001), all of which are necessary for efficient assembly of the TERT and TR components. Recent experiments done in the laboratory have suggested, although not proven, that the HOP and hsp40 components are not strictly required for assembly but serve to accelerate the process (Rapp, Elmore, and Holt, unpublished data).

The RNA subunit, hTR, contains a template that is used for the priming of DNA synthesis by the hTERT subunit. The template within the TR is approximately a 9-11 bases, and varies between species (Chen et al., 2000). Interestingly, mutations made in the hTR template were incorporated into the resulting telomeric DNA following transfection of cells with mutated hTR, and even more striking was the finding that mutations in the yeast TR in *K. lactis* causes a fatal telomere elongation, which could be

the result of the 25 base template sequence and the non-repetitive nature of their telomeres (Reviewed by Blackburn, 1999).

Telomerase Activity

In mice and humans, telomerase is active primarily during fetal development. Telomerase is subsequently turned off in somatic cells around the time of birth, presumably during tissue differentiation, and telomeres shorten with every cell generation in these cells. Stem cells, such as those in the bone marrow, as well as germ cells and activated lymphocytes, are exceptions, as telomerase remains active in these cells (Reviewed by Holt and Shay, 1999). Clearly, this activity is necessary to avoid premature senescence in highly proliferate cell types and provide regenerative capabilities and immunological response throughout the course of life.

In cancers, telomerase is usually re-activated and seems to be a key step in allowing cells to become immortal. As discussed above, immortal cells generally require the reactivation of telomerase to continue proliferation and overcome the telomere-induced crisis typical of preimmortal cells. Because more than 85% of human malignant cancers have telomerase activity (Shay, 1997), telomerase activity can be used as a clinical marker in the detection of cancer, although it is unlikely to replace conventional pathological determinations. Even so, having such a prominent molecular marker should provide an additional tool for those cases that are difficult to diagnose and may provide some suggestive evidence for prognosis as well. However, the level and distribution of telomerase activity has still not been proven to be a 100% reliable tool for

diagnosis and prognosis of cancers (Reviewed in Holt and Shay, 1999). As telomerase activity is not found in all cancers, it is not required for tumorgenesis; yet, without a mechanism for telomere length maintenance, the cells will be susceptible to senescence. What this means is that certain cancer types may be mortal, having long telomeres and no need for a telomere maintenance mechanism. In addition, the traditionally used "85%" number is likely artificially low given the number of laboratories that have contributed to this figure and the variability in sample handling and processing, as well as the inability (at least in the beginning) to distinguish true negatives from false negatives. When the PCR-based telomere repeat amplification protocol (TRAP assay) was first developed, there was no positive control for PCR amplification and truly negative samples could not be easily distinguished from those that had PCR or Taq polymerase inhibitors in the samples. Certainly, there were ways to handle that, like mixing experiments or phenol/chloroform extractions to remove the inhibitors, but it was not until the advent of an internal PCR standard that the numbers could be accurately counted. As such, the number that should be used to telomerase activity in cancers is more accurately expressed as "over 90% of all human cancers" and advanced malignancies likely express telomerase around 99% of the time. Thus, unless a mortal tumor, telomeres in cancer cells are generally observed to undergo no net loss of telomere length, suggesting that either telomerase is active or the minor ALT, recombination-based pathway for telomere length has been activated in these cells.

Introduction of telomerase into normal cell lines has been shown to increase population doublings significantly, more than triple their normal lifespans (Bodnar et al., 1998; Holt

and Shay, 1999). Further, these cells were found to be "indistinguishable" from younger cells of the same lineage (Holt and Shay, 1999). Several groups have studied the effect of introducing telomerase into normal cells, and in each of these studies, it was suggested that while telomerase activity extends the possible population doublings, this activity alone is not sufficient for cancer development (Reviewed in Holt and Shay, 1999).

Some cell types exhibit telomerase activity even though their telomeres continue to shorten. This was the subject of investigation in a seminal paper by Masutomi and colleagues (2003), where telomerase was shown to maintain telomere structure in normal cells that have long been thought to lack any telomerase expression. Following observations that telomere length, structure, and telomerase activity's effects on replicative lifespan and senescence appear correlative rather than directly related, they sought to identify a specific relationship between these elements (Masutomi et al., 2003). Based on the observation of telomere shortening and telomerase activity in keratinocytes, lymphocytes and some hematopoetic progenitor cells, a study was designed to understand the physiologic purpose of telomerase activity in these cells. Using two inhibition systems, a dominant negative hTERT (DN-hTERT) or a hTERT shRNA (short hairpin RNA), they observed that when telomerase activity was blocked in normal human fibroblast cell lines, the cell's entry into S phase was delayed and an accumulation of cells in the G2/M phase occurred (Masutomi et al., 2003). Cell death was not observed in these cells, yet they observed that cell cycle progression in these cells was delayed and continued to proliferate for "many" population doublings. They

concluded that these observations indicate that telomerase activity in these cells was necessary for cell cycle progression of normal cells. Further, they observed that cells with the DN-hTERT mutant had a 50% reduction in population doublings when compared to controls. When observed following cell division, the DN-hTERT infected cells appeared "vacuolated and flattened" compared to normal controls and failed to become confluent in culture even after many weeks. Further investigation indicated that the cells expressing the DN-hTERT entered replicative senescence sooner than controls. These studies indicate that inhibiting hTERT activity in these normal cells prematurely initiates senescence.

The authors further conclude, "the regulation of hTERT and telomerase is a dynamic process even in normal human cells and suggest that active maintenance of the telomere is necessary for the proliferation of normal human cells" (Masutomi et al., 2003). Using cell sorting and cell synchronization, the authors were able to find telomerase activity expressed during the S phase of the cell cycle, using TRAP and immunofluorescence with an hTERT antibody generated in their laboratory. Their findings also suggest that there is physiologic expression of hTERT in "most if not all human cells as they pass through S phase", although they only showed it for a handful of cell strains. They conclude that inhibition of telomerase function during S phase somehow interferes with its role in stabilizing the 3' telomeric overhang, and loss of the 3' overhang is strongly correlated with entry into senescence.

As has previously been discussed, it has been shown by multiple groups that telomere shortening is also associated with the initiation of cellular senescence and may be correlated with the loss of the 3' overhang (Masutomi et al., 2003). To identify a correlation between the telomerase inhibition in these cells and telomere shortening, telomere length was examined. They did not observe a difference between telomere lengths in control or telomerase inhibited cells. An analysis of the integrity of the 3' overhang in the telomerase-inhibited cells was also conducted. When observed around 18 population doublings, they observed a significant reduction in the amount of 3' telomeric overhang DNA (Masutomi et al., 2003). From all of these experiments, they conclude, "modulated telomerase activity is involved in the regulation of the cell cycle, cell proliferation, and lifespan" (Masutomi et al., 2003). What is curious about all of this work is that this is the only group to have shown this, and they use cell strains that have been consistently used by a number of groups, including our own, for over a decade. While the data presented is clear, if not somewhat overstated, there remains serious doubt about the reality of the expression of telomerase (specifically hTERT) in normal human cell strains.

The ALT Pathway

As previously mentioned, some immortal cells are able to maintain telomere length in the absence of telomerase activity. In fibroblasts immortalized by transfection of SV40 Large T-antigen, the ALT pathway is present a significant amount of the time (Reviewed by Bechter et al., 2004). In these cells, the ALT pathway is generally indirectly identified by the absence of telomerase activity and telomeres are found to have a wide variety of

mostly heterogeneous lengths. It is hypothesized that the ALT pathway is enabled by a homologous recombination mechanism. Bechter et al. (2004) found that in cells maintaining telomerase length via the ALT pathway, homologous recombination occurred in equal amounts to cells with telomerase activity. These recombination events appear to occur in the telomeric repeat regions rather than sub-telomeric regions. Bechter et al. (2004) discuss a model whereby "elongation in ALT cells involves a break-induced replication", which is hypothesized to be marked by invasion of a critically short telomere into a donor telomere via homologous recombination leading to "nonreciprocal transfer of DNA onto the shorter telomere". Fewer recombination events appear to occur in telomeric regions containing non-conical sequence variants, which are possibly binding sites for telomere binding factors necessary for recombination inhibition (Bechter et al., 2004). Further investigation into specific factors involved in the ALT pathway could lead to potential therapeutic methods for cancer treatment.

The ALT pathway has been well studied in yeast, where telomerase is constitutively expressed. Generally, yeast cells have been shown to enter senescence following mutation of the telomerase genes. In yeast cells without active telomerase, senescence usually occurs after 70 cell divisions, except when the ALT pathway is activated (Lundblad and Blackburn, 1993). Yeast cells have been shown to have two types of ALT pathways, type I and type II, both of which use recombination mechanisms to maintain telomere length (Teng and Zakian, 1999). Type I and type II ALT pathways are

differentiated by the repeated sequence, where type I repeats in yeast are marked by the repeated Y elements, while type II telomeres have vast arrays of TG1-3 repeats.

Overall, the ALT pathway appears to be a minor cancer pathway, and only a single study has shown that recovery after telomerase inhibition occurs as a result of the activation of the ALT pathway (Bechter et al., 2004). This manuscript shows that a mutation in the mismatch repair gene, MSH6, is the only way that recovery by ALT can occur, and the mutation frequency of mismatch repair genes is generally extremely low. Thus, in terms of cancer development, the ALT pathway is minor compared to the telomerase pathway; even so, studies on ALT will be important for defining additional mechanisms for telomere-based therapeutic interventions.

Telomerase Gene Regulation

Transcriptional regulation of the TERT gene is believed to be the critical control of telomerase expression as the RNA subunit is generally ubiquitously expressed (Bodnar et al., 1998; Cong et al., 2002). The core of hTERT gene promoter is a 330 base pair sequence upstream of the translation start site. The maximum promoter activity was mapped to a 59 base pair region between -208 and -150 (Horikawa et al., 1999). The hTERT promoter does not contain a TATA or CAAT box (Horikawa et al., 1999). The promoter is GC rich, which is consistent with methylation-based regulation (Horikawa, 1999). The E-box, characterized by the sequence CACGTG, which is activated by the bHLHZ family of transcription factors, was identified to be vital to hTERT transcription and that c-Myc expression increases the transcriptional activity of this box (Horikawa et

al., 1999). Direct binding of a c-Myc/Max heterodimer was demonstrated by Xu et al (2001). Upregulation of the hTERT gene by c-myc is consistent with the activation of c-myc by mutant p53 and other viral oncoproteins.

Binding sites for Sp1 and MAZ are found in the hTERT promoter as well (Kyo, 2000). Sp1 binds the GC-boxes thought to be important for transcriptional initiation. These Sp1 binding sites are required for transcriptional activity of the hTERT gene (Kyo et al., 2000). Klingelhutz identified the human papillomavirus 16 E6 as an activator of hTERT transcription (Klingelhutz et al., 1996), which may actually serve to derepress hTERT by inactivation of p53 (discussed more below). Another interesting observation of telomerase transcription in endometrial tissue suggests that sex hormones are possibly involved in the regulation of telomerase activity (Reviewed by Cong et al., 2002). The hTERT promoter does have an estrogen receptor element (ERE), although significantly upstream of the core promoter, but the ERE is thought to be activated when endometrial tissue is proliferating (Kyo et al., 1999).

As telomerase is not expressed in most human somatic cells, the repression of telomerase transcription is of particular interest. Two independent laboratories showed a series of experiments where specific chromosomes from normal human cells were transferred to telomerase-expressing cells demonstrated a likely repressor of hTERT expression (Oshimura et al., 1995; Nishimoto et al., 2001). Oshimura and colleagues suggest that, after microcell-mediated transfer of chromosome 3 in renal cell carcinoma, there is a repressor of telomerase on the p arm of chromosome 3 (Oshimura et al.,

1995). Nishimoto also demonstrated that transfer of chromosome 10 into telomerase-positive human hepatocellular carcinoma cells caused a repression of telomerase activity and eventual telomere shortening (Nishimoto et al., 2001). These findings have lead to additional studies to identify specific factors that repress hTERT transcription.

One case of repression of hTERT transcription involves the competitive inhibition of expression through the E-box, which is bound by the c-Myc/Max heterodimer. Mad1, a member of the Mad family of proteins known for being negative repressors of cell growth, is found to repress telomerase expression when found as a heterodimer with Max (Xu et al., 2001). Mad1 had previously been shown to negatively repress cell cycle progression and to repress other genes controlled by E-boxes in their promoters (Reviewed by Xu et al., 2001).

Repression of hTERT transcription by p53 has been demonstrated by Kanaya et al. (2000). These studies demonstrate that repression of telomerase expression through transfection of p53 preceded cell growth inhibition and apoptosis typically induced by p53 over expression (Kanaya et al., 2000). They found that the transcriptional repression of hTERT by p53 over expression was dependent upon the upstream Sp1 binding sites in the hTERT promoter (Kanaya et al., 2000). Additional negative effectors of telomerase transcription include pRB, E2F, Wilms' tumor 1 tumor suppressor, and myeloid cell-specific zinc finger protein 2 (Reviewed by Cong et al., 2002).

The post-translational modification of telomerase is also considered a significant mechanism for telomerase regulation. The phosphorylation of the telomerase catalytic subunit appears to play the most important role in posttranslational modification of telomerase. Through phosphatase treatment of nuclear extracts, telomerase activity is down-regulated, while kinase treatment of nuclear extracts leads to increased telomerase activity (Bodnar et al., 1996). *In vivo* experiments have shown that tyrosine kinase c-Abl phosphorylates hTERT leading to down-regulation of telomerase activity in contrast to the *in vitro* results discussed above (Reviewed in Cong et al., 2002). There are numerous studies related to potential phosphorylation pathways involved in hTERT regulation, most of which are indirect and fail to provide the critical data or experiments that conclusively show regulation of telomerase via post-translational modification. As such, they will not be discussed for this literature review.

Chapter 5

Phylogenetic Analysis of Telomerase Subunits

The subunits of telomerase have been cloned in a number of organisms. The following discussion will focus on the cloning, homology, and structure of known TERT and TR homologs.

The Functional Telomerase RNA Component

Telomerase RNA subunits (hTR in humans) have been identified in many organisms. These include 24 ciliates, two yeast, mouse, cow and human (Chen, et.al. 2000), as well as 32 new telomerase RNA genes cloned by Greider's group (Chen et al., 2000). They cloned 18 different mammals, 2 birds, 1 reptile, 7 amphibians, and 4 different fish species. There is quite a bit of heterogeneity in the size and sequence of the identified genes in the Telomerase RNA subunit family, but there is also significant sequence homology in critical structural domains.

Phylogenetic analysis of these genes was performed, and eight conserved regions, notated CR1-CR8, were identified in these genes. A conserved region was identified when base pairs shared 90% or greater homology. The first conserved region represented the template region, critical for telomere elongation, with a consensus sequence of 5'-CUAACCCU-3'. CR-1 is 45 nucleotides downstream of the 5' end of the human gene. Interestingly, the mouse and other rodent genes only contain two nucleotides of sequence upstream of CR-1. Mammals with larger sequences upstream

of the first conserved region contain transcriptional promoter sites including a CCAAT and TATA site, suggesting that the TR gene is an RNA polymerase II transcript even though it is not an mRNA. There is some controversy over whether this is true, but given that most mRNAs code for proteins, it is unusual that a functional RNA like TR could be an RNA polymerase II transcript.

CR-2 is one of two longer conserved regions, along with CR-5, with an approximately 25 base region of homology that was useful as an anchor point in analysis and degenerate primer design for PCR amplification and subsequent cloning of the TR genes. The other conserved regions, 3, 4, 6, and 7 are shorter regions of conservation and are critical for the formation of TR's secondary structure.

The following species telomerase RNA subunits were cloned; Class Mammalia includes *Homo sapiens* (Human), *Trichechus manatus* (Manatee), *Elephas maximus* (Elephant), *Dasypus novemcinctus* (Armadillo), *Oryctolagus cuniculus* (Rabbit), *Tupaia glis belangeri* (TreeShrew), *Chinchilla brevicaudata* (Chinchilla), *Cavia porcellus* (Guinea Pig), *Equus caballus* (Horse), *Bos taurus* (Cow), *Sus scrofa* (Pig), *Felis catus* (Cat), *Procyon lotor* (Raccoon), *Mustela putorius furo* (Ferret), *Suncus murinus* (Shrew), *Geomys breviceps* (Gopher), *Microtus ochrogaster* (Vole), *Cricetulus griseus* (Hamster), *Mus musculus* (Mouse), *Rattus norvegicus* (Rat), and *Dasyurus hallucatus* (Quoll). Class Aves includes *Gallus gallus* (Chicken) and *Anodorhynchus hyacinthinus* (Macaw). Class Reptilia includes *Chelydra serpentina* (Turtle). Class Amphibia includes *Xenopus laevis* (Xenopus), *Bombina japonica* (Toad), *Ceratophrys ornata* (Horned Frog),

Pyxicephalus adspersus (Bullfrog), *Dermophis mexicanus* (Dermophis), *Herpele squalostoma* (Herpele), and *Typhlonectes natans* (Typhlonectes). Class Chondrichthyes includes *Dasyatis sabina* (Stingray), *Rhinoptera bonasus* (Cownose Ray), *Rhizoprionodon porosus* (Sharpnose Shark), and *Mustelus canis* (Dogfish Shark).

The telomerase RNA subunit's secondary structure is critical in its interaction with the catalytic subunit, TERT, and the telomere. The secondary structure of the RNA subunit has been looked at by a variety of groups, but it is believed that nucleotides that interact in secondary confirmations will evolve while maintaining Watson-Crick pairing capability, a phenomena referred to as co-variation (Chen et al, 2000) (Figure 8). As such, the telomerase RNA subunit genes sequences are able to maintain their helical secondary structures. Even with different bases, the predicted secondary structures are maintained due to conserved base pairing within the conserved regions of the transcribe RNA component.

Chen and colleagues (2000) identified eight paired regions, separated by junction regions (Figure 8). P1 is 5' of CR-1 and pairs with a region downstream of CR-3. P2 has two regions, P2a and P2b, separated by junction 2, which pairs with a region just downstream of CR-2. P3 is within CR-2 and pairs with a region within CR-3. P4 is downstream of CR-3 and just upstream from the 5' hyper variable paired region. The hyper variable regions are extremely heterogeneous across species. The upstream hyper variable region pairs with a region just downstream of CR-5. P4 pairs with a

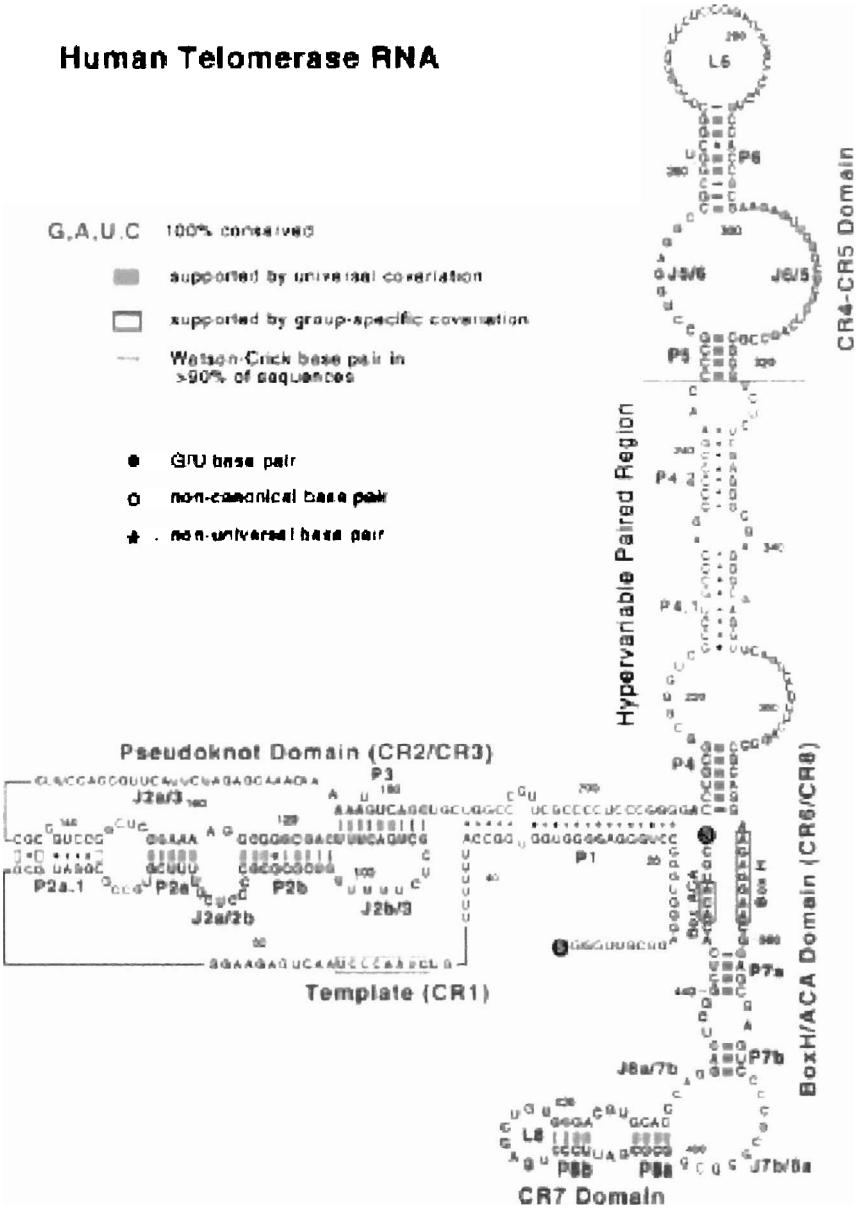


Figure 8. Hypothesized hTR structure. hTR is believed to form a complex secondary structure including several stem loops. This structure is conserved among a variety of very divergent species, illustrating the importance of this complex structure. Taken from Chen et al., 2000.

region in CR-6. P5 is just upstream of CR-4 and pairs with a region at the 3' end of CR-5. P6 is in CR-4 and pairs with the 5' region of CR-5. P7 is just downstream of CR-6 and pairs with a region 5' of CR-8 while P8 is just upstream of CR-7 and pairs with bases just downstream of CR7. All of this pairing creates a rather complex secondary structure with a variety of helices as is illustrated in Figure 8.

Chen proposes 4 distinct domains in vertebrates, including the Pseudoknot domain, the CR-4/-5 Domain, the CR-7 Domain and the Box H/ACA Domain. The Box H/ACA Domain is similar to the Box H and Box ACA seen in snoRNA's (Reviewed in Chen et al., 2000). It is believed that the H/ACA region is necessary in vertebrates for 3' end processing of the RNA (Ganot et al., 1997; reviewed by Chen et al., 1999). This region is not found in lower eukaryotes (Reviewed by Cong et al., 2002). The CR-7 domain contains two helices, as does the CR-4/-5 domain. The Pseudoknot contains the CR-2 and CR-3 regions, helices and the single stranded template region. The Pseudoknot is conserved in ciliates as well as vertebrate species. The Pseudoknot has been shown to be essential for assembly of telomerase in ciliates and is required for the function of human telomerase (Gilley and Blackburn, 1999). Although a rather complicated secondary structure, the sequence and homology of the TR transcript is highly conserved among a wide variety of species, from human to lower eukaryotes.

TERT structure and binding

The TERT enzyme structure has been closely studied. The hTERT gene is located on chromosome 5, on the p-arm. It is 40 kilobases in length and consists of 16 exons

Figure 9). hTERT is located only 2 megabases from the telomere, opening the possibility of a position effect, where reversibility silences genes proximal to telomeres. It is not clear if the effect can span the approximately 2 MB between the hTERT gene and the telomere (Cong et al., 2002). The proximity of the hTERT gene to the telomere raises the intriguing possibility that in successive generations of daughter cells, progressively shorter telomeres bring the hTERT gene closer to the end of the chromosome, allowing a position effect to play a role as cells age.

There are severally differentially spliced hTERT transcripts (Cong et al., 2002). These different transcripts are expressed in a "tissue-dependent and gestational-age dependent manner" (Cong et al., 2002), and only the full length hTERT transcript is found as a protein in cells. A possible dominant negative function of the splice variants was observed in a study by Colgin et al. (2000), but may serve as a dominant negative by preventing transcription of the less prominent wild-type mRNA.

The primary catalytic domain is more than a third of the protein. This domain is characterized by a series of reverse transcriptase motifs that catalyze the synthesis of telomeric repeats. A variety of organisms that have been studied shared homology in this centrally located region of the TERT protein, as well as other reverse transcriptases from viral origins. The C-terminus is quite divergent, with yeast and human sequences sharing almost no similarities in sequence or function. Overall, the TERT genes from various organisms are phylogenetically conserved, though they share more homology

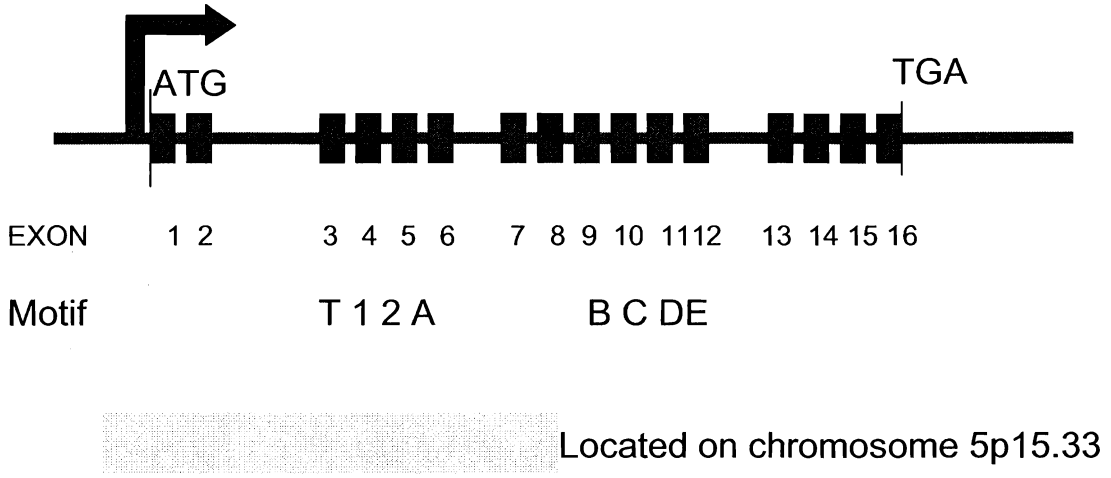


Figure 9. hTERT Genomic Structure. The hTERT gene is located on the p arm of chromosome 5. It includes 16 exons as well as T1, T2 and A, B, C, D, and E reverse transcriptase motifs.

with themselves than they do with other reverse transcriptase genes (Reviewed by Cong et al., 2002).

The N terminus of the telomerase gene has four domains termed I, II, III, and the T-motif. Several groups have shown that these domains are essential for yeast viability (Lingner et al., 1997). Domain III and T-motif are necessary for TERT's ability to associate with the RNA subunit (Bryan et al., 2000). Telomerase activity can be abolished by deleting the first 350 amino acids in human TERT (Reviewed in Armbruster et al., 2001). Alignments of approximately 500 amino acids of the N terminus of several TERT proteins from different organisms "identified 5 identical residues, clustering in three regions termed GQ, CP, and QFP, which overlap with yeast domains I, II, and III, respectively" (Armbruster et al., 2001). It appears that evolutionarily conserved regions of the TERT gene are essential for RNA binding and telomerase activity (Reviewed in Armbruster et al., 2001). Experiments in Tetrahymena also demonstrated that the N-terminus is required for enzymatic activity, namely the T-motif and domains II and III (Lai et al., 2001). The N-terminus has also been shown to be required for interaction with hTR (Bachand and Autexier, 2001).

Chapter 6

Model Systems for the study of Cancer and Aging

The search for effective model systems for the study of cancer and aging has included the use of a number of *in vitro* and *in vivo* systems. A variety of tissue culture, mammalian, and non-mammalian vertebrate model systems have been used to study cancer and aging, and many new model systems are actively being developed. Each system has its own limitations, some of which will be discussed and compared in this chapter.

With regard to the mouse as a model for the role of telomerase in cancer and aging, unique mouse genomic characteristics affect the value of it as an effective model system. As described above, mouse telomeres are extremely long, with lengths ranging from 50 to 100 kilobases in length, making it relatively impractical to study the effect of telomere shortening in mouse aging in a single generation. As such, we will discuss alternative models that could be used to study telomere biogenesis and telomerase regulation during the aging process.

Mouse Knockout

The mouse knockout experiments illustrate some of the challenges of the mouse as a model system for human telomere biology. Mice have been created with the mouse homolog of hTR, the mTR gene, knocked out. These mice were shown to be viable for at least six generations (Blasco et al., 1997), even though cells from these mice had no

measurable telomerase activity following approximately 300 population doublings. Telomere shortening was observed in these animals. Blasco and colleagues observed that telomeres shortened approximately 5 kilobases per generation (Blasco et al., 1997). After 5 generations, the average telomere distributions ranged from no measurable telomere to telomeres of 50 kilobases in length. Because inbred strains of mice have telomeres with lengths averaging between 30 and 80 kilobase pairs, there was no discernible phenotype for 6 generations.

Further, Blasco et al. (1997) observed that mouse embryonic fibroblast (MEF) cultured from the telomerase knockout mice have similar growth and senescence patterns as wild-type mice. Some cells were observed to grow for more than 200 population doublings without telomerase activity. Additionally, nude mice developed tumors after implantation of telomerase knockout embryonic fibroblasts, which had been transformed by oncogenes, suggesting that telomere lengths were sufficient to produce tumors in the absence of telomerase activity.

The telomeres of the telomerase knockout mice were shown to shorten approximately 100 bases per cell division. Because of the dramatically larger mouse telomeres as compared to humans, the shortening of mouse telomeres in the telomerase knockout mouse does not directly lead to cell cycle instability. The large mouse telomeres provide critical subtelomeric regions with substantial protection. Blasco et al. (1997) observed a higher frequency of karyotype abnormalities after 6 generations of breeding

of telomerase knockout mice compared to earlier generations, including aneuploidy and end-to-end fusions.

As evidenced from the mouse knockout experiments, researchers must be prepared to invest significant time and resources to study the cumulative effects of these alterations to mouse aging and cancer in many generations. With telomerase activity not required for the maintenance of telomere length in tumorigenesis, and as the mouse telomeres are able to protect mouse chromosomes from *de novo* changes that cause telomere erosion, the mouse is not an effective model for understanding telomere shortening and its related pathology in order to gain insight into human cancer and aging. An effective model for the study of telomerase inhibition and telomere shortening with a closer relationship to human biology is still being sought, and some suggestions are listed below.

Fish Models

A variety of fish have been looked at as potential model systems for the study of cancer and aging. Fish species such as zebrafish, Japanese medaka, minnows, guppy, trout and others have been studied. It has been demonstrated that fish cancers share similar structure and development to humans (Reviewed in McChesney et al., 2005). Further, response to knockdown of the p53 tumor suppressor gene in zebrafish caused a change in apoptotic response following the induction of DNA damage (Reviewed in McChesney et al., 2005).

Work conducted by the Holt laboratory has demonstrated that telomerase is present in the majority of fish tissues in a variety of marine species. Telomerase was also shown to be expressed in American eel, zebrafish, medaka, dogfish shark, flounder, as well as marine invertebrates such as sea cucumber, green sea crab and lobster (Reviewed in McChesney et al., 2005). Further, this work has demonstrated that fish telomere lengths are consistent with human telomere lengths (McChesney et al., 2005), perhaps making fish a better model for telomere studies related to aging and age-related diseases such as cancer.

The Chicken as a model system for the study of aging and cancer

The chicken, *Gallus gallus*, is considered one of the premier non-mammalian vertebrate models for the study of disease. Though it is considered a critical agricultural commodity, seminal scientific breakthroughs have and continue to be made in the chicken. Critical findings in embryology and development were made using chicken as the model system. The *in ovo* development of chicken embryos as compared to *in utero* development of mammals provides for a closer examination of development. The role of the chicken in the study of immunology has also been pivotal. The distinction between B-cells and T-cells was made in the chicken, with B-cells gaining their name based on the chicken's Bursa of Fabricius (Cooper et al., 1966).

The chicken lifespan, up to 30 years, is substantially longer than the average mouse lifespan. Also, as described above, chicken telomeres are more similar in size to humans than mice. The chicken has three classes of telomere, with Class I ranging

from 0.5 to 10 kb in size, Class II ranging between 10 to 40 kb in size, and Class III ranging between 40 kb to 2 Mb in size. The class I and II chicken telomeres are similar in length to those of human telomeres at varying stages of life. Primary chicken cell cultures are shown to undergo replicative senescence and have low rates of spontaneous immortalization (Hay, 1970; Reviewed in Venkatesan and Price, 1998).

The Chicken genome was sequenced by International Chicken Genome Sequencing Consortium in 2004. The chicken chromosomes share similar size heterogeneity to chicken telomeres as described above. Chromosome 1 of Chicken is 150 to 200 megabases in length while 30 of the 38 chicken autosomes are called "microchromosomes", with sizes between 5 and 20 megabases. The total size of the haploid chicken genome is roughly half the size of the human genome, with $1.2x10^9$ base pairs of DNA. The maintenance of the chicken microchromosomes is interesting in researching both genomic stability as well as the creation of artificial chromosome models. Importantly, comparative analysis of chicken genes with homologs in mouse and human has shown significantly more compact gene sequences in the chicken with smaller introns and intergenic regions (Regnier et al., 2003).

The study of cancer in the chicken has also produced many important findings. The first "tumor virus", Rous sarcoma, as well as the first oncogene, src, were identified in chicken. Further, the avian leukosis virus (ALV) is the subject of significant investigation.

Chicken Telomerase Activity

The expression of telomerase in the chicken was the subject of a paper from Carolyn Price's laboratory in 1998 (Venkatesan and Price, 1998). They studied tissues from 1-day-old chickens, 9-month-old chickens, and a 2-year-old rooster. They looked at samples from the testis, kidney, spleen, liver, lung, pancreas, thymus, brain, ovary, and heart. Activity was detected in both germ-line and somatic cell types. They also observed that telomerase activity was detected in more of the 1-day-old tissues than the rooster tissues, though they did not test a significant number of older animals to confirm this conclusion. Activity was not detected in the heart or brain of the chicken tissue samples, suggesting that similar to human, telomerase is repressed in highly differentiated organs. These observations were also consistent with the telomerase activity profile that has been observed in the mouse, where telomerase activity is found in most tissues other than brain and muscular organs (Venkatesan and Price, 1998).

Chicken embryonic fibroblast telomerase activity was also analyzed. As previously mentioned, chicken embryonic fibroblasts have a limited lifespan and undergo replicative senescence like human primary cell cultures (Reviewed in Venkatesan and Price, 1998), which typically are able to undergo 25-35 population doublings. During these studies, chicken cells were passaged until they underwent replicative senescence, which occurred between 22 and 32 population doublings. They observed a decline in the telomere length with increased population doublings at an average loss of 60 bases per population doubling (Venkatesan and Price, 1998). Telomerase activity was found to be down-regulated when cultures were established from chicken embryos,

raising concerns regarding the study of senescence in these chicken embryonic cells where telomerase up-regulation has typically been observed. Upregulation of telomerase activity was shown to occur in transformed cells and tumors from chicken origins (Forsyth et al., 2002).

Chicken Telomerase Genes

The chicken telomerase RNA subunit, cTR, was cloned by Greider. (Chen, 2000). It was identified in a shotgun approach, using degenerate PCR. The cTR, accession NR 001594, is 465 bases in length, which is very similar to the human TR component of 451 bases. In fact, the cTR shares significant conservation with hTR, including all of the functional and conserved regions.

At the beginning of this investigation, the chicken telomerase catalytic subunit had not yet been identified. Using an *in silico* approach, a 204 AA open reading frame was identified (Figure 10), which shared 73% similarity to hTERT. By comparing the chicken TERT (chTERT) homolog sequence to hTERT, several of the critical regions of the peptide were found to share conserved sequence, including the E Reverse Telomerase motif, which is critical for TERT's catalytic activity. In addition, sequence towards the C-terminus of the TERT protein was present in this EST.

The hypothesized chTERT cDNA was identified through a BLAST search of the BBSRC Chicken EST Database. "This web-site provides access to 339,314 *Gallus gallus* ESTs which were sequenced from 64 cDNA libraries generated from 21 different embryonic

Alignment of hTERT and cTERT Amino Acid Sequence

```
Score =  222 bits (566), Expect = 7e-56
Identities = 112/184 (60%), Positives = 137/184 (73%)

hTERT:   YARTSIRASLTFNRGFKAGRNMRRKLFGVLRLKCHSLFLDLQVNSLQTVCTNIYKILLLQ 1008
         YA TSIR+SL+FN    AG+NM+ KL  VL+LKCH L LDL++NSLQTV  NIYKI LLQ
chTERT:  YAFTSIRSSLSFNSSRIAGKNMKCKLTAVLKLKCHPLLLDLKINSLQTVLINIYKIFLLQ 80

hTERT:  AYRFHACVLQLPFHQQVWKNPTFFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLPSE 1068
        AYRFHACVLQLPF+Q+V  NP FFLR+ISDTAS CY ILKAKN G+SLG+K A+G  P E
chTERT: AYRFHACVLQLPFNQKVRNNPDFFLRIISDTASCCYFILKAKNPGVSLGSKDASGMFPFE 140

hTERT:  AVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQLSRKLPGTTLTALEAAANPALPSDFK 1128
        A +WLC+ AF++KL+ H+V Y  LL  L+  +  L  K+P  T+  L+    P+L  DFK
chTERT: AAEWLCYHAFIVKLSNHKVIYKCLLKPLKVYKMHLFGKIPRDTMELLKTVTEPSLCQDFK 200

hTERT:  TILD 1132
        TILD
chTERT: TILD 204
```

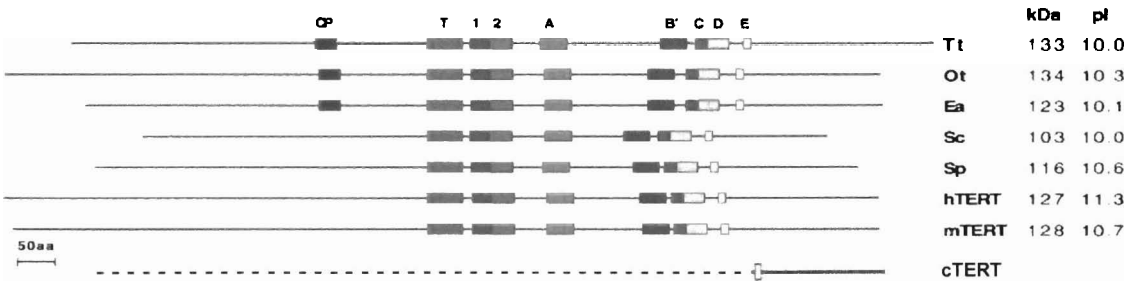
Figure 10. Chicken Telomerase Reverse Transcriptase (chTERT) homolog amino acid alignment with hTERT. The chicken est, 050049.1, was identified and translated into a hypothesized amino acid sequence. This sequence was then compared to the hTERT amino acid sequence. An alignment of more than 73 percent similarity was identified. This est was later confirmed by Delany et.al. to be the chTERT gene.

and adult tissues (sequence stats + tissue list). These ESTs were clustered into 64,760 gene-bins, which were assembled into 85,486 contiguous sequences (contigs)." The database was blasted using hTERT sequence. (http://www.chick.umist.ac.uk/).

Using the hTERT gene for comparisons, 1 unique EST, 050049.1 (Blast results shown in Figure 10) was identified that had significant homology to hTERT. This sequence was cloned from adult kidney + adrenal gland, as well as from ovary. Translation of the DNA sequence using the ExPASy Proteomics tools at http://au.expasy.org/tools/#translate produced an open reading frame of 204 amino acids, which was used for alignment analysis(Figure 11).

Following the identification of this EST, which likely represented the chicken homolog of hTERT, the Delany group confirmed the identity of the chTERT gene (Delany and Daniels, 2004). Delany identified the chTERT mRNA from gastrula stage embryos and found a 4497 base pair mRNA, which produced a protein of 1346 amino acids residues in length. This sequence was submitted to, and accepted by, NCBI and has received the accession NM_001031007.

A thorough analysis of the chTERT gene reveals that the protein sequence of chTERT is 45% identical to hTERT (Delany and Daniels, 2004). The chTERT protein has a longer N-terminal flexible linker region, which is 144 amino acids longer than what is found in the human protein (Delany and Daniels, 2004). Delany mapped chTERT to chromosome 2q21 near an interstitial telomere site (Delany and Daniels, 2004).



Tt=Tetrahymena, Ot=Oxytrica, Ea=Euplotes, Sc=yeast (cerevisiae), Sp=yeast (pombe)

Figure 11. Comparison of various organisms TERT sequence, with newly identified chicken EST sequence aligned. The chTERT est was aligned along with the TERT homologs from Tetrahymena, Oxytrica, Euplotes, yeast, mouse and humans. The chTERT shared homology to these other TERTs across the E RT motif as well as the C terminus.

Chapter 7

Discussion and Conclusions

The study of telomere structure and telomerase biology has been an exciting and enlightening field for over 2 decades. The study of how and why we age touches the root of the human condition. For thousands of years, humans have sought to evade or overcome the reality of fate and old age. Myths such as the fountain of youth or the Holy Grail have provided inspiration for those hopeful that someday we will conquer the diseases of old age or aging altogether.

Early insights into telomere biology have given hope that perhaps we have uncovered the molecular clock that determines human lifespan. Normal cells, which are normally capable of undergoing a finite number of cell divisions, can occasionally extend their lifespan and either undergo a process of programmed cell death or activate some type of immortalization mechanism. With this came the hope that perhaps we could affect longevity through manipulation of molecular processes that lead to telomere shortening, i.e. activation of immortalization. With the discovery of telomerase came additional insight into how telomeres maintain their lengths and regulate the process of aging. This maintenance of telomere length was observed in cells that intuitively would have been expected to maintain telomere lengths, such as germ cells and cancer cells.

The observation of telomerase activity in neoplasia, though intuitive, has lead to a large and promising field in cancer research and the development of potential therapies

targeted at inhibiting telomerase function. Insight into the molecular process leading to telomerase re-activation in neoplastic cells has and will continue to lead to new understandings of cancer biology in order to define mechanisms and pathways involved in telomerase activation. In addition, the process for telomere stabilization in immortal cells has allowed for interesting insight into the molecular process of aging. With the knowledge that telomerase activity alone is not tumorgenic, there is hope that exogenous telomerase activity could artificially extend lifespan. Tissue culture studies from a variety of laboratories substantiate this hypothesis in that normal telomerase-negative cell strains transfected with exogenous telomerase have substantially increased life spans (Bodnar et al., 1998).

The development of effective models systems for the study of aging and cancer is vital to furthering this field of research. Though the mouse has served as an incredibly effective model for the study of most human pathology and molecular processes, it poses significant challenges in the study of telomere biology, especially as it relates to the aging process. As such, it is critical to the advancement of the telomere field to identify new animal models for the study of telomeres and telomerase activity with respect to aging and progression to cancer.

The chicken has proven to be a valid model for the study of vertebrate embryology and development. With several classes of telomere with varying lengths, the chicken has telomeres suited to make direct comparisons to humans. The extremely large, class III telomeres are similar to those of the mouse, posing the same challenges to single

generation analysis of telomere shortening and cancer development. Of interest is the possible chromosome specific effects that are likely to be seen in the chicken chromosomes where class II and particularly class I telomeres are present. These chromosomes would trigger the damaged DNA response sooner than their counterparts with class III telomeres. These studies would be particularly interesting if studied in the absence of telomerase activity.

As discussed, the chicken, like the mouse, constitutively expresses telomerase in some somatic cells. With the observations by the Hahn laboratory that telomerase maintains telomere stability independent of telomere elongation, perhaps the telomerase-negative chicken may not be required if the constitutive telomerase activity found in some chicken somatic cells serves this same stabilizing function.

The Hahn lab's observations (Matsutomi et al., 2003) are particularly interesting when considering the mouse and chicken as animal models. If the constitutive telomerase activity serves primarily to maintain telomere stability rather than overall length, then perhaps both animals have greater promise as models for the study of telomere and telomerase biology. The chicken, with its multiple classes of telomeres would be a better model than the mouse in that the first two classes (class I and II) of telomere are so similar to the length of human telomeres.

Studies of telomere shortening in the chicken would provide interesting insight into the effect of shortening of specific chromosomes. With the varying classes of telomere

lengths in chickens, the DNA damage response would only be expected to be observed in the shorter classes. Dysfunction of the shorter chromosomes, including the microchromosomes of the chicken would provide interesting information about specific genomic changes caused by specific chromosomal damage.

Developing a new chicken model for the study of telomeres as they relate to cancer progression and potentially aging is a worthy pursuit. The chicken lifespan, telomere lengths and time between generations all provide a reason for the development of a chicken model. The chicken provides advantages over the traditional mouse model for telomere studies and is a worthy alternative model for the study of cancer.

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Vita

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