CHAPTER 1:

Telomeres in Aging: Birds

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Handbook of Models for the Study of Human Aging

(In press 2005)

I. Abstract

This chapter describes the use of avian species (the domestic chicken G*allus domesticus* in particular) as model organisms for research in telomere biology and aging. Presented here are key concepts of avian telomere biology including characteristics of the model: the karyotype, telomere arrays, telomere shortening as a measure of the senescence phenotype or organismal aging, and telomerase activity in avian systems including chicken embryonic stem cells, chicken embryo fibroblasts, the gastrula embryo and DT40 cells. Key methods used to measure telomere shortening, telomerase activity, and to conduct expression profiling of selected genes involved in telomere length maintenance are noted as are methods for conducting gain- and loss-of-function studies in the chicken embryo. Tables containing references on general topics related to avian telomere biology and poultry husbandry as well as specific information regarding chicken orthologs of genes implicated in telomere maintenance pathways are provided. Internet resources for investigators of avian telomere biology are listed.

II. Glossary

Telomere-The chromosome end which in vertebrates is composed of many copies of the hexanucleotide repeat 5' TTAGGG 3' along with associated DNA binding proteins.

Replicative senescence-After a finite and typically species and tissue-specific number of divisions, cells are no longer able to proliferate. Cell morphology changes such as increased cell size, increase in the size of the nucleus and nucleoli, increased vacuolation, and expression of senescence markers are typically observed in senescent cells.

Proliferation-The expansion of a population of cells by repeated rounds of cell division.

Culture arrest-A condition observed in senescence wherein cells cease proliferating.

Hayflick limit-The barrier to further cellular proliferation represented by culture arrest of senescent cells.

End-replication problem-The inability of DNA polymerase to replicate the 5' end of a linear chromosome causes loss of telomeric (TTAGGG) repeats upon successive divisions.

Telomerase-The enzyme, composed of an RNA template and a specialized reverse transcriptase, which can add telomere repeats to the ends of a chromosomes.

Transformation-In the context of the *in vitro* cellular phenotype, the process whereby a cell loses contact inhibition and anchorage dependence, acquires the ability to form colonies in semi-solid media, exhibits decreased requirements for growth factors and overcomes the Hayflick Limit to become immortalized.

Helicase-An enzyme which separates DNA strands in preparation for replication.

Transcription factor-A protein, including an enzyme or coenzyme, a vitamin or other organic molecule which controls or affects the process of gene transcription, usually through the binding of a specific DNA sequence motif.

Progeroid disorder-A disorder characterized by premature aging in a child.

Terminal restriction fragment (TRF) analysis-In the context of telomere biology, the analysis by gel electrophoresis of telomere fragments obtained by digestion

of genomic DNA with a restriction enzyme. The TRF includes the telomere repeat plus adjacent sequence up to the restriction site. TRFs are typically resolved as an overlapping smear, following Southern blotting using a telomere probe, reflective of the species-specific diversity of sizes of telomere arrays.

TRAP-Telomerase Repeat Amplification Protocol also known as the TRAP assay. An assay which measures the presence of telomerase in a cell or tissue extract usually by primer extension wherein TTAGGG repeats are added to a synthetic oligonucleotide by the telomerase enzyme.

Chicken embryo fibroblast (CEF)-A fibroblast cell usually derived from 7-11 day-old (E7-11) chicken embryos. A cell from which connective tissue develops.

DT40-A transformed chicken B-cell derived from an Avian Leukosis Virus (ALV)-induced bursal lymphoma (tumor) of a female chicken.

cES-chicken embryonic stem cells derived from Stage X embryos, which are pre-blastula embryos collected from unincubated eggs consisting of about 40,000 cells.

Electroporation-A technique by which polar molecules are introduced into a host cell through the cell membrane. An electric pulse temporarily disturbs the phospholipid bilayer, allowing molecules such as DNA to pass into the cell.

RNAi-A naturally occurring, double-stranded, RNA-mediated mechanism for silencing of gene expression which can be exploited for loss-of-function experiments.

siRNA-Small, interfering RNA fragments used in RNAi experiments to silence gene expression.

Morpholino-A specialized antisense oligonucleotide used to silence gene expression.

III. Introduction: The chicken as a model organism

The versatility and utility of the domestic chicken as a developmental model was recently celebrated in a special issue of the journal Developmental Dynamics [(2004) 229, 413-712.] The chicken is one of the primary models for vertebrate developmental biology and a model organism for the study of virology, immunology, cancer and gene regulation (Tickle, 2004; Antin and Konieczka, 2005). With a 6.6X draft sequence of its genome completed, the chicken is poised to become even more valuable in traditional fields of study and also in aging research.

The earliest recorded descriptions of the chicken as a model for biological processes are attributed to Hippocrates and Aristotle who both wrote about embryonic development in fertilized chicken eggs. Twentieth century embryologists authored numerous treatises describing, diagramming, and providing detailed photographs of the chicken during development (Hamburger and Hamilton, 1951; Romanoff, 1960; Eyal-Giladi and Kochev, 1976) which promoted use of the chicken embryo as a model for study of mechanisms including morphogenesis; neurogenesis; somatogenesis; limb, limb-digit and craniofacial development; left-right symmetry; axis development and others. The extensive use of the chicken as a model for early vertebrate development and its role in biomedical research has of necessity produced a detailed and comprehensive body of knowledge about basic chicken biology (Stern, 2005; Scanes et al., 2004). Add to all of this the accessibility of the chicken embryo, the relative economy of breeding and maintaining chickens and the ease of

manipulation of embryonic and adult tissues and the chicken becomes an obvious choice as a model for the study of organismal and cellular senescence.

IV. Aging and Replicative Senescence

Cellular or replicative senescence (*in vitro*) is often utilized as a model for the aging process (*in vivo*) due to the hypothesis that cellular aging recapitulates organismal aging (Wadhwa et al., 2005). The central dogma of replicative senescence holds that cultures of vertebrate fibroblasts have a limited capacity for proliferation. After a finite number of cell divisions, proliferation slows and culture arrest ensues. The barrier represented by culture arrest, termed the Hayflick Limit, is accompanied by a number of morphological changes including increased cell size, increase in the size of the nucleus and nucleoli, increased vacuolation of the cytoplasm and endoplasmic reticulum, expression of senescence-associated markers such as beta-galactosidase, and other changes in morphology and gene expression (Cristafalo et al., 2004 and references therein).

A genomic alteration associated with cellular or replicative senescence in a variety of organisms, including the chicken, is the shortening of telomeres (Prowse and Greider, 1995; Taylor and Delany, 2000; Swanberg and Delany, 2003). Shortened telomeres may induce a DNA damage response, signaling cell cycle arrest. If the damage cannot be repaired, a checkpoint response results in further arrest or apoptosis. An alternative or complementary model for telomere-induced replicative senescence, is loss of the protective effect of accessory proteins, such as TRF2, at the telomeres (Karlseder et al., 2002). Reactivation of

telomerase or induction of the ALT (alternate lengthening of telomeres) pathway may provide protection against apoptosis or senescence and facilitate transformation and immortalization by stabilizing telomeres (Swanberg and Delany, 2003 and references therein).

The prevailing explanation for telomere shortening, the end-replication problem, is due to the inability of DNA polymerase to replicate the ends of a linear chromosome, resulting in the incomplete replication of the 5′ end of a daughter strand. Telomerase is able to offset telomere shortening by adding telomere repeats to the parent strand which generates a longer telomere in the daughter strand. The telomerase holoenzyme is composed of two elements, *T*elomerase *R*NA, TR, which contains the template for addition of telomeric repeats (Greider & Blackburn, 1989) and *TE*lomerase *R*everse *T*ranscriptase, TERT, the component which catalyzes the addition of repeats to the parent-strand chromosome end (Lingner et al., 1997). Most normal, adult vertebrate somatic cells, with the exception of cells from the lab mouse (*Mus musculus*), exhibit little or no telomerase activity (Levy et al., 1992; Kim et al., 1994; Wright and Shay 2002; Levy et al., 1992). Not only does telomerase maintain telomeres of proliferating cells, it is also implicated in oncogenesis (Greider and Blackburn, 1989).

In addition to the end-replication problem and the compensating function of telomerase, telomere length is impacted by proteins which bind to and contribute to the architecture of the telomere. The thousands of duplex DNA telomere repeats are, for the most part packaged in closely-spaced nucleosomes

(Blackburn, 2001). However, the G-rich 3' overhang assumes a terminal loop (t-loop) which displaces one of the duplex strands forming a related structure (D-loop). The D-loop t-loop is stabilized by telomere-binding proteins and their interaction partners (Greider 1999; Griffith et al., 1999; Wei and Price, 2003). Closed chromatin loops resembling t-loops have been observed in chicken using electron microscopy (Nikitina and Woodcock, 2004).

Telomere-repeat-binding factors 1 and 2 (TRF1 and 2) bind to double-stranded telomeric DNA (Wei and Price, 2003). TRF1, which induces telomeric DNA strands to bend, loop and pair (Bianchi et al., 1997; Smogorzewska et al., 2000) may produce shortening of telomeres by sequestering the 3' overhang from telomerase (van Steensel and de Lange, 1997). TRF2 is described as protective of telomeres in some studies (Karlseder, 2003) and as a negative regulator of telomere length in other studies (Smogorzewska et al., 2000; Stansel et al., 2001). Overexpression of TRF1 or TRF2 produces a progressive shortening of telomeres (Ohki and Ishikawa, 2004 and references therein). Tankyrase 1 and 2 have the ability to bind TRF1, resulting in the ADP-ribosylation of TRF1, and the release of TRF1 from telomeric DNA. Overexpression of tankyrase 1 results in the removal of TRF1 from the telomeres followed by telomere elongation (Smith and de Lange, 2000).

In addition to the tankyrases and TRF1 and TRF2; Rap 1 and Pot 1 are involved in telomere maintenance. Rap1 interacts with TRF2 and Pot 1 may coat and protect both G-strand overhangs and the displaced G strand of a t loop (Bauman and Cech, 2001; Tan et al., 2003). Other proteins known to be relevant

to telomere length regulation include c-myc, an oncogenic transcription factor which regulates cell proliferation, differentiation and apoptosis (Piedra et al., 2002). Down-regulation of c-myc is believed to be a prerequisite to differentiation (Skerka et al., 1993; Baker et al., 1994) and c-myc re-activates telomerase in transformed cells by inducing expression of its catalytic subunit TERT (Wu et al., 1999).

Chicken orthologs of TRF1 and 2, tankyrase 1 and 2, TR, TERT , c-myc, Rap 1 and Pot 1 have been characterized. In addition, chicken orthologs of the helicases which are missing or mutated in the progeroid disorders, Werner and Bloom Syndrome, have been identified but not studied. The Werner (WNR) and Bloom (BLM) proteins, both Req-Q helicases, have been implicated in telomere maintenance pathways (Du et al., 2004). Table 1 lists chicken genes related to telomere length regulation, their human orthologs and relevant references.

V. The chicken as a paradigm for aging research

Organisms frequently used in aging studies include yeast, drosophila, *C.elegans* and *M. musculus*, the laboratory mouse. With all of these well-characterized models available, particularly a mammalian vertebrate as well-studied as the lab mouse, why use an avian model? The advantages of using a vertebrate are obvious and the mouse would at first glance appear to be a better choice than the chicken except for shortcomings of the mouse vis-à-vis the study of aging and oncogenesis. For example, mice have a very short lifespan. In contrast, maximum life expectancies of many species of birds approach the human life expectancy (Forsyth et al., 2002; Austad, 1997). Lifespan is

significant, as cellular and genetic mechanisms governing cell proliferation are likely conserved in longer-lived species.

In addition to the issue of lifespan, laboratory mouse somatic cells retain telomerase activity and do not appear to display division-dependent telomere shortening (Prowse and Greider, 1995; Forsyth et al., 2002; Kim et al., 2002). Mouse models of telomere shortening have been developed, but it takes several generations of a telomerase knockout mouse (TR-/TR-) to achieve a phenotype which demonstrates division-dependent telomere shortening (Cheong et al., 2003). In contrast, human and chicken somatic cells possess little or no telomerase, with down-regulation of telomerase occurring early in development. Division-dependent telomere shortening is established in chicken chromosomes (*in vivo* and *in vitro)* and human chromosomes. In human, mouse and chicken, highly proliferative tissues such as embryonic cells and intestine as well as transformed cells exhibit telomerase activity (Taylor and Delany, 2000; Forsyth et al., 2002; Swanberg and Delany 2003; Delany et al., 2003).

Unlike mouse fibroblasts, both chicken and human primary fibroblast cells are generally refractory to spontaneous immortalization (Lima and Macieira-Coelho, 1972; Lima et al., 1972; Macieira-Coelho and Azzarone, 1988; Prowse and Greider, 1995). In addition, critically short human telomeres induce senescence either by activating p53 or by inducing the p16/RB pathway, and suppression of both pathways is required to suppress senescence of aged human cells. In mouse, the p16/RB response to telomere dysfunction is not active (Smogorzewska and de Lange, 2002). In contrast, the senescence

pathways of chicken and human fibroblast systems thus far seem to share more similarities than differences (Kim et al., 2002) see Table 2. For an excellent review of the developmental regulation of telomerase activity in human, mouse, chicken and flowering plants see Forsyth et al., (2002).

VI. Features of the chicken genome relevant to the study of aging

The chicken karyotype consists of 39 pairs of chromosomes, which is typical of most avian species. The genome is organized as eight pairs of cytologically distinct macrochromosomes, the Z and W sex chromosomes and thirty pairs of small cytologically indistinguishable microchromosomes (ICSGS, 2004). As in other vertebrates, chicken telomeres consist of a highly conserved hexanucleotide repeat, 5' $TTAGGG_{(n)}$ 3'. The cytogenetic features of the telomere repeat were first described in chicken by Nanda and Schmid (1994). Molecular features of telomeric DNA in the chicken genome were described in 2000 (Delany et al.). Although the avian genome is one third the size of the human genome (1.25 pg versus 3 pg/haploid cell), the amount of telomeric DNA sequence is five to ten times more abundant in birds than in humans (Delany et al., 2000; Nanda et al., 2002). Higher telomere repeat content in the chicken is likely due to the high number of chromosome ends (2n=78 or 156 chromosome termini), the load of interstitial telomeric DNA and the presence of an unusual category of ultra-long telomeric arrays (see Figure 1).

Telomeric DNA in the chicken can be categorized into three main array size classes. Class I telomere repeats are 0.5 – 10 kb in length and exhibit discrete and genotype-specific banding patterns. Class I repeats are interstitially

located and show no evidence of telomere shortening. Class II repeats are 10 - 40 kb and appear on Southern blots as the typical overlapping smear of TRFs; Class II arrays show evidence of terminal location based on digestion by *Bal* 31 and exhibit division-dependent shortening in somatic tissues. Class III telomeres are hundreds of kilobases in size, and range to 3 megabases. Shortening of these arrays has not been established because of the inability to resolve changes of 100s of nucleotides (typical telomere erosion) in the context of 100's to 1000's of kilobases of the Class III arrays (Delany et al., 2000). In order to resolve Class III arrays on a gel, special pulse field gel electrophoresis parameters are required (Delany et al., 2000).

Not all avian species exhibit the Class III arrays (Delany et al., 2000; Nanda et al., 2002). Current models suggest that the Class III arrays of the chicken map to a subset of microchromosomes, perhaps serving to protect these small genetic elements from erosion and/or contributing to high microchromosome recombination rates (Delany et al. 2000; Delany et al. 2003). It is important to note that the existence of megabase telomere arrays in chicken does not diminish the power of the chicken as a model for division-dependent telomere shortening as it appears to be the shortest telomere or the unprotected telomere which triggers genome instability (Hemann et al., 2001; Karlseder et al., 2002).

Telomerase activity and telomere-shortening profiles in avian cells *in vivo* and in *vitro* mirror what is observed in human cells. Telomerase activity is developmentally regulated *in vivo* with high levels of telomerase in early stage

chicken embryos (preblastula through neurula) and during organogenesis all organs surveyed up to 10 days of embryonation (E10) followed by down-regulation for most somatic tissues. Constitutive telomerase activity continues for “renewable” tissues including intestine, spleen, and organs or cells of the reproductive system. An average decrease of 3.2 kb in telomere length was observed from the early embryo to the adult (Taylor and Delany, 2000). *In vitro* observations include absence of telomerase from non-transformed primary cells (CEFs) contrasted with telomerase activity in cultured blastodermal cells, cES cells and in every transformed avian cell type surveyed to date (Table 3).

As measured by mean TRF, the *in vitro* rate of telomere shortening observed in Class II arrays in CEFs is approximately 50 bp of telomeric DNA per population doubling. Yet calculation of percent telomeric DNA at representative passages revealed that an average of 63% of the terminal telomeric DNA was eroded in CEFs by senescence. The greatest loss of telomeric DNA occurred precipitously in later passages. These data suggest two mechanisms of telomere shortening: (1) telomere attrition due to the end-replication problem and (2) catastrophic erosion preceding culture arrest (Swanberg and Delany, 2003).

VII. Tools for utilizing the chicken in aging studies

A variety of techniques for the study of telomere biology are available including telomere terminal restriction fragment (TRF) analysis, fluorescence *in situ* hybridization (FISH), variations of polymerase chain reaction (PCR) and the telomere repeat amplification protocol (TRAP), For an excellent summary of selected methods utilized to measure telomere length, see Nakagawa et al.,

(2004). A list of references pertaining to techniques used in the study of telomere biology is contained in Table 4.

A. Telomere terminal restriction fragment analysis

First described in Harley et al., 1990, telomere terminal restriction fragment analysis establishes mean telomere length in a tissue or cell sample or percent of telomeric DNA present in one sample relative to another. To measure mean telomere length, genomic DNA is first digested with a restriction enzyme or a cocktail of restriction enzymes followed by electrophoretic separation through an agarose gel. It is essential that DNA concentration be equivalent in each lane. The gel is Southern blotted and hybridized to a TTAGGG(n) probe labeled with a radionuclide or a fluorochrome producing a smear of fragments. Densitometry readings taken at a number of locations along the smear are summed and averaged. Mean telomere length is defined as $\sum(OD_i)/\sum(OD_i/L_i)$ where OD_i is the densitometer output and L_i is the length of the DNA at position i. Sums are calculated over the range of lengths covered by the smear of TTAGGG-hybridized DNA (Harley et al., 1990, Swanberg and Delany, 2003).

In order to measure percent telomeric DNA present in one sample relative to a calibrator sample (Harley et al., 1990), DNA is restricted, separated by gel electrophoresis, Southern blotted and hybridized as with the determination of mean TRF length. However, rather than taking densitometry readings at discrete locations along the length of the smear of telomeric DNA, total telomeric DNA is measured by calculating the total integrated signal ($\sum OD_i$) over the same range of fragment sizes used for mean TRF analysis. Total integrated signal in this

range is measured in each lane of any given gel and results are expressed as a percentage of the signal from the earliest passage (Harley et al., 1990; Swanberg and Delany, 2003). The measurement of TRFs reveals a high degree of variability within cell lines prepared from single embryos of a highly inbred line and mean TRF measurements are also subject to variability resulting from drift in the subpopulations within a culture. Therefore it is advisable to assay using more than one method to obtain a biologically relevant picture of telomere attrition or erosion (Swanberg and Delany, 2003).

B. FISH

Telomere arrays have been examined in a wide sampling of avian species, including chicken, using fluorescence *in situ* hybridization (FISH) (Nanda and Schmid, 1994; Nanda et al., 2002). While the Nanda study was not quantitative, the existence of large telomere arrays in birds was quite apparent using traditional FISH techniques. Telomere quantitative fluorescence *in situ* hybridization (telomere Q-FISH) is a variation of this method which has been utilized effectively in several organisms. Using Q-FISH, telomere length is expressed as a ratio of telomere fluorescence in cells that have undergone erosion to telomere fluorescence in cells in the same tissue section with intact telomeres. The inherent disadvantage of Q-FISH is that only a small subset of telomeres can be examined at any one time relative to the bulk methods (e.g., TRF analysis) (Nakagawa et al., 2004 and references therein).

C. PCR-Based Methods for Telomere Length Measurement

A technique which addresses some of the limitations of Q-FISH, is single telomere length analysis (STELA). Using STELA, a 20-mer non-complementary oligonucleotide with a TTAGGG tail is linked to the G-rich 3' overhang of the telomere. The TTAGGG tail is then ligated to the complementary 5' strand of the telomere. PCR is performed using one primer for the linked oligo and a second primer recognizing unique subtelomeric sequence. Use of this technique requires identification of subtelomeric sequences, which has not yet been accomplished in avian species, but should be possible in chicken now that the genome is sequenced (Nakagawa et al., 2004 and references therein). Edges of telomeric DNA were identified in the draft sequence for the macrochromosomes (ICGSC, 2004, see supplementary information).

A second PCR-based technique which can be used to compare the abundance of telomere repeats is quantitative real-time PCR (Q-PCR). This technique quantifies the fold-difference between telomere-repeat copy number in an experimental sample compared to a reference DNA sample. Disadvantages of this method are that it does not determine absolute telomere length and that interstitial telomere sequences, present in avian species, will be measured as well as terminal repeats (Nakagawa et al., 2004 and references therein). This should not be a problem if telomere shortening is being measured, because the number of interstitial repeats should not change relative to terminal repeats unless dramatic genome reorganization such as a breakage-fusion-bridge cycle is occurring.

D. TRAP assay

The telomerase repeat amplification protocol (TRAP) assay, first described by Kim et al. (1994), relies upon primer extension of an oligonucletide by telomerase. Cells are lysed and cellular protein extracts are incubated with an oligonucletide to which a series of TTAGGG repeats will be added when telomerase is present in the cell extract. Variations of the TRAP assay exist including radioactive or non-radioactive gel-based detection, ELISA-based detection and semi-quantitative or quantitative protocols. For an excellent review of the TRAP assay and many of its iterations, see Saldanha et al. (2003).

E. Gene Expression Analysis

Real-time fluorescence-based PCR and RT-PCR have emerged as powerful methods for examining gene expression patterns in many contexts. In traditional PCR, an amplicon which accumulates after a predetermined number of cycles is analyzed by gel electrophoresis. In real-time PCR, reactions are characterized by the PCR cycle at which amplification of a target molecule is first detected by release of a fluorescent signal in real-time. The greater the quantity of the target molecule in the reaction mix, the earlier a significant increase in fluorescence will be measured. Quantitation is accomplished with reference to a threshold cycle, (C_t), defined as the fractional cycle number at which fluorescence, generated by the increase in PCR product, exceeds a set threshold above the baseline. For an excellent treatise on fluorescence-based real-time PCR, refer to Bustin “A-Z of Quantitative PCR” (2004).

Recently, real-time quantitative TaqMan PCR was utilized to look at expression of genes involved in chicken telomere maintenance pathways. Chicken primers and fluorescent probes were developed for six target genes (tankyrase 1, tankyrase 2, TRF1, TRF2, cTERT, cTR and c-myc) as well as for three housekeeping genes for normalization purposes. In cell culture, chicken GAPDH mRNA levels were found to show the least standard deviation for all samples examined and therefore GAPDH values were used to normalize the target gene values.

Analysis of mRNA expression patterns of the target genes in CEFs, DT40, the gastrula embryo and cES cells revealed up-regulation of tankyrase 2, TRF1, TRF2, c-myc, cTERT and cTR in DT40 cells, with c-myc levels up-regulated 184-fold in DT40 relative to the gastrula and 282-fold in DT40 relative to CEFs and cES cells. Telomerase holoenzyme components (cTERT and cTR) were present, although at low levels, in CEFs and were up-regulated in DT40, cES cells and the gastrula relative to CEFs. Down-regulation of TRF1, c-myc, cTERT and cTR appeared to be a feature of senescing CEFs which had survived an average of 30.5 PD (Swanberg et al., 2004; Swanberg and Delany, 2005). For a detailed discussion of these expression patterns as well as primer and probe sets, for target and housekeeping genes, see Swanberg et al., (2004) and Swanberg and Delany (2005).

F. Electroporation, RNAi and Morpholinos

One of the requirements for a good model system is the ability to do gain- and loss-of-function experiments. Techniques exist to perform such experiments

in chicken. A number of investigators utilize electroporation to introduce exogenous DNA into the chicken embryo *in ovo* (Muramatsu et al., 1997). For an excellent review of electroporation techniques *in ovo*, see Krull (2004). Loss-of-function experiments can be conducted by introducing short, interfering RNAs (siRNAs) or morpholinos into the chick embryo.

Double-stranded RNA-mediated interference (RNAi), a naturally occurring mechanism which results in the silencing of gene expression, has become a very powerful tool for experimental gene suppression in a number of organisms. The phenotypes observed with RNAi silencing of gene expression range from knockdown to knockout (Agrawal et al., 2003). RNAi was successfully exploited in chicken (*in ovo*) for gene silencing (Bourikas and Stoeckli, 2003; Pekarik et al., 2003; Krull 2004; Sato et al., 2004). In addition to siRNAs, RNAi morpholinos were used in loss-of-function studies in chicken. For example, Sheng et al. (2003) used morpholino oligonucleotides to knock down expression of genes in the future neural plate of the chicken embryo.

G. Genomic Tools

Information regarding web-based tools for sequence and bioinformatics analysis of avian species, BAC and cDNA libraries, chicken gene chips and a number of other websites of interest to researchers are contained in Table 5. For further detail on cDNA arrays for chicken gene expression analysis see Burnside et al. (2005). Tutorials oriented toward the biologist new to bioinformatics can be found in Antin and Konieczka (2005). Both Antin and Konieczka (2005) and

Dequeant and Pourquie (2005) describe additional resources for the study of chicken genomics.

H. Telomeres as a Tool for Age Determination in Birds

Estimating age in unmarked bird populations is of primary interest to many disciplines. The relationship between telomere shortening and chronological age was studied recently by determination of the telomere rate of change (TROC) or "telomere clock" in a number of bird species. Measuring the length of TRFs in DNA from erythrocytes and plotting mean telomere length against the maximum lifespan in years for each species, a correlation between TROC and lifespan was indicated. In most of the species studied, telomeres appeared to shorten more slowly in long-lived birds than short-lived birds. Interestingly, in a particularly long-lived bird, Leach's storm petrel, telomeres did not shorten with age, but lengthened (Vleck et al., 2003, Haussmann et al., 2003).

In another study examining DNA from erythrocytes it was found that while telomere length in blood cells declined between the chick stage and the adult in two species of long-lived seabirds, telomere length in adults was not related to age. This study cautioned that rates of telomere loss were not constant with age and that there was a great deal of inter-individual variation in the magnitude of telomere loss (Hall et al., 2004). It should be noted that avian erythrocytes are the product of erythroid progenitor cells capable of extended self-renewal (Beug et al., 1994) and therefore are likely to possess a significantly different telomere-length maintenance pathway than the majority of somatic cells whose telomeres typically demonstrate division-dependant telomere shortening. It would not,

therefore, be surprising to find that telomere shortening profiles in this renewable cell population would bear a greater resemblance to the profiles of other renewable tissues than to the telomeres of non-renewable cell populations such as fibroblasts.

VIII. Research Resources: stocks and lines, cells and cell lines

A variety of avian stocks and lines are available to the investigator of telomere biology. Genetic stocks and mutant lines are listed in an Avian Stocks Database linked to the Poultry and Avian Research Resources: Living Stock Populations website of the Animal Science Department, University of California, Davis (see Table 5 for URL). In addition, a selection of transformed and non-transformed avian cells and cell lines are available through the American Type Culture Collection (see Table 5 for URL). Protocols for primary culture of isolated chicken tissues can be found in Fresheny, Culture of Animal Cells A Manual of Basic Technique (2000).

IX. Husbandry

A number of excellent resources on basic chicken biology and husbandry are available including Sturkie's "Avian Physiology" (2000) and Scanes' "Poultry Science" (2004). In addition, the United States Department of Agriculture and several other agencies or associations provide both web-based and written materials on poultry husbandry and animal welfare. Table 5 contains web-based resources on poultry husbandry and related topics.

X. Conclusions

The study of telomere biology and telomere maintenance pathways has provided and will continue to provide a great deal of insight into the processes of replicative senescence, the relationship between cellular senescence and organismal aging, the genesis of cancer and the regenerative potential of embryonic stem cells. Use of *in vivo* and *in vitro* avian systems to facilitate research in these fields can only add to our body of knowledge. With the 6.6X draft sequence of the chicken genome now available, the chicken is a much more powerful model.

Investigation of telomere maintenance pathways in the chicken and other birds establishes, among other things, that non-renewable cells and tissues exhibit little or no telomerase activity accompanied by division-dependent telomere shortening; that embryonic cells and tissues as well as transformed cells exhibit high levels of telomerase; and that many telomere-associated genes are expressed differentially in pluripotent, differentiated and transformed cell systems, much as is seen in human systems. TERT and TR genes are transcribed in at least one telomerase-negative cell type, which suggests that the regulation of telomerase activity is more complex than merely switching the genes for telomerase enzyme components on and off. While telomere shortening profiles are unlikely to be the equivalent of rings on a tree for the determination of chronological age, comparisons of telomere status in pluripotent vs. differentiated, transformed vs. non-transformed and early passage vs. senescent cells are informative. Considerable work is necessary to fill in gaps,

but the chicken model for telomere biology offers the opportunity to study a vertebrate system free from many of the issues inherent in the murine model. Chickens, therefore, have the potential to become the new “lab rat” for aging research.

XI.

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Table 1. Chicken and human orthologs of genes involved in telomere maintenance pathways

Gene	Description	Accession	chromosome or locus	References
hTERT	H. sapiens telomerase reverse transcriptase	AF015950	5p15.33	Nakamura et al., 1007
cTERT	G. gallus telomerase reverse transcriptase	AY502592	2q21	Delany and Daniels, 2004
hTR	H. sapiens telomerase RNA	NR_001566	3q26	Feng et al., 1995
cTR	G. gallus telomerase RNA	AY312571	9q-terminal	Delany and Daniels, 2003
tankyrase 1	H. sapiens tankyrase 1	NM_003747	8p23.1	Broccoli et al, 1997
tankyrase 1	G. gallus tankyrase 1	AY142108	4	DeRyker et al., 2003
tankyrase 2	H. sapiens tankyrase 2	AF438201	10q23.3	Kaminker et al., 2001
tankyrase 2	G. gallus tankyrase 2	AY142107	unknown	DeRyker et al., 2003
TERF1/TRF1	H. sapiens telomeric repeat binding factor 1	NM_003218	8q13	Zhong et al., 1992
TERF1/TRF1	G. gallus telomeric repeat binding factor 1	AY237359	2	DeRyker et al., 2003
TERF2/TRF2	H. sapiens telomeric repeat binding factor 2	BC024890	16q22.1	Broccoli et al., 1997
TERF2/TRF2	G. gallus telomeric repeat binding factor 2	AJ133783	11	Konrad et al., 1999

Table 1. (continued)

Gene	Description	Accession	chromosome or locus	References
Rap 1	H. sapiens TRF2-interacting telomeric RAP1 protein	NM_204468	4	Tan et al., 2003
Rap 1	G. gallus TRF2-interacting telomeric RAP1 protein (RAP1) mRNA	AY083908	11	Tan et al., 2003
Pot 1	H. sapiens protection of telomeres 1	NM_015450	7q31.33	Bauman and Cech, 2001
Pot 1	G. gallus POT1 single-strand telomeric DNA-binding protein	AY555718	1	Wei and Price, 2004
WRN	H. Sapiens Werner Syndrome (WRN) protein	NM_000553	8p12-p11.2	Gray et al., 1997
WRN	G. gallus Werner Syndrome protein (WRN)	NM_001012888	4	Caldwell et al., 2005
BLM	H. Sapiens Bloom Syndrome (BLM) protein	NM_000057	15q26.1	Ellis et al., 1995
BLM	G. gallus Bloom Syndrome (BLM) protein	NM_001007087	10	Caldwell et al., 2005
c-myc	H. sapiens c-myc oncogene	V00568	8q24	Watt et al., 1983
c-myc	G. gallus c-myc oncogene	X68073	2	Harris et al., 1992

Table 2. Telomerase activity, telomere shortening and ease of immortalization in vertebrate model systems

Mouse	Human	Chicken
Moderate to high telomerase in somatic cells	Low or no telomerase in most somatic cells	Low or no telomerase in most somatic cells
No division-dependent telomere shortening	Division-dependent telomere shortening	Division-dependent telomere shortening
Fibroblasts spontaneously immortalize	Fibroblasts refractory to spontaneous immortalization	Fibroblasts refractory to spontaneous immortalization

Table 3. Telomerase Positive Transformed Avian Cell Lines

(Adapted from Swanberg and Delany, 2003)

Cell name	Description
RP-19	turkey B cell
DT40	chicken B cell (bursal lymphoma)
RP-9	chicken B cell (lymphoblastoid)
MSB-1	chicken T cell (spleen tumor cells in vitro)
MQ-NCSU	chicken macrophage (peripheral blood)
QT6	quail fibroblast (fibrosarcoma)
QT35	" " "
LMH & LMH/2A	chicken hepatocyte (hepatocellular carcinoma)

Table 4. References for the Study of Avian Telomere Biology

Telomere cytogenetics

Nanda and Schmid, 1994

Nanda et al., 2002

Delany et al., 2000

Replicative senescence in chicken cell culture

Lima et al., 1972

Lima and Macieira-Coelho, 1972

Measuring telomeres

Harley et al., 1990

Nakagawa et al., 2004

Telomere shortening in birds

Taylor and Delany, 2000

Delany et al., 2003

Swanberg and Delany, 2003

Telomeres as Tools for Age determination

Vleck et al., 2003

Haussmann et al., 2003

Hall et al., 2004

TRAP assay

Kim et al., 1994

Saldanha et al., 2003

Table 4. (continued)

Telomerase in birds

Taylor and Delany, 2000

Delany et al., 2000

Swanberg and Delany, 2003

Venkatesan and Price, 1998

Haussman et al., 2004

Gene expression patterns: telomere maintance pathways

Swanberg et al., 2004

Swanberg and Delany, 2005

Quantitative PCR

Bustin, 2004

Gain- and loss-of-function techniques in chick embryo

Krull, 2004 (electroporation)

Bourikas and Stoeckli, 2003 (RNAi)

Pekarik et al., 2003 (RNAi)

Sato et al., 2004 (RNAi)

Kos et al., 2003 (morpholinos)

Chicken Genome Sequence and Genomic Resources

ICGSC, 2004

Antin and Konieczka, 2005

Dequeant and Pourquie, 2005

Table 5. Internet resources for researchers in avian telomere biology

Taylor and Delany, 2000

Delany et al., 2000

Swanberg and Delany, 2003

Venkatesan and Price, 1998

Haussman et al., 2004

Gene expression patterns: telomere maintance pathways

Swanberg et al., 2004

Swanberg and Delany, 2005

Quantitative PCR

Bustin, 2004

Gain- and loss-of-function techniques in chick embryo

Krull, 2004 (electroporation)

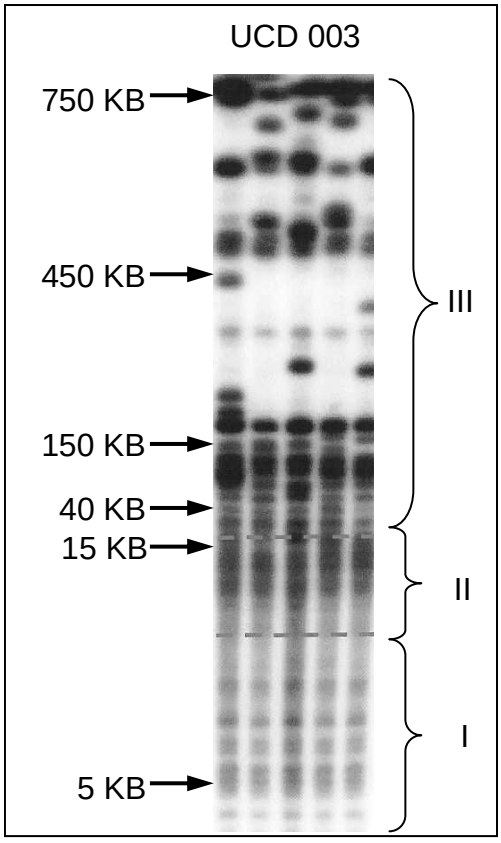
Bourikas and Stoeckli, 2003 (RNAi)

Pekarik et al., 2003 (RNAi)

Table 5. (Continued)

Ensemble Chicken Genome Browser	http://www.ensembl.org/Gallus_gallus/
Gallus gallus EST and in situ hybridization analysis database	http://geisha.biosci.arizona.edu/
Gallus gallus Trace Archive	http://www.ncbi.nih.gov/Traces/trace.cgi
GENEfinder Genomic Resources (Red Jungle and Chicken BAC Libraries) TAMU	http://hbz.tamu.edu/bacindex.html
NCBI Clone Registry	http://www.ncbi.nlm.nih.gov/genome/clone/query.cgi?EXPR=chicken
NCBI's compendium of chicken genomic resources	http://www.ncbi.nlm.nih.gov/projects/genome/guide/chicken/
NetVet	http://netvet.wustl.edu/birds.htm
Sanger Institute Chicken Genome Sequencing Project	http://www.sanger.ac.uk/Projects/G_gallus/
UD Chicken EST Database	http://www.chickest.udel.edu/
US Poultry Genome Website	http://poultry.mph.msu.edu/
Chicken Genome Array (Affymetrix)	http://www.affymetrix.com/products/arrays/specific/chicken.affx

Figure 1. Image of pulse-field gel showing chicken Class I, II and III telomere arrays. Class II arrays are analyzed for telomere shortening.



CHAPTER 2

Dynamics of Telomere Erosion in Transformed and Non-transformed Avian Cells in Vitro

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Published in *Cytogenetic and Genome Research* (2000)102:318-325

I. ABSTRACT

Although vertebrate telomeres are highly conserved, telomere dynamics and telomerase profiles vary among species. The objective of the present study was to examine telomerase activity and telomere length profiles of transformed and non-transformed avian cells in vitro. Non-transformed chicken embryo fibroblasts (CEFs) showed little or no telomerase activity from the earliest passages through senescence. Unexpectedly, a single culture of particularly long-lived senescent CEFs showed telomerase activity after over 250 days in culture. Transformed avian lines (six chicken, two quail and one turkey) and tumor samples (two chicken) exhibited telomerase activity. TRF profiles of non-transformed CEF cultures derived from individual embryos of an inbred line (UCD 003), exhibited cycles of shortening and lengthening with a substantial net loss of telomeric DNA by senescence. The TRF profiles of several transformed cell lines resembled TRF profiles of senescent CEFs in that they exhibited little of the typical smear of terminal restriction fragments suggesting that these transformed cells may possess a reduced amount of telomeric DNA. These results show that avian telomerase activity profiles are consistent with the telomerase activity profiles of human primary and transformed cells. Further, monitoring of telomere lengths of primary cells provides evidence for a dynamic series of changes over the lifespan of any specific cell culture ultimately resulting in net telomeric DNA loss by senescence.

II. INTRODUCTION

Telomere shortening is believed to occur due to regulation of telomerase, one of its components or accessory proteins (Forsyth et al., 2002; Karlseder et al., 2002; Chan et al., 2003). Shortening of telomeres can result in a loss of growth potential characteristic of replicative senescence, an increase in genome instability and cell death by induction of the DNA-damage response and apoptosis pathways. The length of the shortest telomere may trigger telomere dysfunction and loss of growth potential (Hemann et al., 2001a; Hemann et al., 2001b). In this model, short telomeres are recognized as damaged, signaling a G2/M cell cycle arrest affording the cell time to repair the damage. If the damage is not repaired, a checkpoint response results in further cell cycle arrest or apoptosis (Lee et al., 1998; Hemann et al., 2001b). Induction of the DNA-damage response by telomere shortening may be a protective genetic mechanism that prevents the proliferation of abnormal, aging cell lineages. Alternatively, senescence may be induced not by shortening of telomeres *per se* but by loss of the protective effect of accessory proteins or telomerase on capped telomeres (Chan et al., 2003; Karlseder et al., 2002). Regardless of the mechanism involved, a hallmark of tumorigenesis is the re-emergence of telomerase activity which enables tumor cells to evade DNA-damage pathways. Persistence of telomerase in immortalized cells may prevent apoptosis by stabilizing shortened telomeres. Conversely, re-activation of telomerase appears to induce resistance to apoptosis (Hahn et al., 1999; Herbert et al., 1999; Holt et al., 1999).

Although the vertebrate telomere repeat sequence is highly conserved, telomere organization, telomere dynamics and telomerase profiles vary among species (see Delany et al., 2000; Forsyth et al., 2002; and Delany et al. 2003 for review). *Gallus gallus domesticus*, the domestic chicken, has a long history as a model organism in developmental biology; is a significant resource for human vaccine production and research; and is also a globally important food-animal species.

Telomere array abundance, size, and location have been examined in the chicken genome (2n = 78, diploid size of 2.5 pg). Chicken telomere arrays range from 0.5 to 2 Mb and have been classified based upon size, chromosome location, Southern blot banding pattern, and age-related shortening. Class I arrays range from 0.5 to 8/10 kb, exhibit a Southern blot pattern of discrete bands and do not appear to shorten in a division-dependent manner. These arrays resist digestion by *Bal* 31 exonuclease suggesting an interstitial location. Class II arrays range from 8/10 kb to 35/40 kb, exhibit a smeared Southern blot pattern of overlapping telomere fragments and demonstrate division-dependent shortening. Class III arrays range in size from 40 kb to ~2 Mb, are rapidly digested by *Bal* 31 indicating a terminal location, exhibit hypervariable patterns of discrete bands when Southern blotted (even between individuals within a highly inbred line) and are the longest telomere arrays reported to date for any vertebrate (Delany et al., 2000; Taylor and Delany, 2000; Delany et al., 2003).

The objectives of the present study were to examine telomerase activity profiles and telomere length dynamics in transformed and non-transformed avian

cells in vitro within the context of the unusual features of the avian genome. The results indicate that the telomeres of chicken embryo fibroblasts in vitro undergo a dynamic series of events as evidenced by measurement of shorter and longer mean telomere restriction fragments over the life span of the cultures followed by a precipitous erosion of telomeric DNA at senescence. This dramatic erosion of telomeric DNA may be attributable to some as yet unknown active mechanism rather than passive attrition of telomeric sequence due to incomplete DNA replication. The results also suggest that the telomere profiles of at least some telomerase-positive transformed avian cell lines may provide evidence of catastrophic pre-transformation erosion of telomeres.

Our findings, which are consistent with studies of human cells in vitro wherein telomerase activity is absent from normal fibroblasts (which show a net loss of telomeric DNA over the lifetime of the culture) and present in transformed cell populations, provide evidence that chickens and other avian species possess telomere clock mechanisms and, in spite of the unique features of its genome, establish that the chicken is an appropriate and biologically relevant system for studies of human replicative senescence.

III. MATERIALS AND METHODS

A. Cell Culture

Chicken embryo fibroblasts (CEFs) were purchased from the American Type Culture Collection (ATCC) or isolated from E11 embryos to create pooled-embryo or individual-embryo cell cultures. For the pooled cell culture (CEF2), E11 commercial layer-type embryos (n=12) were utilized (Lima and Macieira-Coehlo, 1972). For individual-embryo cell cultures (CEF3 1 to 6), six E11 embryos (UCD 003 line; Pisenti et al., 2001) were utilized. Cell cultures maintained in DMEM with L-glutamine, 10% FBS, and 5% penicillin-streptomycin were split 1:3 or 1:4 until senescence. Senescence was determined by growth dynamics, cellular morphology and, in the case of CEF3 cultures, by a β-galactosidase assay (Dimri et al., 1995). Population doubling was determined for each passage of CEF3 cultures using the following equation:

$$\text{Population doubling} = \log N_t - \log N / \log 2$$

with N the number of cells seeded and N_t the number of viable cells at the end of the passage. (Patterson, 1979; Venkatesan and Price, 1998). Senescence staining was performed using the Senescence *β*-galactosidase Staining Kit (Cell Signaling Technology). Cultures were deemed senescent when >90% of the cells were positive for *β*-galactosidase. Cultures were maintained until no further samples could be extracted.

B. Transformed Cells

Transformed cells were acquired from a variety of sources. DT-40, LMH, and QT6 cells were obtained from the ATCC. DT-40's were also acquired from Dr. Jean-Marie Buerstedde at the Department of Cellular Immunology, Heinrich-Pette-Institute, Germany. LMH/2A, RP19, MSB1 and RB1B spleen and thymus tumor cells were donated by Dr. Carol Cardona of the University of California School of Veterinary Medicine, Davis CA. MQ-NCSU cells were acquired from Dr. Muquarrab Qureshi of the Department of Poultry Science North Carolina State University, Raleigh, NC. RP-9 cells were provided by Dr. Henry Hunt, USDA-ARS-ADOL, Michigan via Dr. Marcia Miller, Beckman Research Institute Duarte, CA. See Table 1 for a description of these cells and cell lines.

C. DNA Isolation and Analysis of Terminal Restriction Fragments (TRF)

Genomic DNA was extracted from CEFs spanning the earliest passages through senescence. DNA samples were isolated and purified using the AquaPure Genomic DNA Isolation Kit (BIORAD). Purified DNA samples were digested overnight with *Hae* III and quantified by fluorometry (Molecular Dynamics Fluorimager 595). Fifty ng of DNA per lane were separated by electrophoresis in 0.6% agarose gels for four hours at 55 volts. Using this protocol, high molecular weight Class I telomeric DNA is retained (i.e. does not migrate) and only Class II fragments are analyzed. Mean telomere length and percent telomeric DNA were determined for all lanes of each gel. To examine telomere shortening in a typical telomere restriction fragment smear, molecular weight markers were run on each gel. Prior to hybridization, each gel was stained

with ethidium bromide and photographed. The gels were destained, Southern-blotted and hybridized with a ^{32}P-labeled $TTAGGG_{(7)}$ probe as previously described (Taylor and Delany, 2000). Blots were exposed to Kodak BioMax MR film and the resulting autoradiographs were compared to the gel photographs. Molecular weight markers, determined with reference to the gel photographs, were noted on the autoradiographs. Autoradiographs were scanned and analyzed with Kodak 1D image analysis software version 3.6. Mean telomere length was defined as $\sum(OD_i \times Li)/\sum OD_i)$ (Taylor and Delany, 2000; Ramirez et al., 2003) with OD_i the net intensity (intensity – background) of the DNA at a given position on the gel and L_i the DNA length at that same position as measured by the image analysis software. OD_i and L_i measurements were made at 12 points along each lane of a typical blot. To supplement mean TRF analysis, total telomeric DNA, consisting of the total integrated signal ($\sum OD_i$) over the same range of fragment sizes used for mean TRF analysis, was determined for each lane by densitometry. Integrated signals from each lane were expressed as a percentage of the signal from early passage DNA as previously described (Harley et al., 1990).

D. Preparation of Cell Extracts and Telomerase Assays

Telomerase activity was assayed using the Telomeric Repeat Amplification Protocol (TRAP) (Kim et al., 1994) in which telomerase adds telomeric repeats to a synthetic oligonucleotide primer followed by PCR amplification. Cell extracts were prepared and analyzed using the TRAPeze

Telomerase Detection Kit (Intergen). Two micrograms of protein were used in each TRAP assay, with protein concentration determined by Bradford assay.

IV. RESULTS

A. Non-transformed Cells

i. Telomerase Profiles of Chicken Cells in Vitro

Typically, the CEF cultures derived from pooled or individual E11 embryos showed no telomerase activity from the earliest passages through senescence (see Table 1 and Figure 1A). Exceptions included low activity detected in CEF3 cultures 3 and 4 (the first 50 bp band of the telomerase oligonucleotide ladder was faintly evident) at PD_4 and PD_8 respectively, the earliest timepoints for which protein extract samples were prepared for these cultures (see Figure 2D, lane 3). Additionally, CEF3 culture 1 displayed a faint 50 bp band at $PD_{23,}$ a point in the culture's lifespan wherein more than 90% of the cells displayed a senescent phenotype as indicated by cell morphology and a positive β-galactosidase assay (data not shown). Notably, a single flask of senescent CEF2 cells which survived for over 250 days in culture showed telomerase activity (Figure 1B). CEFs ($PD_{18)}$ which were obtained from ATCC exhibited no telomerase activity (Figure 1A). Non-transformed chicken kidney fibroblasts and early stage chicken embryos were positive for telomerase (Table 1).

ii. Telomere Length Profiles and Loss of Telomeric DNA in Chicken Embryo Fibroblasts

The TRF Southern blots exhibited the expected smeared hybridization signal consisting of a series of overlapping TRF fragments ranging from about 8 to 23 kb, plus a broad band at 25-35 kb (which was excluded from this analysis). These TRF fragments represent the chicken Class II terminal telomere arrays previously determined to display division-dependent shortening (Delany et al., 2000; Taylor and Delany, 2000). Telomere length profiles derived from TRF smears of cultures 1 to 6 (CEF3) were unexpectedly variable, with mean telomere length increasing and decreasing throughout the lifespan of these cultures (see Figures 2B and C and 3A and B). In five cultures, mean telomere length from early passages to senescence showed a net decrease ranging in size from 621 to 2191 bp. However, as shown in Figure 3 and Table 2, a net increase in mean telomere length of 564 bp for CEF3 culture 3 was observed. These changes in mean telomere length produced a loss rate of 28 to 88 bp per cell division in the cultures with a decrease in mean TRF and a rate of increase of 25 bp per cell division in CEF3 culture 3. To supplement this data, a second measure of telomere shortening, loss of terminal telomeric DNA throughout the lifespan of each culture, was also examined by measuring integrated lane intensity over the same range of fragment sizes used to determine mean TRF length. Interestingly, in three cultures, terminal telomeric DNA exhibited increases ranging from 14 to 27% over the earliest PD's for which data were taken (data not shown). Subsequently all of the cultures demonstrated a striking loss of terminal telomeric DNA by senescence, with losses for all the cultures ranging from 40 to 85% (see Table 2, Figures 2B and 3).

B. Transformed Cells

i. Telomerase activity in transformed avian cells and cell lines

As indicated in Table 1 and Figure 1C, eleven transformed cell lines from three avian species (chicken, turkey and quail) representing five cell types including B and T cells, macrophages, hepatocytes and transformed fibroblasts, showed telomerase activity.

ii. Telomere Length Profiles and Percent Telomeric DNA in Transformed Avian Cells and Cell Lines

Notably, TRF profiles of MQ-NCSU cells, ATCC DT-40 cells, RP9 cells and LMH cells showed very little of the typical TRF smear of overlapping terminal restriction fragments (Figure 4). In some cases, the smear was nearly indiscernible by eye and only detectable by densitometry. In fact the profiles of transformed cells resembled the profiles of senescent CEFs in this respect (see Figure 4). Mean TRF lengths for the four transformed cell lines were longer than the mean TRF lengths of CEF3 cultures 1 to 6 at senescence (Table 3).

V. DISCUSSION

The present study examined telomere length dynamics of Class II avian telomere arrays and telomerase activity in avian cells and cell lines, illustrating variability in telomere length patterns and documenting that a number of transformed avian cells possess telomerase activity. In contrast, primary CEFs exhibit little or no telomerase activity with rare exceptions, including early or late passages of normal CEFs and one flask of senescent CEF2 cells that survived over 250 days in culture. An earlier study (Venkatesan and Price, 1998) provided evidence for down-regulation in vitro. Telomerase activity at an early point in the lifespan of a culture would be consistent with the presence of a small number of cells from a telomerase-positive population that had yet to be supplanted by the dominant telomerase-negative fibroblast cells. Theoretically, a small number of cells can provide a positive TRAP assay result (Kim et al., 1994). Alternately, down-regulation of telomerase activity in CEFs in culture might occur because telomerase activity could require factors not present in vitro. Telomerase activity in a senescent culture would be consistent with the establishment of a post-crisis population of cells exhibiting a dysregulated telomerase expression profile, perhaps as a precursor to transformation.

Six CEF3 cultures derived from embryos of a highly inbred line and possessing little or no telomerase activity, exhibited variable telomere profiles including lengthening and shortening throughout their lifespan (Table 1 and Figure 2). Most notably, all six cultures exhibited dramatic and potentially catastrophic loss of telomeric DNA by senescence (Table 2 and Figure 2). The

TRF blots for CEF3 cultures 1-6 reflected this loss of telomeric DNA in a diminished TRF smear. Interestingly, the TRF blots of transformed cells, all of which exhibited telomerase activity, exhibited longer mean TRF lengths than CEF3 cultures 1 to 6. However the TRF blots of transformed cells also showed little or no TRF smear, which may be evidence of pre-transformation telomere erosion.

The end-replication problem, wherein telomere shortening occurs passively as a result of incomplete replication of the parental DNA strands, explains only relatively small losses of telomeric DNA over the lifespan of a primary cell culture. Mean TRF length profiles provided evidence for losses of telomeric DNA in the range of 28 to 88 bp per population doubling, a relatively low rate of telomere attrition. However, by another measure, percent telomeric DNA, losses were dramatic. These losses of telomeric DNA, ranging from 40-85%, suggest that a mechanism other than incomplete end-replication is operating. Such a dramatic erosion of telomeric DNA may precede chromosomal end fusions (Chan et al., 2003). Catastrophic telomere erosion is an early event in DNA damage-induced apoptosis that may be induced by the release of reactive oxygen species due to loss of mitochondrial membrane potential (Ramirez et al., 2003) or by loss of the protective effect accessory proteins or telomerase afford telomeres (Chan et al., 2003).

Apparent increases in telomere length, as measured by mean TRF values, can be explained either by shifts in clonal populations or by critically short telomeres prompting a recombination pathway leading to telomere elongation

which in turn allows a few cells to regain proliferative potential and reestablish the culture (Ijpma and Greider, 2003 and references therein). Cells utilizing the mechanism referred to as alternative lengthening of telomeres or ALT (Reddell, 2003) may invoke recombination-mediated lengthening of critically shortened telomeres by strand invasion; annealing of a DNA strand from one telomere to the complementary strand of another telomere which acts as a template for synthesis of new telomere repeats (Dunham et al., 2000; Varley et al., 2002). A hallmark of tumorigenesis is either the persistence of telomerase or an ALT mechanism that enables tumor cells to evade DNA-damage pathways. Persistence of telomerase or induction of an ALT mechanism in immortalized cells may prevent apoptosis by stabilizing telomeres. The existence of an ALT mechanism in non-transformed CEFs is purely speculative as no normal cells with such a mechanism have been detected (Reddel 2003).

The inconsistency between the two measures of telomere shortening used in this study, mean TRF and percent telomeric DNA, may be explained in the context of emerging and declining cell lineages with varying TRF distributions. For example two cell populations, one with TRF values falling in a normal distribution over a broad range and the second with a skewed TRF distribution falling over a narrow range, can produce the same mean TRF. Thus the elimination of clonal populations (signaled by critically short telomeres) from the replicating pool of cells, followed by a second clonal population with different parameters assuming dominance within the culture, could eventually shift the mean TRF length upward or downward. This shift, however, might be

accompanied by an overall loss of telomeric DNA as a growing proportion of cells in the culture reach a telomeric crisis. The inconsistency between different measures of telomere shortening suggests that relying only upon mean TRF when analyzing telomere dynamics can produce misleading results.

Based upon our results, it is proposed that two distinct modes of telomere shortening were observed in the six CEF3 cultures. The first mode, telomere attrition due to the end-replication problem, induced cycles of telomere shortening with the demise of one lineage followed by the emergence of a new dominant lineage. This cycling of lineages within a culture could produce waves of lengthening and shortening. When all of the lineages of cells making up a culture had achieved critically short telomeres, senescence occurred followed by crisis. Crisis was accompanied by massive telomere loss due to a second mode of telomere shortening, telomere erosion, perhaps induced by oxidative DNA damage or down-regulation of telomere-associated proteins, followed ultimately by the demise of the culture. The lack of an intense Class II TRF smear in the blots of telomerase-positive, transformed cells examined in this study suggests that, due to the induction of a telomere-stabilizing mechanism such as the up-regulation of telomerase, these lineages were able to survive and proliferate despite loss of the normal chicken telomere length profile.

An alternate explanation for both the cycling in TRF length as well as the dramatic loss in telomeric DNA near the end of the lifespan of the CEF3 cultures might be a pattern of “breakage-fusion-ring-bridge-breakage” caused by end-fusion of chromosomes with short telomeres (McClintock, 1942). The shifting of

terminal telomeric sequences to an interstitial location due to end fusions followed by breakage of the fused chromosomes could produce the pattern of shortening and lengthening detected by changes in TRF smears for the CEF3 cultures.

The study of avian telomere biology is in its infancy relative to the knowledge base of other model systems, but holds much promise for the future. Rodents have long been used as model organisms for the study of human aging. However, the murine model may not be optimal for studies of human replicative senescence for a number of reasons. Wild-type rodent somatic cells can retain telomerase activity and do not appear to display division-dependent telomere shortening (Prowse and Greider, 1995; Forsyth et. al., 2002; Kim et al., 2002). Both human and chicken somatic cells lack telomerase; with down-regulation of telomerase activity occurring early in development (Forsyth et al., 2002). Recent studies have demonstrated division-dependent telomere shortening in chicken chromosomes both in vivo and in vitro and the ontological down-regulation of telomerase in most chicken somatic tissues in vivo (Delany et al., 2000, Taylor and Delany, 2000). Chicken and human primary fibroblast cells are generally refractory to spontaneous immortalization, in contrast to mouse fibroblasts. (Lima and Macieira-Coelho, 1972; Lima et al*.,* 1972; Macieira-Coelho and Azzarone, 1988; Prowse and Greider, 1995). Also, there is a fundamental difference between human and mouse telomere damage signaling mechanisms (Smogorzewska and de Lange, 2002). Many of the known telomere proteins, including TRF1, TRF2, Pot1, RAP1 and tankyrase are highly conserved between

chicken and human (Konrad et al., 1999; Price, 2001; Wei et al., 2002 and references therein).

The results of this study further establish the similarities between human and avian telomere biology by demonstrating that telomere dynamics in non-transformed and transformed chicken cells are consistent with what has been observed in human cells. The significance of these similarities is not diminished by the existence of megabase telomere arrays in chicken as it appears to be the shortest telomere, not average telomere length that is critical for cell viability and chromosome stability (Hemann et al., 2001a). It is foreseeable, therefore, that the chicken may prove to be an extremely important model for studies of human cellular senescence and cellular transformation. Further research utilizing molecular and cytogenetic techniques, protein expression profiles and apoptosis assays will provide valuable insight into telomere-mediated pathways to senescence and oncogenesis in chickens and other birds.

VI.

ACKNOWLEDGMENTS

The authors thank Drs. Buerstedde, Cardona, Qureshi, Hunt and Miller for donations of cells and cell lines. Research was funded by the USDA-NRI-CGP (99-35205-8369, 02-35205-11568).

VII.

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Table 1. Telomerase profiles of avian cells and cell lines

Designation	Telomerase	Description	Agent of Transformation
Non-transformed			
SL-29	-	ATCC[1] CEF[2] PD^{3}_{18}	
CEF2	-	P_0[4] - P_{15}	
CEF2[5]	+	> 250 days in culture	
CEF3-1	PD_{23} only	PD_1 - PD_{23}	
CEF3-2	PD_4 only	PD_3 - PD_{30}	
CEF3-3	PD_8 only	PD_4 - PD_{27}	
CEF3-4	-	PD_8 - PD_{26}	
CEF3-5	-	$PD_{0.4}$ - PD_{24}	
CEF3-6	-	$PD_{1.5}$ - PD_{26}	
kidney	+	fibroblast	
pre-blastula (Stage X)	+	embryo	
gastrula or neurula	+	embryo	
Transformed			
in vitro			
RP-19[6]	+	turkey B cell	MDV[7]
DT40[8]	+	B cell (bursal lymphoma)	ALV[9]
RP-9[10]	+	B cell (lymphoblastoid)	ALV
MSB-1[11]	+	T cell (spleen tumor cells in vitro)	MDV
MQ-NCSU[12]	+	macrophage (spleen phagocyte)	MDV
QT6[13]	+	quail fibroblast (fibrosarcoma)	MC[14]
QT35[15]	+	" " "	MC
LMH & LMH/2A[16]	+	hepatocyte (hepatocellular carcinoma)	DEN[17]
293 cells	+	human embryonic kidney	HAdV-5[18]
in vivo			
spleen	+	tumor cells[19]	MDV (RB1B)[20]
thymus	+	"	"

All of the above are chicken cells except where indicated.

[1] American Type Culture Collection

[2] CEF = Chicken embryo fibroblast

[3] PD_n = Population doubling

[4] P_n = passage number

[5] non-transformed at culture initiation, earlier passages were telomerase negative

[6] Nazerian et al. (1982); Nazerian (1987)

[7] Marek's disease virus

[8] Humphries and Zhang (1992)

[9] Avian leukosis virus

[10] Okazaki et al. (1980)

[11] Akiyama and Kato (1974)

[12] Qureshi et al. (1990)

[13] Moscovici et al. (1977)

[14] Methylcholanthrene

[15] Moscovici et al. (1977)

[16] Kawaguchi et al. (1987)

[17] Diethylnitrosamine

[18] Human adenovirus type 5

[19] Personal communication from Dr. Carol Cardona of the UC Davis School of Veterinary Medicine

[20] Ross (1989)

Table 2. Telomere shortening and loss of telomeric DNA in six primary CEF cultures. Five of the six cultures showed an overall decrease in mean TRF length. The decrease in mean TRF for these five cultures ranged from 28 to 88 bp per cell division. The total telomeric DNA losses for all six cultures ranged from 40% to 85%.

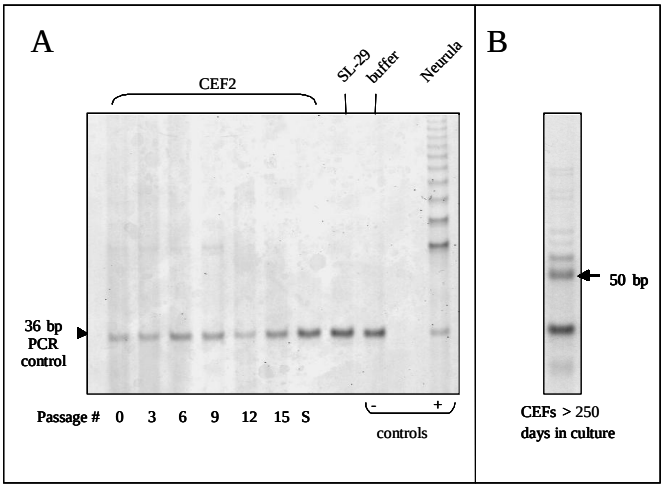
Culture	Mean TRF Length (bp)		Δ Mean TRF Length (bp)	Δ Mean TRF/ Population doubling (bp)[1]	Loss of Telomeric DNA[2]
	Early	Senescent			
CEF3-1	13,832 (PD_1)	13,211 (PD_{23})	-621 (PD_1 : PD_{23})	-28.0 bp	69% (PD_1 : PD_{23})
CEF3-2	13,934 (PD_3)	12,840 (PD_{30})	-1094 (PD_3 : PD_{30})	-40.5 bp	69% (PD_3 : PD_{30})
CEF3-3	10,468 (PD_4)	11,032 (PD_{27})	+564 ($PD_{8.5}$: PD_{27})	+ 24.5 bp	54% (PD_4 : PD_{27})
CEF3-4	16,999 (PD_8)	15,205 (PD_{26})	-1794 (PD_{10} : PD_{26})	-87.5 bp	85% ($PD_{5.5}$: PD_{24})
CEF3-5	17,097 (PD_{04})	15,655 (PD_{24})	-1442 ($PD_{0.5}$: PD_{24})	-61.0 bp	63% (PD_{04} : PD_{24})
CEF3-6	13,569 ($PD_{1.5}$)	11,378 (PD_{26})	-2191 ($PD_{1.5}$: PD_{26})	-88.0 bp	40% (PD_1 : PD_{26})

[1] Based on TRF smear analysis.

[2] Based on total net lane intensities. Integrated signals from each lane were expressed as a percentage of the signal from early passage DNA as previously described (Harley et al., 1990).

Figure 1. Telomerase activity is lacking in primary CEFs and present in transformed avian cell lines and tumors. (A) Representative TRAP assay results showing lack of telomerase activity in the CEF2 culture in passages 0-15, senescent CEF2 cells (S) and in ATCC CEFs (SL-29). (B) Positive TRAP assay results of CEF2 cells >250 days in culture. (C) TRAP assays of transformed avian cell lines and tumors. Lane 12:human embryonic kidney cells (positive control). Lane 13:chicken neurula (positive control). Negative controls not shown. See Table 1 for details on cell cultures and tumors.

Figure 1.
Swanberg and Delany (2003)



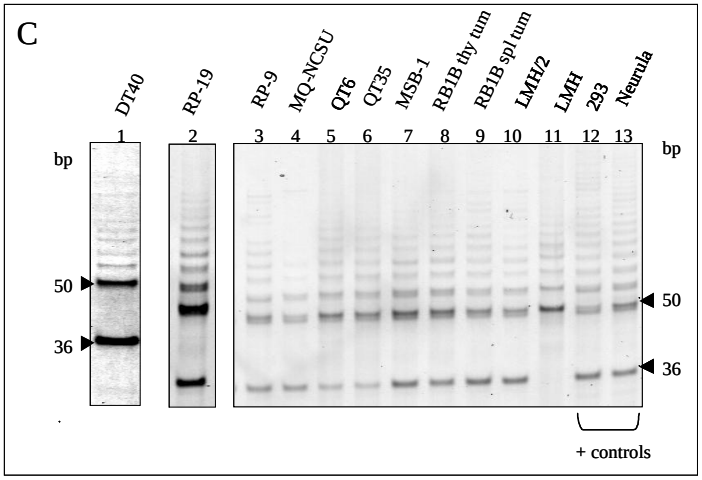


Figure 2. Telomere dynamics, telomerase profiles and growth data for a representative CEF culture. (A) Growth curve for CEF3-4 culture showing cumulative increase in population doubling over the lifetime of the culture and plateau as population doublings decrease (see Materials and Methods). (B) Telomere shortening profile illustrates division-dependent telomere shortening (utilizing mean TRF values) from PD $_{5.5}$ to PD_{26} and loss of telomeric DNA (percent telomeric DNA derived from the ratio of the values for PD_n to PD_{26}) for the same timepoints. (C) Terminal restriction fragment (TRF) blot for $PD_{5.5}$ to PD_{26} with 50 ng of *Hae* III-digested genomic DNA in each lane. (D) TRAP assays for CEF3-4 from PD_8 to PD_{26} assay exhibit lack of telomerase activity except at PD_8 (lane 3) where there is a faint 50 bp band visible. Lane 1: 10 bp DNA ladder, Lane 2: blank, Lanes 3-9: PD_8 to PD_{26}, Lanes 10 and 11: human 293 cells (positive control), Lane 12: CHAPS buffer with no cell extract (negative control), Lane 13: chicken neurula (positive control).

Figure 2.
Swanberg and Delany (2003)

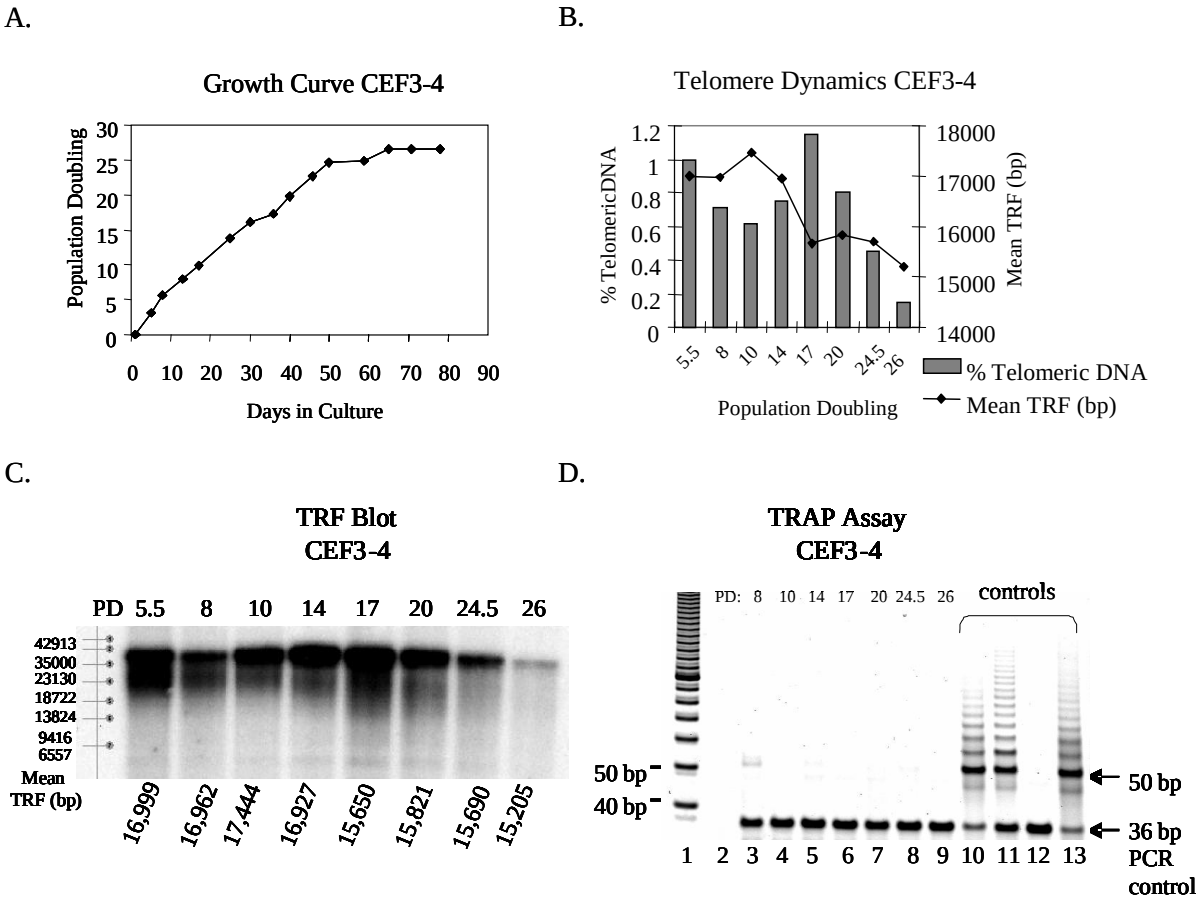
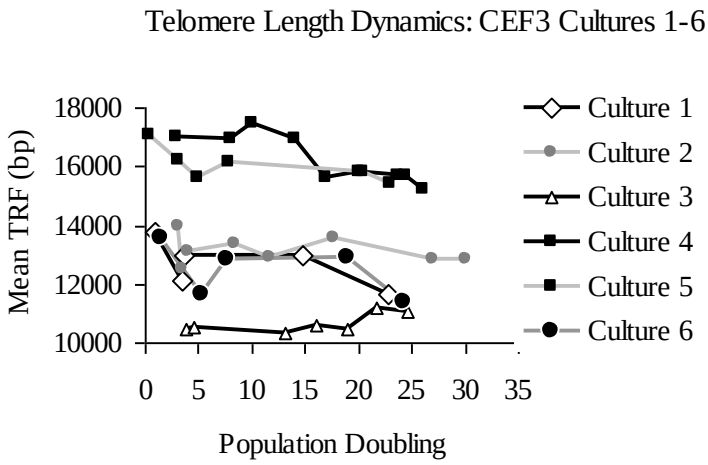


Figure 3.
Swanberg and Delany (2003)

A.



B.

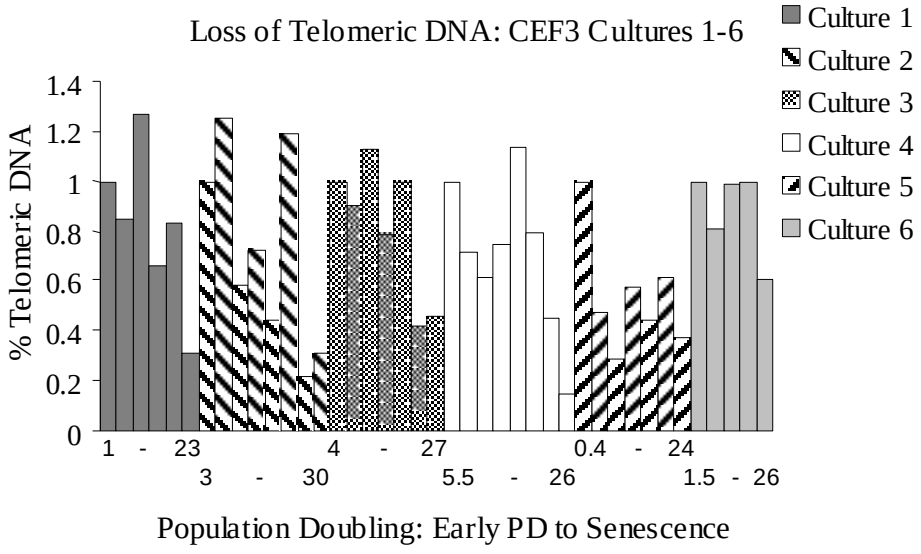
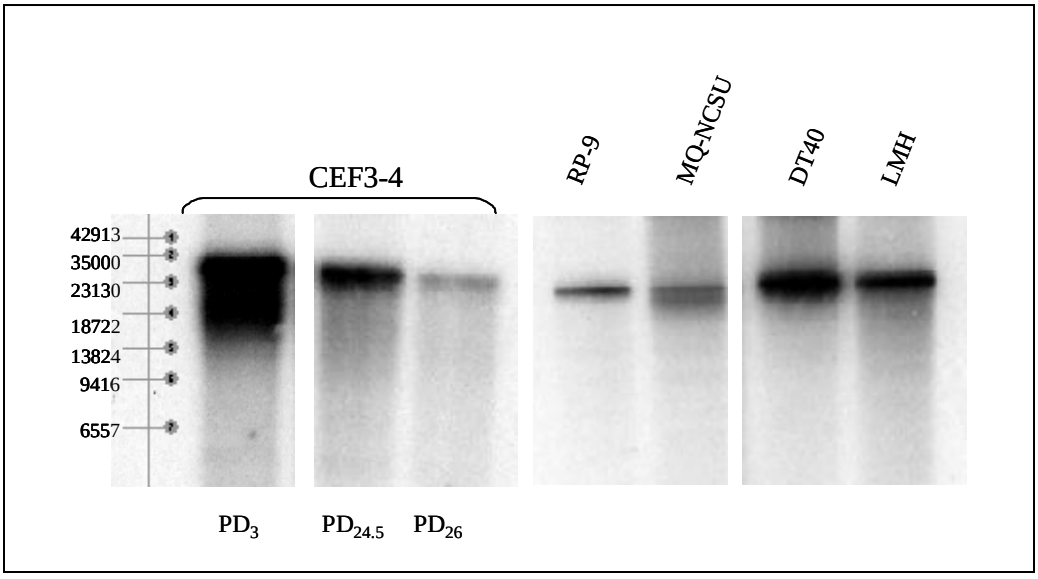


Figure 4. TRF profiles of transformed cells resemble those of senescent CEFs lacking the typical smear of overlapping TRF fragments. Representative TRF blots of samples from CEF3-4 cell culture and transformed cell lines. By senescence, much of the telomeric DNA smear in the CEF culture has disappeared. Interestingly, TRF blots of the transformed cell lines also show little or no TRF smear indicating that, relative to early passage CEFs, these transformed cells may possess a reduced amount of terminal telomeric DNA.

Figure 4.
Swanberg and Delany (2003)



Telomerase Activity and Differential Expression of Telomerase Genes and c-myc in Chicken Cells *in vitro*

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Published in *Developmental Dynamics* (2005) 231(1):14-21

I. ABSTRACT

This study examined telomerase activity and gene expression profiles for three genes in *Gallus gallus domesticus*: telomerase reverse transcriptase (chTERT), telomerase RNA (chTR), and c-myc. Expression of these genes was studied in chicken embryonic stem (chES) cells, chicken embryo fibroblasts (CEFs), and DT40 cells using quantitative real-time RT-PCR. Our results establish that, relative to transcription levels in telomerase-negative CEFs, chTERT and chTR are up-regulated in telomerase-positive chES cells. Transcription levels of chTERT, chTR and c-myc are dramatically up-regulated in telomerase-positive DT40 cells, relative to CEFs and chES cells. These results are consistent with a model in which telomerase activity is up-regulated in proliferating embryonic stem cells requiring stable telomeres to endure multiple rounds of cell division, down-regulated in differentiated, lifespan-limited cells and up-regulated in immortalized, transformed cells where uncontrolled proliferation is correlated with c-myc dysregulation and telomerase activity.

II. INTRODUCTION

The completion of the chicken genome sequence has intensified the level of interest in this organism and increased the need for basic and applied research in chicken including the study of embryonic stem (ES) cells. ES cells are undifferentiated pluripotent cells derived from an embryonic cell population prior to commitment of the cells to a particular developmental pathway. These cells are an important tool for developmental biology research, creation of transgenic systems for production of human therapeutic biologics in animal species and for basic applications such as knock in/out gene function studies. Further, ES cells are a critical component of regenerative tissue and engineering systems for human medicine (Rippon and Bishop, 2004). Mammalian ES cells are derived from a cluster of cells inside the pre-implantation blastocyst, the inner cell mass or ICM, which is the population of cells from which the entire embryo is generated (Evans and Kaufman, 1981; Nichols et al., 1990).

Despite their common ICM origin, ES cells from different species exhibit variations in morphology, phenotype and potency (Pain et al., 1996 and references therein; Verfaillie et al., 2002; Rippon and Bishop, 2004). Therefore, applying the designation “ES” to new cell lines is typically deferred until key characteristics are established. For example, features of mammalian ES cells include a normal karyotype *in vivo* and *in vitro* and indefinite propagation under a diverse set of favorable conditions which may include murine feeder layers and cytokines or growth factors. Species-dependent expression of embryonic or primordial germ cell biomarkers is observed in ES cells, including alkaline

phosphatase activity and cell surface antigens such as ECMA-7, SSEA-1,-3, and EMA-1,-6 (Strickland et al., 1980; Kemler, 1981; Hahnel and Eddy, 1987; Urven et al., 1988). Verfaillie et al., (2002) suggest that distinguishing criteria for ES cell designation should include (1) the capacity to differentiate into multiple cell types representing ectoderm, endoderm or mesoderm *in vitro*, (2) the capacity to contribute to all cell lineages including germ line upon implantation into a host embryo, and (3) demonstration that a cell line established from a single ES cell (i.e., clonally derived) possesses the pluripotent character of the original ES culture from which it was derived.

In the chicken, the “blastoderm” embryo (Stages IX- XI: Eyal-Giladi and Kochav, 1976) found within the egg at the time it is laid, consists of ~50,000 cells and is equivalent to the mammalian morula. Chicken blastodermal-derived cells cultured *in vitro* exhibit many of the features of mammalian ES cells and therefore are considered chicken embryonic stem cells (chES cells). These features include amenability to long-term culture as well as alkaline phosphatase activity and antigenic marker expression (e.g., EMA-1, SSEA-1,-3). Evidence for the capacity of chES cells to contribute to all lineages has been shown by the generation of somatic and germline chimeras and the condition-dependent *in vitro* differentiation of chES cells into diverse cell types [Pain et al., 1996; Dinsmore et al., 1998; Pain et al., 1999; Tsai et al., 1999 (US Patent 6,140,118)].

Constitutive telomerase activity is a feature of mammalian ES cell systems (Thomson et al., 1998; Amit et al., 2000; Xu et al., 2001; Verfaillie et al., 2002; Carpenter et al., 2003). Telomerase is the enzyme responsible for replicating and

maintaining the telomeres of proliferating cells (Blackburn and Gall, 1978). Telomere erosion due to a lack of telomerase is a well-studied mechanism which can promote cellular senescence or induce apoptosis (Forsyth et al., 2002; Swanberg and Delany, 2003 and references therein). Constitutive telomerase activity in ES cells may enable them to evade the telomere clock mechanism which might otherwise impose a limitation on the proliferative potential of individual cells and lineages.

The status of telomerase activity is unknown in long-term cultures of chES cells maintained in the absence of feeder layers or cytokine supplementation. Therefore, in this study we sought to establish telomerase activity profiles in chES cells cultured up to 181 days without feeder layers or cytokines. Further, to explore the underlying basis for these telomerase activity profiles, we studied mRNA expression levels of the genes which encode the components of the telomerase holoenzyme, chicken telomerase RNA (chTR) and chicken telomerase reverse transcriptase (chTERT). We also examined the transcript levels of c-myc, a transcription factor known to be involved in the regulation of TERT expression. The gene expression profiles from the chES cultures were compared to corresponding profiles of telomerase-negative primary chicken embryo fibroblasts (CEFs) and telomerase-positive transformed DT40 cells.

This study represents the first time these genes have been studied utilizing a highly sensitive quantitative real-time RT-PCR assay and the first time chTERT and chTR expression have been studied in any context. Our results reveal distinct gene expression patterns among c-myc, chTERT, and chTR in

three *in vitro* chicken systems: chES cells, CEFs and DT40 cells, thereby providing insight into the mechanisms that may contribute to cellular immortalization in non-transformed and transformed cells in chicken.

III. RESULTS

A. TRAP Assay

Table 1 summarizes the TRAP results and Figure 1 shows representative TRAP gels for the blastodermal embryos and chES cultures. Five of six pooled blastodermal embryo samples collected from unincubated, embryonated eggs, exhibited a positive telomerase activity profile as measured by the PCR-based telomere repeat amplification protocol (TRAP) (Kim *et al*., 1994). The remaining sample exhibited no telomerase activity. Eighteen of twenty-one samples of chES cells in culture for periods ranging from seven to 181 days exhibited telomerase activity. A definitive conclusion regarding telomerase activity in the remaining three samples could not be drawn due to technical artifacts which could not be resolved. The results for these samples were therefore designated as inconclusive (Table 1). Representative TRAP results are illustrated in Figure 1. Of all the samples tested, 10 were from cultures older than 100 days. Nine of these samples were telomerase positive and the result for the remaining sample was inconclusive. Whether or not supplements such as chicken embryo extract (CEE) or turkey navel extract (TNE) were added to the media, chES samples were telomerase-positive (Table 1). As a check on antigenic status of the cells, a commonly used antigenic cell marker for ES cells, EMA-1, was examined in two long-term chES cultures. Cells from both samples were positive for EMA-1 (see Table 1 and Figure 2).

Because mouse STO cells are commonly employed as feeder cell layers for ES systems, including chES cells, and since mouse cells are typically

telomerase positive, irradiated and non-irradiated STO cells were examined for telomerase activity to determine the potential impact of this cell layer on telomerase assay results. In both cases, the STO samples were positive for telomerase activity and exhibited a TRAP ladder consistent with the non-processive nature of mouse telomerase (Prowse et al., 1993), see Figure 1.

B. Gene Transcript Analysis

Relative transcript levels of chTERT, chTR, and c-myc mRNAs were examined using quantitative real-time RT-PCR in telomerase-positive chES cultures #24 and #25 (Table 1), cultured for 148 and 153 days, respectively. Gene-specific values from three replicate samples per culture were averaged and an overall average for the two cultures was calculated for each gene. The chES results were compared to those from early passage (~ 5 population doublings) CEFs and results from DT40 cells as described in the methods below.

Figure 3 illustrates the relative mRNA levels of chTERT, chTR and c-myc among the three cell systems examined. Surprisingly, detectable levels of chTERT were observed in telomerase-negative CEFs. The chES cells exhibited moderately high levels of both chTR and chTERT (11-fold and 15-fold elevation, respectively) in comparison to early passage CEFs. In contrast, the mRNA levels of c-myc were similar in chES cells and CEFs. The DT40 mRNA levels for all three genes were dramatically higher when compared to the CEFs, 155-, 282- and 324-fold higher for chTERT, c-myc and chTR, respectively. When compared to chES cells, the DT40 values for c-myc, chTERT and chTR were 10-, 30- and 282-fold higher, respectively. Interestingly, the chTERT/chTR ratios for chES

cells and CEFs were the same, 6.4 to 1, while the chTERT/chTR ratio in DT40 cells was 3.1 to 1 (Figure 4).

IV. DISCUSSION

In this study we examined the expression of telomerase and telomerase-related genes in chES cells in culture, comparing and contrasting chES expression patterns to patterns seen in CEFs and DT40 cells, thereby contributing new information regarding telomerase activity and the telomere clock mechanism to the field of chicken stem cell biology. A majority of the blastoderm embryo samples derived from unincubated eggs (*in vivo* samples i.e., no culturing) exhibited telomerase activity which is consistent with previous reports (Taylor and Delany, 2000; Swanberg and Delany, 2003). A majority of cultured chES cells derived from the blastodermal embryos also exhibited telomerase activity. The chES cells exhibited telomerase activity, regardless of which of several conditions were used to grow the cells. The results support the conclusion that telomerase activity is a general feature of chES cells in culture, as also observed for mammalian ES cells.

Our results are consistent with a prior study that reported positive telomerase activity in chicken embryonic cells (designated CECs) grown *in vitro* long term (Pain, et al., 1996). However, in that study, the CECs were grown on STO feeder cell layers and, in some cases, in the presence of specific growth factors such as LIF (leukemia inhibitory factor). ES culture systems often utilize feeder cell layers, such as mouse STO cells, to provide growth factors which maintain ES cells in an undifferentiated state (Park, et al., 2003; Rippon and Bishop 2004). In our study, STO cells were found to exhibit telomerase activity (see Figure 1). Therefore, the use of mouse STO cells as feeder layers in chES

culture systems should be regarded as a confounding factor when utilizing telomerase activity as a biomarker for ES cell status.

During embryonic development both human and chicken tissues exhibit down-regulation of telomerase activity coincident with organogenesis and somatic differentiation (Forsyth et al. 2002; Delany et al., 2003). Down-regulation of telomerase is considered a sign of differentiation (Sharma et al., 1995). Here we found that telomerase is active in proliferating, telomerase-positive chES cells that exhibit moderately elevated levels of TERT and TR expression compared to telomerase negative CEFs (Swanberg and Delany, 2003) which are derived from cells of E11 embryos wherein organogenesis and differentiation of cell layers is essentially complete. We also found that c-myc does not appear to be dysregulated in telomerase-positive chES cells that exhibit elevated levels of chTERT and chTR mRNA (compared to telomerase negative CEFs). In fact, the levels of c-myc expression were identical in the CEFs and chES cells. In contrast, c-myc mRNA is considerably elevated in telomerase-positive, transformed DT40 cells, accompanied by 10- and 30-fold increases in chTERT and chTR (compared to chES cells).

Levels of c-myc mRNA in DT40 cells were dramatically higher than in either chES cells or CEFs (282-fold higher). High expression of c-myc mRNA in DT40s was reported previously (Baba et al., 1985; Neiman et al., 2001) and is attributed to activation of the c-myc gene by the transforming agent, avian leucosis virus. A number of studies suggest that c-myc re-activates telomerase in transformed cells by inducing expression of the catalytic subunit TERT (Wu et al.,

1999). The c-myc protein mediates transcriptional activity of a target gene through binding to E-box elements with the core sequence CACGTG located in the promoter or enhancer region of the target (Kuramoto et al., 1999). Interestingly Falchetti et al., (1999) found that telomerase activity emerged in v-myc-transformed avian cells that had previously been telomerase negative.

Chicken TERT was only recently cloned, sequenced and analyzed for regulatory features. The chTERT regulatory sequence was found to contain one E-box and chTR 3′ sequences contain c-myc binding sites suggesting that TR may also be subject to regulation by c-myc (Delany and Daniels, in press; Delany and Daniels, 2003). DT40 chTR levels in this study were 324-fold higher than found in CEFs and the chTERT/chTR ratio in DT40 cells was 3.1 to 1 as opposed to the 6.4 to 1 ratio observed in CEFs and chES cells. These ratios are noteworthy as TR up-regulation is a hallmark of some human cancers (Avilion et al., 1996, Soder et al., 1997; Soder et al. 1998; Naito et al. 2001).

Chicken and human share many features of the telomere clock mechanism, including telomere shortening and down-regulation of telomerase in most somatic cells (Delany et al., 2003). Regulated expression of c-myc, chTERT and chTR is a feature of normally proliferating human cell populations, including embryonic stem cells. On the other hand, dys-regulation of c-myc accompanied by re-activation or over-expression of telomerase is a common attribute of the transformed phenotype in many human cancers. Substantial evidence in human suggests that c-myc can induce transcription of TERT, which is thought to be a rate-limiting determinant in the activation of telomerase. The results of this study

are consistent with the human model in that telomerase activity is moderately up-regulated in proliferating embryonic stem cells (e.g., chES) which require stable telomeres in order to endure multiple rounds of cell division, down-regulated in differentiated, lifespan-limited cells (e.g., CEFs) and dramatically up-regulated in immortalized, transformed cells (e.g., DT40 cells) where uncontrolled proliferation is associated with c-myc dysregulation and telomerase activity.

V. EXPERIMENTAL PROCEDURES

A. Chicken Embryonic (chES) Cells

Blastoderm embryos from unincubated SJ line chicken eggs (Stages IX-XI, Eyal-Giladi and Kochav, 1976) were harvested in unsupplemented LM medium (8.8 g/l Leibowitz L-15, 5.0 g/l McCoy 5A, 1.5 g/l $NaHCO_3$), gently agitated, and then spun at 200 x g for 5 minutes to remove yolk material. The supernatant was withdrawn and the blastodermal embryos were resuspended in 25 ml of LM in a 50 ml tube. After adding LM, the embryos were manually shaken for 30 seconds to dissociate the tissue into individual cells or small clumps of cells, poured through a 100 µm cell strainer (Falcon) and plated at ~ 2 x 10^5 cells/ml. Cell cultures were grown in 100 milimeter cell culture plates at 39^0C with 5% CO_2 in LM medium with 20% FBS, 2.0 mM Glutamax (GIBCOBRL), 8.0 mM additional NaHCO3, 1.0 mM sodium pyruvate, 5 µg/ml Gentamycin and 4µg/ml Fungizone. In some cases, cells were grown on a Costar Transwell 75mm polycarbonate membrane insert with 0.4 µm pores for maximum exposure to the medium.

In some culture systems 5% chicken embryo extract (CEE) and/or turkey navel extract (TNE) [Tsai et al., 1999 (US Patent 6,140,118)] were added to the culture medium to assess their effect on cell growth. Samples from chES cultures #24 and #25 (Table 1), cultured for 148 and 153 days, respectively, were used in the transcript analysis.

B. Chicken Embryo Extract (CEE) Preparation

Three hundred E10 chicken embryos were added to 100 ml unsupplemented LM medium. The medium was poured off, centrifuged briefly to remove red blood cells, and then added back to the tissue. This mixture was homogenized in a blender for 1 min, transferred to a plastic centrifuge bottle, incubated at 4^0 C for 1 hr, and frozen at -80^0 C. The extract was thawed and re-frozen an additional two times. The extract was centrifuged at 7,000 rpm in a Sorvall GS3 rotor for 1 hr, dispensed into 50 ml aliquots, and frozen at -80^0C. After adding CEE to the growth medium, the complete medium was sterilized by filtration.

C. Turkey Navel Extract (TNE)

The small opening in the abdominal area of an avian embryo, sometimes referred to as the “navel”, is in reality the opening to the yolk sac stalk. TNE was prepared from hatching turkey embryos by excising and homogenizing the skin around the umbilical cord as described in detail in US Patent 6,140,118 (Tsai et al., 1999).

D. EMA-1 Detection

Status of the primordial germ cell surface marker EMA-1 was examined on chES culture by indirect immunofluorescence. A mouse anti-EMA-1 (IgM) was obtained from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, 007 Biology Building East, Iowa City, IA 522542. Biotinylated goat anti-mouse IgM (□-chain specific) and FITC-conjugated Streptavidin were obtained from Sigma (St. Louis, MO). The EMA-1 antibody was used at 1:100 dilution. The remaining antibodies were used at the manufacturer's recommended dilution. The cells were stained and analyzed in the dish in which they were grown. Cells were incubated with the anti-EMA-1 monoclonal antibody for 20 minutes, washed with media three times, incubated with the goat anti-IgM for 20 minutes, washed three times with media, incubated with FITC-Strepavidin for five minutes, and washed three times with PBS. The cells were examined using a Leica DM1RB (Inverted UV microscope) fitted with an Optronics DE1-750 CE digital output image capture system.

E. Chicken Embryo Fibroblasts (CEFs)

Chicken embryo fibroblasts (CEFs) were isolated from six E11 embryos from the UCD 003 line and maintained as described in Swanberg and Delany, 2003. Values from six cultures were averaged for the transcript analysis.

F. DT40 Cells

DT-40 cells were obtained from the American Type Culture Collection (ATTC CRL-2111) and from Dr. Jean-Marie Buerstedde (GSF-National Research Center for Environment and Health, Neuherberg, Germany). Culture conditions

were followed as outlined previously (see Media I formulation in Chang and Delany, 2004). Three cultures from each DT40 source were used for transcript analysis; a final value was derived from the average of the six cultures as the individual culture results were similar.

G. Telomerase Activity Detection

Cell extracts for the telomerase assay were prepared from cell pellets (blastodermal embryos, CEFs, DT40) or directly from aliquots of irradiated and non-irradiated STO cells on receipt from ATCC (catalog nos. CRL-1503 and 56-X). Extracts were prepared and analyzed according to manufacturer's directions using the TRAPeze Telomerase Detection Kit (Serologicals Corporation) which is based upon the Telomeric Repeat Amplification Protocol (TRAP) (Kim et al., 1994).

H. Gene Transcript Analysis by Real Time Quantitative TaqMan® RT-PCR

i. Theoretical Basis

In traditional PCR, reactions are characterized by analysis of a product which has accumulated after a predetermined number of cycles. With real-time PCR, reactions are characterized by the point at which amplification of a target molecule is first detected. The greater the quantity of the target molecule in the reaction mix, the earlier a significant (above background) increase in fluorescence is observed. The threshold cycle (C_t) is defined as the fractional cycle number at which fluorescence generated by cleavage of a probe for the target molecule exceeds a set threshold above the baseline (Bièche et al., 2000).

ii. Primers and Probes

Primers and probes were developed for each of the three target genes (chTERT, chTR and c-myc) as well as for three housekeeping genes for normalization purposes [housekeeping genes including GAPDH (Genbank CB018343), chicken transferrin receptor (Genbank X55348.1) and chicken ribosomal protein L10 (Genbank CB271063)]. Chicken GAPDH mRNA levels were found to show the least standard deviation for all samples examined and therefore GAPDH values were used to normalize the target gene values. Primers and probes were selected with Primer Express software (Applied Biosystems, Foster City, CA) and are shown in Table 2.

iii. Sample Preparation

Cells (chES, CEFs, DT40) were washed with ice-cold PBS and lysed in 0.8 ml of 1x AB lysis buffer (Applied Biosystems, Foster City, CA). Total RNA was extracted from the lysates using a 6700 automated nucleic acid workstation (Applied Biosystems) according to the manufacturer's instructions.

iv. RT-reaction and real-time PCR

Complementary DNA (cDNA) was synthesized using 100 units of SuperScript III, 600 ng random hexamer primers, 10 U RNaseOut, and 1 mM dNTPs (all reagents from Invitrogen, Carlsbad, CA) in a final volume of 40 μl. The reverse transcription reaction proceeded for 120 min at 50°C. After addition of 60 μl of water, the reaction was terminated by heating for 5 min to 95°C and cooling on ice.

Each PCR reaction contained 400nM primer and 80nM TaqMan® probe for the respective TaqMan® system and commercially available PCR mastermix (TaqMan® Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 μl of the diluted cDNA sample in a final volume of 12 μl. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescence signals were collected during annealing and C_t values were extracted with a threshold of 0.04 and baseline values of 3-15.

v. Relative quantitation of gene transcription

Transcript values were derived using the comparative C_t method (User Bulletin #2, Applied Biosystems) and are reported as relative transcription levels. In brief, the housekeeping gene, GAPDH was used to normalize the C_t values of the target genes (ΔC_t). The ΔC_t was calibrated against target gene values for early passage CEFs or chES cells ($\Delta\Delta C_t$). The relative linear amount of target molecules relative to the calibrator, was calculated by $2^{-\Delta\Delta Ct}$. Therefore, all gene transcription is expressed as an n-fold difference relative to the calibrator chosen for a particular analysis.

VI.

ACKNOWLEDGEMENTS

We thank Dr. Christian Leutenegger of the Lucy Whittier Molecular and Diagnostic Core Facility, University of California-Davis, for expertise pertaining to the TaqMan® experiments. SES was supported by an Austin Eugene Lyons Fellowship (University of California-Davis) and a Glenn/AFAR Scholarship for Research in the Biology of Aging. Research was funded by the USDA-NRI-CGP (02-35205-11568, MED) and by subcontract 23XS002 (JBD) with the Fredrick Cancer Research Facility (FCRF) of the National Cancer Institute, NIH. Support and advice from Dr. Stephen Hughes, FCRF, is acknowledged.

VII.

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Figure 1. TRAP assay results illustrating telomerase activity profiles in cultured chicken ES cells and mouse STO cells. Representative TRAP assay results for chES cultures at 7, 14, 126, 153 and 181 days in culture. The positive telomerase activity of non-irradiated mouse STO cells is also shown. The PCR-based TRAP assay detects the stepwise addition of TTAGGG repeats to an oligonucleotide primer by primer extension if telomerase is present in the sample extract. Controls include buffer only (negative control -) and Hamburger and Hamilton (1951) Stage 4 embryo (positive control +). Arrowheads indicate the 36 bp PCR internal control amplicon. Presence of the control amplicon indicates that the PCR reaction was successful and thus a “negative” TRAP assay result was not due to faulty PCR.

Figure 1.
Swanberg et al. (2004)

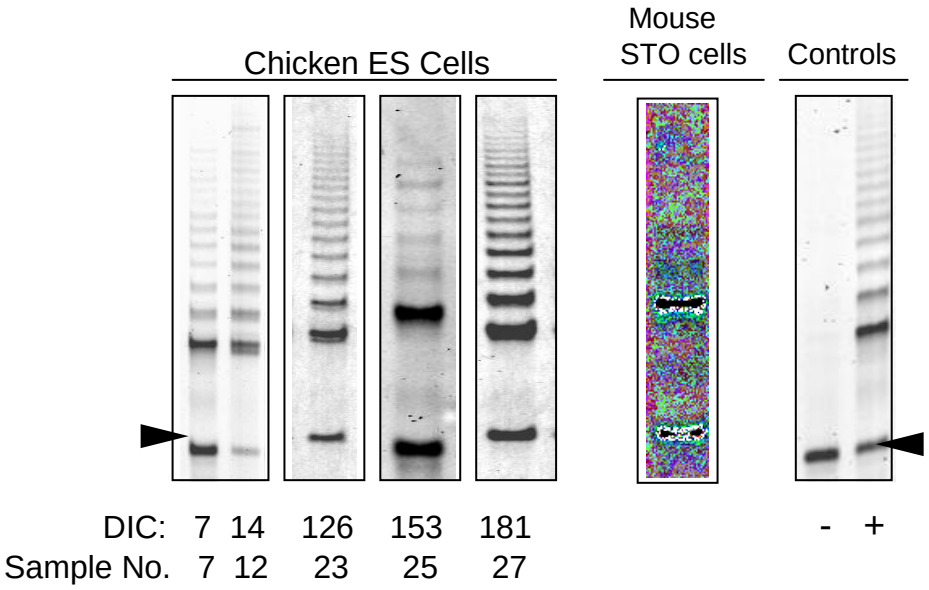


Figure 2. EMA-1 status of chicken embryonic stem (chES) cells in culture. Panel (a) shows chES cells by phase optics illumination and panel (b) shows the same cells with a positive staining pattern detected by indirect immunofluorescence using a monoclonal antibody against EMA-1. EMA-1 is a primordial germ cell surface marker used to identify ES cells.

Figure 2.
Swanberg et al. (2004)

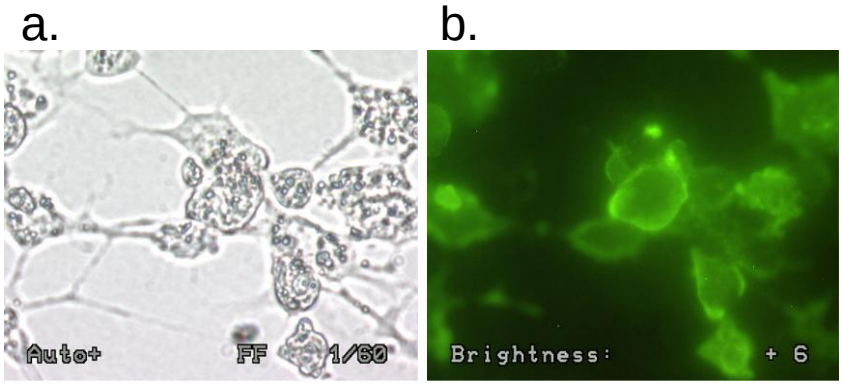
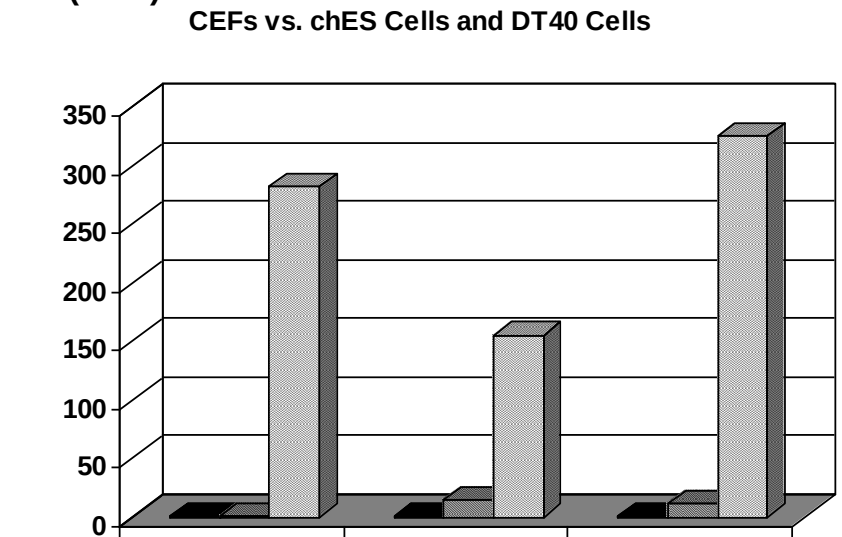


Figure 3. Relative transcript levels of c-myc, chTERT and chTR mRNA in chicken embryo fibroblasts (CEFs), chicken embryonic stem cells (chES cells), and DT40 cells. Analysis was conducted by TaqMan® real-time RT-PCR using the comparative C_t method. Results shown in panel (a) indicate fold differences in mRNA levels among the cell types. Six chES replicates (148 or 153 DIC) were averaged. Values from six DT40 cultures were also averaged. These averages for chES and DT40 cultures were calibrated against early passage CEFs which were set to a value of 1. In panel (b) the fold differences in mRNA levels for DT40 and chES cells are compared (chES values set to 1).

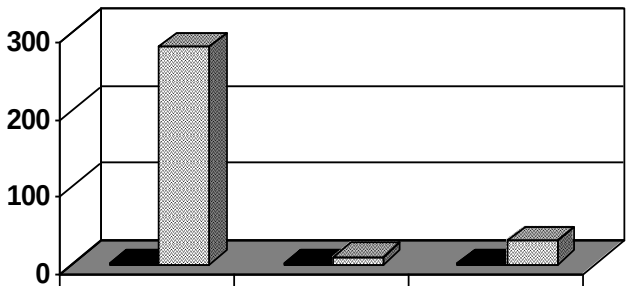
Figure 3.
Swanberg et al. (2004)

a.



	c-myc	chTERT	chTR
CEFs	1	1	1
chES	1	15	11
DT40	282	155	324

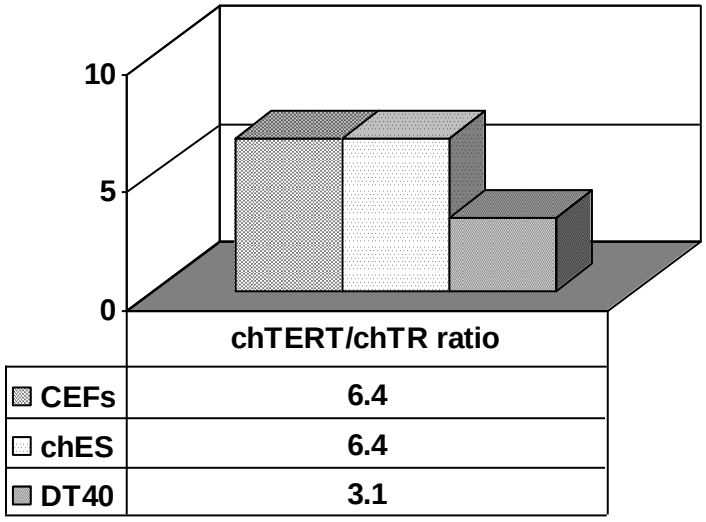
b.



	c-myc	chTERT	chTR
chES	1	1	1
DT40	282	10	30

Figure 4. Ratios of telomerase gene transcripts in chES cells, CEFs, and DT40 cells. Ratio of chTERT to chTR transcripts is 6.4 to 1 in chES cells and CEFs. In contrast the ratio in DT40 cells is 3 to 1. Both chTERT and chTR are up-regulated in DT40s but this up-regulation is not proportional.

Figure 4.
Swanberg et al. (2004)



Differential Expression of Genes Associated with Telomere Length Homeostasis and Oncogenesis in an Avian Model

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In Press Mechanisms of Ageing and Development (2005)

I. ABSTRACT

Telomere-binding proteins, their interaction partners and transcription factors play a prominent role in telomere maintenance and telomerase activation. We examined mRNA expression levels of tankyrase 1 and 2, TRF1 and 2, c-myc, TERT and TR in *Gallus domesticus*, the domestic chicken, by quantitative real-time PCR, establishing expression profiles for three contrasting cell systems: the pluripotent gastrula, differentiated embryo fibroblasts and transformed DT40 cells. All seven genes were up-regulated in DT40 cells compared to telomerase-negative CEFs and a majority of the genes were also up-regulated in the gastrula relative to CEFs. Surprisingly, we found TERT and TR transcripts in CEFs, albeit at low levels. TRF1 was down-regulated in the six CEF cultures by the time of culture growth arrest. A marked increase in the TRF2:TRF1 ratio occurred at or near senescence in all of the CEF cultures studied, with the most elevated ratio found in a short-lived culture in which TRF1 mRNA levels decreased 2-fold and TRF2 levels increased 21-fold. This culture also showed highly reduced, degraded telomeres by Southern blot analysis. These data suggest that genes involved in telomere maintenance and telomerase induction are expressed differentially in pluripotent, differentiated and transformed cell systems.

I. INTRODUCTION

Telomeres, the ends of eukaryote linear chromosomes consisting of tandem arrays of telomeric repeats, protect the genome from degradation. In vertebrates, telomeres are composed of thousands of duplex DNA repeats of the sequence 5' TTAGGG 3', with the G-rich strand extending as a 3' overhang. A major part of the vertebrate telomere is packaged in closely spaced nucleosomes (Blackburn, 2001). However, the 3' G-rich overhang assumes a terminal loop configuration (t-loop) displacing one of the duplex strands and forming a related structure (D-loop). The D-loop t-loop is stabilized by telomere-binding proteins and their interaction partners (Wei and Price, 2003; Greider 1999; Griffith et al., 1999).

The end-replication problem, the incomplete replication of the 5' end of each daughter strand, results in progressive shortening of telomeres leading to genome instability. Telomerase provides a means to replace telomere repeats which are lost during replication as a result of the inability of DNA polymerase to replicate to the end of a linear chromosome. Telomerase activity not only maintains the telomeres of proliferating cells but is implicated in the process of cellular immortalization and oncogenesis (Greider and Blackburn, 1989). *T*elomerase *R*NA, TR, contains the template for addition of telomeric repeats (Greider & Blackburn, 1989) and is generally believed to be constitutively expressed (Yi et al., 2001). T*e*lomerase *r*everse *t*ranscriptase, TERT, the component which catalyzes the addition of these repeats to the parent-strand chromosome end, has been described as the rate-limiting molecule in the

assembly of the telomerase holoenzyme (Zou et al., 2005). Transfection of non-transformed, telomerase-negative human cells with a vector encoding the human telomerase catalytic subunit resulted in the elongation of telomeres and extension of the lifespan of the cells which would otherwise have undergone replicative senescence (Bodnar et al., 1998).

Telomere-associated proteins involved in the regulation of telomere length include telomere-repeat-binding factors 1 and 2 (TRF1 and 2) each of which binds as a homodimer to double-stranded telomeric DNA or also, in the case of TRF2, as an oligomer (Wei and Price, 2003). TRF1 induces bending, looping and pairing of double-stranded telomeric DNA (Smogorzewska et al., 2000; Bianchi et al., 1997) and may induce shortening of telomeres by sequestering the 3' overhang from telomerase (van Steensel and de Lange, 1997). TRF2 is described both as protective of telomeres (Karlseder, 2003) and as a negative regulator of telomere length (Stansel et al., 2001; Smorgorzewska et al., 2000). Long-term expression of both TRF1 and TRF2 by stable transfection or overexpression of TRF1 or TRF2 produces a progressive shortening of telomeres (Ohki and Ishikawa, 2004; Karlseder et al., 2002; Smogorzewska et al., 2000; van Steensel and de Lange, 1997). In contrast, expression of a dominant negative TRF1 mutant which does not bind telomeric DNA results in telomere elongation (Ohki and Ishikawa, 2004; van Steensel and de Lange, 1997). New studies suggest that TRF2 and TRF1 function cooperatively as both proteins can be found linked to a third protein, TIN2, which may stabilize the binding of TRF1 and TRF2 to the telomere (Ye et al., 2004; Houghtaling et al.,

2004). Interaction partners of TRF1 include the tankyrases (de Rycker et al., 2003). The binding of tankyrase 1 or 2 to TRF1, resulting in the ADP-ribosylation of TRF1, may attenuate the affinity of TRF1 for telomeric DNA. Consistent with this relationship, overexpression of tankyrase 1 results in the removal of TRF1 from the telomeres followed by telomere elongation (Smogorzewska and de Lange, 2004).

In addition to the telomere-binding proteins and their interaction partners, other proteins play a role in telomere length regulation including c-myc, an oncogenic transcription factor known to regulate cell proliferation, differentiation and apoptosis as well as cell size (Piedra et al., 2002). The expression of c-myc is down-regulated in quiescent and differentiated cells and, in fact, c-myc down-regulation might be a necessary prerequisite to differentiation (Baker et al., 1994; Skerka et al., 1993). Recent research suggests that c-myc re-activates telomerase in transformed cells by inducing expression of its catalytic subunit TERT (Wu et al., 1999).

The chicken has long been recognized as a premier model organism in developmental biology (Antin et al., 2004 and associated papers) and shows promise as a model for research in the biology of aging, including telomere biology. Somatic cells of the domestic chicken, *Gallus domesticus*, share the following telomere-related features with human somatic cells: down-regulation of telomerase activity, division-dependent telomere shortening both *in vivo* and *in vitro* and re-emergence of telomerase in oncogenic cells (Swanberg and Delany 2003; Delany et al., 2003; Taylor and Delany, 2000). Interestingly, both human

and chicken cells are refractory to transformation. In contrast, mouse somatic cells exhibit constitutive telomerase activity, show no division-dependent shortening of telomeres and are readily amenable to transformation (Swanberg and Delany, 2003; Forsyth et al., 2002). With the similarity of the chicken and human telomere clocks and the array of new genomic tools including the chicken genome draft 6.6x sequence (Antin and Konieczka, 2005), the chicken is poised to emerge as a powerful new model in aging research.

Orthologs of tankyrase 1 and 2, TRF1 and 2, TERT and TR are described for the domestic chicken, *Gallus domesticus* (Delany and Daniels, 2004; de Rycker et al., 2003; Delany and Daniels, 2003; Chen et al., 2000, Konrad et al., 1999) and c-myc was first discovered in chicken (Hayward et al., 1981). To further our knowledge of chicken telomere biology, we examined mRNA expression of tankyrases 1 and 2, TRF1 and 2, c-myc, TERT and TR by quantitative real-time PCR in the chicken model. Expression patterns were studied in six chicken embryo fibroblast (CEF) cultures with different lifespan phenotypes as well as in the gastrula and DT40 cells. In both the telomerase-positive gastrula and DT40 cells, the genes were more abundantly transcribed than in most of the telomerase-negative CEF samples. Notable differences in transcription patterns, consistent with the proliferative potential of each cell type, were identified.

II. MATERIALS AND METHODS

A. Cell Culture

CEFs were isolated from six E11 (11 days of embryogenesis) embryos from UCD 003, a highly inbred chicken line (Pisenti et al., 1999). Individual cultures (unsynchronized) were derived from single embryos and maintained in DMEM with L-glutamine, 10% FBS, and 5% penicillin-streptomycin in a humidified 95% air, 5% CO_2 atmosphere as previously described (Swanberg and Delany, 2003). The cultures were passaged when they reached 80-90% confluence and split 1:3 or 1:4 until culture arrest. Population doubling (PD) was determined for each passage using the following equation:

Population doubling = [log N_t-log N]/log 2

With N the number of cells seeded and N_t the number of viable cells at the end of the passage (Patterson, 1979; Venkatesan and Price, 1998). Senescence was determined by growth dynamics, cellular morphology and by a β-galactosidase assay (Dimri et al., 1995). Senescence staining was performed using the Senescence β-galactosidase Staining Kit (Cell Signaling Technology). Cultures were deemed senescent when >90% of the cells were positive for β-galactosidase (Swanberg and Delany, 2003).

Four cultures lived for an average of 30.4 PD (range from 29-32 PD), one short-lived culture reached senescence at 24 PD and one culture exhibited a longer lifespan of 36 PD. DNA, RNA and protein samples were extracted from these cultures for Southern blot analysis of telomere length, real-time quantitative PCR for transcript levels, and a telomerase activity assay, respectively. Cultures

were maintained until culture growth arrest at which point no further samples could be extracted.

B. Embryo Samples

Gastrula stage embryos were obtained by incubating fertile UCD-003 eggs 24 hours. Seven gastrulas (Stage 4 or 5, Hamburger and Hamilton, 1951) were collected, pooled and processed for transcript profiling.

C. Transformed Cells

Cells from a transformed avian leucosis virus-induced bursal lymphoma cell line, DT40, were also examined for transcript profiling. DT40 cells were acquired from the ATCC (American Type Culture Collection) and from Dr. Jean-Marie Buerstedde GSF, Institute for Molecular Radiobiology, Ingolstaedter Landstr. 1, D-85764, Neuherberg-Munich, Germany.

D. Telomerase Activity Detection

Cell extracts for the telomerase assay were prepared from CEF cell pellets. Extracts were prepared and analyzed according to manufacturer's directions using the TRAPeze Telomerase Detection Kit (Serologicals Corporation) which is based upon the Telomeric Repeat Amplification Protocol (TRAP) (Kim et al., 1994). Two micrograms of protein were used in each TRAP assay, with protein concentration determined by Bradford assay. Gastrula-stage embryos and DT40 cells were previously shown to be telomerase positive (Swanberg et al., 2004, Swanberg and Delany, 2003; Taylor and Delany, 2000).

E. DNA Isolation and Analysis of Terminal Restriction Fragments (TRF)

For each individual culture, genomic DNA was extracted from CEFs at early passage to culture growth arrest. DNA samples were isolated and purified using the AquaPure Genomic DNA Isolation Kit (BIORad) followed by digestion of each purified sample with *Hae*III and quantification using a Molecular Dynamics Fluorimager 595. Equal amounts of DNA in each experimental lane were separated by electrophoresis, along with a lane for the molecular weight marker, in a 0.6% agarose gel for 4 hours at 55 volts.

For all experimental lanes in each gel, mean telomere length and percent telomeric DNA were determined. The gels were destained, Southern-blotted and hybridized with a ^{32}P-labeled $TTAGGG_{(7)}$ probe (Taylor and Delany, 2000). Autoradiographs were scanned and analyzed with Kodak 1D image analysis software version 3.6. Mean telomere length was defined as $\sum (OD_i \times L_i)/ (\sum OD_i)$ with OD_i the net intensity (intensity - background) of the DNA at a particular position on the gel and L_i the DNA length at that same position as measured by the image analysis software for 10-12 positions along each lane of a typical blot (Taylor and Delany, 2000; Ramirez et al., 2003). Total telomeric DNA was measured by calculating the total integrated signal ($\sum OD_i$) over the same range of fragment sizes used for mean TRF analysis. Integrated signals were expressed as a percentage of the signal from the earliest passage on any given gel (Harley et al., 1990).

F. Gene Transcript Analysis by Quantitative Real-Time TaqMan PCR

i. Primers and Probes

Primers and probes were developed for each of the seven target genes (TERT, TR, TRF1, TRF2, tankyrase 1, tankyrase 2, c-myc) as well as for three housekeeping genes including chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (CB018343), chicken transferrin receptor (X55348.1) and chicken ribosomal protein L10 (CB271063). Primers and probes (see Table 1) were selected with Primer Express software (Applied Biosystems, Foster City, CA). For each system, with the exception of TERT, for which intron and exon boundaries had not been ascertained, and TR which has no introns and is a direct transcript, primers were designed such that one member of the primer spanned an exon/exon boundary. This primer design scheme minimized the chance that any contaminating genomic DNA would be amplified.

A validation experiment was conducted with the primers and probes for the three housekeeping genes using a subset of the samples (24 of 87 samples). GAPDH produced the most consistent profile and was selected as the transcript with which target gene transcripts would be normalized. Normalization of CEF samples using cell numbers produced comparable results, but could not be used in the gastrula analysis, so GAPDH was used to normalize all samples.

ii. RNA Isolation and Reverse Transcription

Media was removed from the flask and the cells were washed with 1x PBS (without $MgCl_2$ and $CaCl_2$). Cells were scraped from the surface of the flask and placed in 800 microliters of lysis buffer (ABI). The pooled-gastrula and DT40

samples were lysed in the same manner. RNA was extracted and reverse-transcribed on an automated ABI 6700 ANA workstation. After reverse transcription, the samples were DNased for 15 minutes at 37^0 C followed by inactivation of the DNase at 85^0 C for 5 minutes.

iii. Real-time TaqMan PCR

Reactions were conducted in 96-well plates with 1x ABI TaqMan Universal PCR Mastermix, a final concentration of 400 nM for each primer, a final concentration of 80 nM for the probe, along with 0.4 microliters of H_2O per reaction. PCR cycles were as follows: 50^0 C for 2 minutes, 95^0 C for 10 minutes, followed by 40 cycles of 95^0 C for 15 seconds and 60^0 C for 1 minute. Fluorescent signals were collected during annealing and a C_t value, the PCR cycle at which non-background fluorescence is first detected, was extracted for each sample utilizing a threshold of 0.04 and baseline values of 3-15.

iv. Quantitation and analysis

The comparative C_t method was used in these experiments as previously described (Swanberg et al., 2004). CEFs from early PDs were used to calibrate the later PDs in several analyses. In other cases, CEF and DT40 cells were calibrated against values for the gastrula. Gene transcription ratios were calculated using the following formula:

$$2^{(-\Delta Ct)}/2^{(-\Delta Ct')}$$

with ΔC_t equal to the normalized cycle threshold value for gene one and $\Delta C_t'$ the normalized cycle threshold value for gene two. For an excellent review of all aspects of relative quantitation of gene expression using TaqMan systems see

ABI Prism 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression (2001).

III. RESULTS

The comparative C_t method facilitates the comparison and analysis of a variety of transcription patterns depending upon which sample is utilized as the calibrator. Of the six CEF cultures analyzed, four (designated intermediate lifespan CEFs) reached an average PD of 30.4 (standard deviation of ± 1.3), one long-lived culture reached PD36 and one short-lived culture reached only PD24. In analysis one, mRNA expression patterns in the six CEF cultures at ~6 PD, ~16 PD and culture growth arrest (henceforth referred to as early passage, middle passage, and endpoint CEFs) were compared to each other with early passage CEFs as the calibrator (Table 2). In analysis two, gene expression in the intermediate lifespan CEFs was compared to the gastrula and DT40 cells, with the gastrula as the calibrator (Table 3). In addition, gene expression ratios were calculated (Figures 1 and 2). A gene was considered to be up- or down-regulated if it showed at least a 2-fold increase or decrease in expression, respectively.

A. Analysis One: Long-lived, intermediate lifespan and short-lived CEFs

In a comparison of long-lived (Culture 1), intermediate lifespan (Cultures 2-5) and short-lived (Culture 6) CEFs, several noteworthy profiles emerged (Table 2). In long-lived and intermediate lifespan cultures, tankyrase 1 mRNA expression showed less than a 2-fold difference. In these same cultures, tankyrase 2 transcripts either showed no net change from early passage to culture growth arrest or were down-regulated 2 to 3-fold. TRF1 transcription was significantly down-regulated in all six cultures, from 2 to 10-fold, as measured by

a two-tailed, paired t-test ($p = 0.002$). In the long-lived and intermediate lifespan cultures, the change in TRF2 mRNA expression was less than 2-fold. TRF2 was up-regulated 21-fold in the short-lived culture. As a result of the down-regulation of TRF1 and, in the case of the short-lived cell culture, a 21-fold up-regulation of TRF2, there were overall increases in the TRF2:TRF1 ratios from early passage to culture growth arrest in all six CEF cultures (Figure 1). Tankyrase 1 and 2 were also up-regulated over the lifespan of the short-lived CEF culture (Table 2). No consistent c-myc transcription profile was observed in the long-lived and intermediate lifespan CEFs. Over the period from early passage to culture growth arrest in these cultures, c-myc transcript levels ranged from a 4-fold down-regulation to a 4-fold up-regulation. However, in the short-lived culture, c-myc was up-regulated 64-fold.

TERT and TR transcripts were amplified in CEF samples at low levels with average C_t values for TERT and TR at 33 and 36, respectively. TR was up-regulated 9-fold in the long-lived CEF culture and down-regulated or unchanged in the intermediate-lifespan cultures. In the short-lived CEF culture, TR transcript levels dropped to zero and TERT transcription was up-regulated 16-fold from early passage to culture growth arrest (Table 2). What appeared to be a trend toward higher levels of TERT transcripts relative to TR was observed in five of the six CEF cultures at senescence, including the culture in which TR transcript levels dropped to zero. The exception, long- lived CEFs, exhibited a TERT:TR ratio more in line with the ratios observed in the gastrula and DT40 cells (Figure 2).

B. Analysis Two: Comparison among the Gastrula, Intermediate Lifespan CEFs and DT40 cells

With the exception of the tankyrases and TRF1 in a few CEF samples, the seven target genes were more abundantly transcribed in the gastrula and DT40 cells than in intermediate lifespan CEFs (Table 3). TRF1 transcript levels were comparable to gastrula levels in early passage and middle passage CEFs. By culture growth arrest, however, TRF1 was down-regulated 8-fold in the intermediate lifespan CEFs. TRF2 transcripts were less abundant in the CEFs relative to both the gastrula and DT40 cells. TRF2:TRF1 ratios in the gastrula and DT40 cells were 115:1 and 137:1 respectively compared to a range of 22-106:1 in early passage and a range of 142-383:1 in endpoint CEFs with intermediate lifespans (Figure 1). A 184-fold increase in c-myc expression was observed in DT40 cells (Table 3). Relative to the gastrula embryo, both TERT and TR were down-regulated in CEFs and up-regulated in DT40s (Table 3). The TERT:TR ratios in the gastrula and DT40 cells were 4:1 and 3:1 respectively, compared to the higher levels of TERT relative to TR in five of the six CEF cultures (Figure 2).

C. Gene Expression/Population Doubling Correlations in the Six CEF Cultures

Correlation of gene expression levels for the seven target genes relative to the lifespan of each of the six CEF cultures revealed that endpoint expression levels of tankyrase 1, tankyrase 2, TRF2, c-myc and TERT were negatively correlated with culture lifespan (correlation coefficients of -0.84, -0.80, -0.78, -

0.80 and -0.79, respectively). That is, the higher the level of c-myc expression for example, the shorter the cell culture lifespan.

D. Telomerase activity

All CEFs were telomerase-negative in contrast with the chicken gastrula and DT40 cells which were previously shown to be telomerase positive (Taylor and Delany, 2000; Delany and Swanberg, 2003; Swanberg et al., 2004).

E. TRF analysis

Analysis of telomere TRF length for the long-lived and intermediate lifespan CEFs by Southern blot showed gradual shortening of telomeres consistent with previous results (Swanberg and Delany, 2003). A typical TRF blot for one of these cultures is shown in Figure 3a. Genomic DNA from the short-lived culture (sample taken at PD 22.5 just prior to culture arrest) lacked the high molecular weight TRF band observed in TRF gels run from other CEF cultures in both this study and a previous study (Swanberg and Delany, 2003) and showed an enhanced smear of relatively low molecular weight TRFs indicative of telomere shortening (Figure 3b).

IV. DISCUSSION

The significance of telomere shortening remains the subject of much interest and debate with studies suggesting that shortening induces replicative senescence and crisis (Wright and Shay, 2002) and others indicating that an altered telomere state rather than shortening *per se* is responsible for the senescence phenotype (Karlseder et al., 2002; Blackburn, 2001). Telomere-binding proteins and other accessory proteins likely play a prominent role in telomere maintenance by switching the telomere from a closed state (D-loop t-loop) to an open state allowing extension of the G-rich strand by telomerase. Closed terminal loops corresponding to telomere t-loops were observed in both mammalian and avian cells (Nikitina and Woodcook, 2004). Disruption of the protective configuration maintained by these proteins leads to telomere degradation and crisis by inducing the DNA damage response (Karlseder et al., 2002; Blackburn, 2001; Stansel et al., 2001; Greider, 1999; Griffith et al., 1999).

A. Expression of tankyrase 1 and tankyrase 2 mRNAs

Tankyrase 2 mRNA was up-regulated in DT40s compared to long-lived CEFs, intermediate lifespan CEFs and the gastrula (Tables 2 and 3). Tankyrase is known to reduce the affinity of TRF1 for telomeric DNA which in turn induces telomere elongation by allowing telomerase to access the 3′ overhang (Smogorzewska and de Lange, 2004). Our finding that tankyrase mRNA transcripts are more abundant in telomerase-positive DT40 cells than in the gastrula or CEFs is consistent with a number of studies which found up-regulation of tankyrase in transformed cells (Gelmini et al., 2004 and references

therein). Up-regulation of tankyrase 1 in transformed cells would increase access of telomerase to the telomere ends, potentially facilitating telomere stability in an immortalized cell line such as DT40.

B. TRF1 and TRF2 mRNA Expression

In light of prior research suggesting that TRF2 is protective of telomeres, high levels of TRF2 mRNA in a short-lived culture with prematurely degraded telomeres and the consistently elevated TRF2:TRF1 mRNA ratios in senescent CEFs were surprising findings. However, there is a growing body of evidence suggesting that TRF2 may either be a negative regulator of telomere length or may activate a telomere degradation pathway (Ancelin et al., 2002; Smogorzewska et al., 2000). Up-regulation of TRF2 was recently shown to be related to telomere shortening and carcinogenesis in human hepatocarcinoma (Oh et al., 2005).

In human cells, TRF2 co-localizes and physically interacts with the Werner (WRN) and Bloom (BLM) helicases which appear to function in the telomere maintenance pathway. TRF2 also recruits the WRN exonuclease which acts upon telomeric DNA. Binding of TRF2 to either the WRN or BLM helicase induces unwinding of telomeric and non-telomeric substrates while TRF1 acts as an inhibitor of this process. The ability of TRF2 to stimulate unwinding of telomeric substrates appears to vary with the relative concentrations of TRF2 and TRF1. At equimolar concentrations of the two proteins or with TRF2 in molar excess, the BLM helicase unwinds telomeric DNA. However, with TRF1 in molar excess, unwinding is prevented (Lillard-Wetherell et al., 2004; Machwe et al.,

2004; Opresko et al., 2004). This suggests that TRF1 and TRF2 can play opposing roles in telomere length regulation, that relative amounts of these binding proteins may have a significant impact on telomere maintenance, and that elevated levels of TRF2 may be implicated in telomere shortening or degradation (Machwe et al., 2004; Lillard-Wetherell et al., 2004; Opresko et al., 2002; Stavropoulos et al., 2002).

Our data are supportive of this model in that an increase in TRF2 mRNA expression and an increasing TRF2:TRF1 mRNA ratio negatively correlated with lifespan in CEFs. Aging CEFs in all cases exhibited a dramatic increase in the TRF2:TRF1 mRNA ratios. In intermediate lifespan and long-lived CEFs, the shifts in TRF2:TRF1 mRNA ratios were due to a significant down-regulation of TRF1 mRNA. In short-lived CEFs, the change in the TRF2:TRF1 mRNA ratio caused by a 2-fold down-regulation of TRF1 mRNA was amplified by the 21-fold up-regulation of TRF2 mRNA all of which was accompanied by a dramatic loss of telomeric DNA. These observations support a connection between changing TRF2:TRF1 ratios and the senescence phenotype. Interestingly, TRF2 and TRF1 were both elevated in the gastrula and DT40.

C. Telomerase Activity and mRNA Expression of TERT, TR and c-myc

The finding that TERT is transcribed in telomerase-negative CEFs, albeit at low levels, was unexpected. TR transcripts were also discovered in many of the CEF samples. Interestingly, TR transcripts were less abundant than TERT transcripts in DT40 cells, the gastrula embryo and CEFs. The mean C_t values for TERT and TR in CEF samples were 33 and 36, respectively, suggesting that

transcript copy number was very low, perhaps down to a single copy in the case of TR (personal communication, Christian Leutenegger). These findings are noteworthy in that human TERT is described as the rate-limiting molecule in the formation of a functional human telomerase holoenzyme, TR is considered to be constitutively expressed and activation of telomerase can be achieved by providing an exogenous source of TERT alone (Masutomi et al., 2000; Bodnar et al., 1998). Since the half-life of TERT and TR RNAs, TERT protein levels and the presence of splice variants that are not distinguishable by our primers are unknown, further research is needed in order to determine the significance of the presence of TERT and TR transcripts in telomerase-negative cell types (Chang and Delany, 2005; Cerezo et al., 2001).

We previously found that c-myc mRNA is considerably elevated in telomerase-positive, transformed DT40 cells relative to chicken embryonic stem (chES) cells in culture and CEFs (Swanberg et al., 2004). In this study, we found that c-myc mRNA is also elevated (184-fold) in DT40s relative to the gastrula. Identified 20 years ago as the cellular homolog of the *v-myc* oncogene, c-myc activates cell cycle machinery, promotes cell proliferation, is essential for cell growth, activates glycolysis and can accelerate the rate of cell death when overexpressed (Dang, 1999 and references therein). All of this suggests that c-myc is a moderator of cell cycle mechanisms and cellular metabolism. Tightly regulated in normal cells, c-myc overexpression increases cell size, and impairs cellular differentiation (Piedra et al. 2002). C-myc may also be a “key switch” for the induction of telomerase activity (Dang, 1999). In light of what is known about

c-myc, it is not surprising that c-myc expression would be low in differentiated CEFs, high in embryonic stem cells and the gastrula, and highest in transformed DT40 cells where c-myc is dysregulated by ALV insertion. The high levels of c-myc mRNA observed in DT40 cells here are similar to levels observed in earlier studies comparing DT40 cells to normal embryonic cells (Nieman et al., 2001; Baba et al., 1985)

Dysregulation of c-myc whether by gene amplification, chromosomal translocation or promoter modification (Baba et al., 1985) is implicated in neoplastic transformation in many vertebrates (Neiman et al., 2001; Iritani and Eisenman, 1999 and references therein; Bouchard et al., 1998; Baba et al., 1985). In our study, c-myc transcription was accompanied by 4-fold increases in TERT and TR in DT40 cells relative to the gastrula which is consistent with other research indicating that c-myc re-activates telomerase in transformed cells by inducing expression of the catalytic subunit TERT (Wu et al., 1999). The chicken TERT gene contains a 5′ E-box motif known to be bound by c-myc (Delany and Daniels, 2004; Kuramoto et al., 1999). In addition, elevated levels of c-myc, TERT and TRF1 in DT40s could reflect, at least in part, the result of a dosage effect as these genes are all located on chicken chromosome 2 which is trisomic in DT40 cells (Chang and Delany, 2004 and references therein).

Results of this study compliment earlier observations of c-myc, TERT and TR transcript levels in chES cells (Swanberg et al., 2004). From the two studies a picture emerges in which expression of these three genes varies depending upon the proliferative capacity of the cell system examined. During embryonic

development in both human and chicken tissues, somatic differentiation is accompanied by down-regulation of telomerase activity (Delany et al., 2003; Forsyth et al., 2002; Sharma et al., 1995). A high level of transcription in telomerase-positive toti/pluripotent embryonic cells is followed by down-regulation in differentiated and senescent cells *in vitro* and *in vivo*. In contrast, the process of cellular transformation involves up-regulation of c-myc and the telomerase holoenzymye components required to stabilize telomeres through the many rounds of replication and cell division attributed to immortalized cells (Table 4).

V. Conclusions

In this study, expression patterns of genes associated with telomere length regulation and telomerase induction were examined by quantitative real-time Taqman PCR. Using this sensitive technique to measure mRNA transcript levels enabled us to explore gene expression profiles in an array of cell types: transformed vs. non-transformed, telomerase negative vs. telomerase positive, early passage vs. senescent and differentiated vs. non-differentiated. Our results reveal TERT and TR transcripts in telomerase-negative CEFs and suggest that TRF1 down-regulation may be a general feature of senescing cells. Further, up-regulation of the tankyrases, TRF2, c-myc and TERT was implicated in early cell culture arrest. The increase in TERT transcripts relative to TR transcripts from early passage to senescence in five of the telomerase-negative CEF cultures, along with low TERT:TR ratios in the telomerase-positive cell types examined suggest that an optimal TERT:TR mRNA ratio may be required for telomerase to

remain activated. TRF2:TRF1 ratios were similar in telomerase-positive embryonic and transformed cells but increased dramatically in senescing CEFs, particularly in one short-lived culture with a high percentage of telomeric DNA loss and an increased rate of telomere shortening, indicating that the ratio of these two telomere binding proteins may be critical to telomere stability. In light of our results and current research regarding the roles of TRF2 and TRF1 in telomere length maintenance, it would not be surprising to discover that TRF2 either protects telomeres or facilitates the unwinding of telomeric substrates depending upon cell type, proliferative status, TRF1 levels or some other as yet unknown factor.

VI.

ACKNOWLEDGMENTS

We thank Dr. Christian Leutenegger of the Lucy Whittier Molecular and Diagnostic Core Facility, University of California-Davis, for expertise pertaining to the TaqMan® experiments. SES was supported by an Austin Eugene Lyons Fellowship (University of California-Davis). Research was funded by a Glenn/AFAR Scholarship for Research in the Biology of Aging (SES) and the USDA-NRI-CGP (02-35205-11568, MED).

VII.

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Table 1. Primers and probes for quantitative real-time PCR of nine genes, six involved in telomere length maintenance or telomerase activation and three housekeeping genes, one of which (GAPDH) was utilized for normalization. Primers and probes were designed using sequences from the accession numbers provided. Except for TERT, for which intron and exon boundaries had not been ascertained, and TR, which is a direct transcript, one of the two primers for each gene spans an mRNA exon/exon and will therefore not align with genomic sequence preventing the amplification of a product from any residual genomic DNA.

Gene	Accession	Primers and Probes (5'-3')
tankyrase 1	AY142108	Forward-GGAGGCCAGCAAGGTACCA Reverse-GGGCAAGGTCAAGGAGAATAGTC Probe-TCCTTATTTAACTTTCCACTGCGTGAGTCAGG
tankyrase 2	AY142107	Forward-ATCTCTGGACAGCAAGGGCTTA Reverse-ATCTTCAGAGGAAAGATCTATGAGAAGAGT Probe-CCCATACCTGACTCTTAATACCTCCAGTAGCGG
TRF1	AY237359	Forward- TGGCGCACGCTGTTTCTA Reverse- AAGCTGACAGAGGTACACCATTTTT Probe-TGGCTTCTCCAAAGTAACAACGCATCAGA
TRF2	AJ133783	Forward-TCAGATGCTGCGCGTCAT Reverse-ATCGAAGGTGCAATCTAGGTTTTC Probe-CAGTTCCTGTCCCGGATCGAGGAA

Table 1. (continued)

Gene	Accession	Primers and Probes (5'-3')
chTR	AY312571	Forward- CTCCGCTGTGCCTAACCCTAAT Reverse- TCGCCCGCTGAAAGTCAG Probe-AATTGATGGTGCTGTCGCCGCG
chTERT	AY502592	Forward-GTCAGAGCGAAGTCATCACAAGAAT Reverse-TGGCAAAACTCTGAAGTGACAAC Probe-ATGGATACTCCTTGCTGGATGAGAA
c-myc	X68073	Forward- AGCGACTCGGAAGAAGAACAAG Reverse-ATCGACTTCGCTTGCTCAGACT' Probe-AGAAGATGAGGAAATCGATGTCGTTACA
GAPDH	CB018343	Forward-TTGTTTCCTGGTATGACAATGAGTTT Reverse-CTCACTCCTTGGATGCCATGT Probe-ATACAGCAACCGTGTTGTGGACTTGATGGT
Transferrin receptor	X55348	Forward-CATGCCCACCTTGGAACTG Reverse-GGTGGAAACTGGTGTGGTT Probe-AGACCCTTACACCCCAGGCTTCCCTT
Ribosomal protein L10	CB271063	Forward-CCGGCGCGGTGTTACA Reverse-CACCCCCCGGCAGAAG Probe-CTGCAAAAATAAACCCTACCCCAAATCGC

Table 2. Transcript profiling of seven target genes over *in vitro* lifespan of six telomerase-negative chicken embryo fibroblast (CEF) cultures. Net change in CEF mRNA expression from early passage to culture endpoint. For each individual culture, its early passage value was used as the calibrator.

CEF Culture #	Tank 1	Tank 2	TRF1	TRF2	c-myc	TERT	TR
1 (36 PD)	↔	3x ↓	4x ↓	↔	4x ↓	↔	9x ↑
2 (32 PD)	↔	3x ↓	2x ↓	↔	↔	14x ↓	20x ↓
3 (30.5 PD)	↔	↔	5x ↓	↔	4x ↑	3x ↑	↔
4 (30 PD)	↔	↔	10x ↓	↔	↔	↔	↔
5 (29 PD)	↔	2x ↓	8x ↓	↔	↔	↔	2x ↓
6 (24 PD)	5x ↑	8x ↑	2x ↓	21x ↑	64x ↑	16x ↑	no transcripts[1]

↔ = less than a 2-fold difference in mRNA expression

↑ = greater than or equal to a 2-fold increase in mRNA expression

↓ = greater than or equal to a 2-fold decrease in mRNA expression

[1]No TR transcripts were detected at culture endpoint therefore no comparison could be made

PD = culture lifespan

Table 3. Transcript profile comparisons among the gastrula-stage embryo, intermediate lifespan CEFs, and transformed DT40 cells. Genes involved in telomere length regulation are down-regulated in telomerase-negative CEFs[1] relative to the gastrula. Six of these genes are up-regulated in telomerase-positive DT40 cells compared to the gastrula.

Cell Type	Tank 1	Tank 2	TRF1	TRF2	c-myc	TERT	TR
Gastrula[2]	1	1	1	1	1	1	1
DT40	↔	3x↑	4x ↑	4x ↑	184x ↑	4x ↑	4x ↑
CEFs: Early passage	3x↓	↔	↔	3x↓	2x↓	41x ↓	54x ↓
CEFs: Middle passage	↔	↔	↔	5x↓	5x↓	19x ↓	61x ↓
CEFs: Endpoint	3x↓	↔	8x ↓	4x↓	2x↓	55x ↓	164x ↓

[1] mean values for the four intermediate lifespan cultures were used in this analysis

[2] the gastrula sample was used as the calibrator and is therefore set at 1

↔ = less than a 2-fold difference in mRNA expression

↑ = greater than or equal to a 2-fold increase in mRNA expression

↓ = less than or equal to a 2-fold decrease in mRNA expression

Table 4. Correspondence of differentiation status, cellular phenotype and transcript profiling in chicken cells.

		Phenotype			Transcript profiling		
Cell Type	Differentiation status	T-ase[2]	Transformed	Immortalized	c-myc	TERT	TR
chES cells	totipotent	+	no	yes	1	1	1
Gastrula	pluripotent	+	no	no	↔	2x ↑	6x ↑
CEFs: Early passage	differentiated	-	no	no	2x ↓	19x ↓	9x ↓
CEFs: Middle passage	differentiated	-	no	no	5x ↓	8x ↓	10x ↓
CEFs: Endpoint	differentiated	-	no	no	↔	26x ↓	27x ↓
DT40[1]	differentiated bursal stem cell	+	yes	yes	282x ↑	10x ↑	30x ↑

[1] B-cell line which retains the ability to diversify its rearranged immunoglobulins despite having matured to the differentiated bursal stem cell stage (Winding and Berchtold, 2001 and references therein; Kim et al., 1990).

[2] Telomerase activity

[3] Swanberg et al., 2004

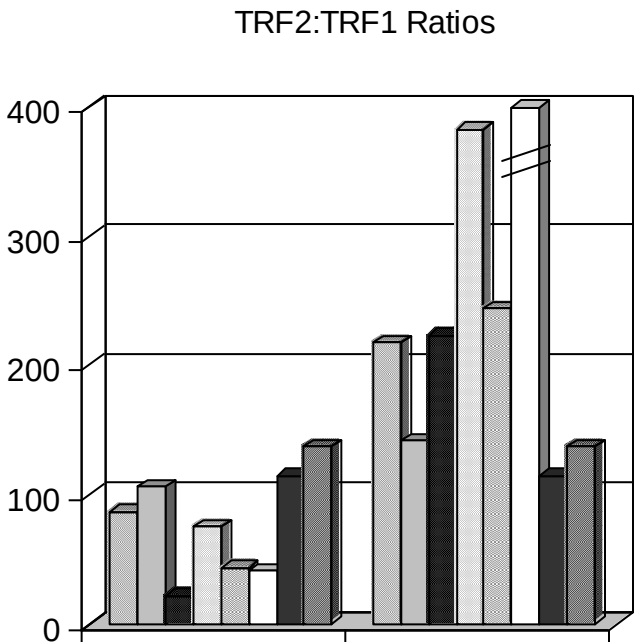
[4] Taylor and Delany, 2000

[5] Swanberg and Delany, 2003

Figure 1. TRF2:TRF1 ratios in endpoint CEFs are higher than ratios in early passage CEFs, the gastrula and DT40 cells. There is a negative correlation (R^2 = -0.87) between TRF2 transcript levels and population doubling. The ratio for the endpoint CEF6 culture (1746) is off the scale of the chart and hatchmarks so indicate.

Figure 1.

Swanberg and Delany (2005)

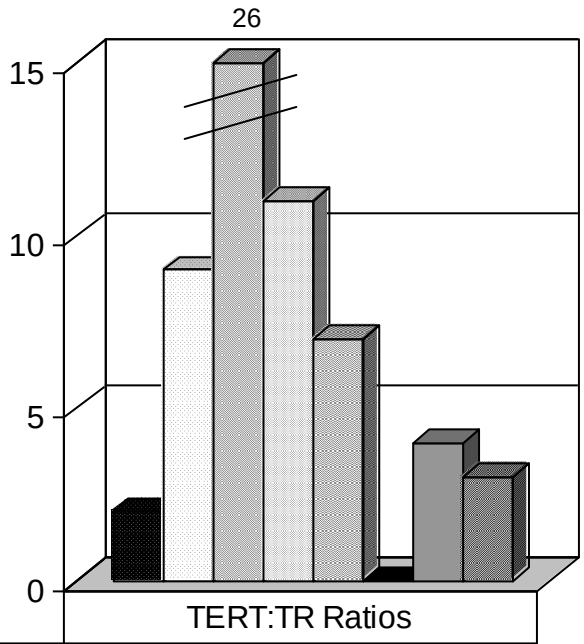


	Early CEFs, Gastrula, DT40	Endpoint CEFs, Gastrula, DT40
CEF1 (36 PD)	87	218
CEF2 (32 PD)	106	142
CEF3 (30.5 PD)	22	223
CEF4 (30 PD)	75	383
CEF5 (29 PD)	43	244
CEF6 (24 PD)	41	1746
Gastrula	115	115
DT40	137	137

Figure 2. TERT:TR ratios are lower in the telomerase-positive gastrula and DT40 cells as well as in long-lived CEFs at senescence. In a comparison of the gastrula, DT40 cells and six CEF cultures at senescence/culture arrest, it appears that TERT transcripts were more abundant relative to TR in the gastrula, DT40 and long-lived CEFs relative to short-lived and intermediate lifespan CEFs. The increase in TERT:TR transcript ratios from early passage to culture arrest in five of the telomerase-negative CEF cultures along with the relatively low TERT:TR ratios in the telomerase-positive cell types suggest that TERT:TR transcript stoichiometry may be crucial to telomerase activation. Note that early and endpoint CEFs are compared to the same gastrula and DT40 values.

Figure 2.

Swanberg and Delany (2005)



	TERT:TR Ratios
CEF 1 at 36 PD	2
CEF 2 at 32 PD	9
CEF 3 at 30.5 PD	26
CEF 4 at 30 PD	11
CEF 5 at 29 PD	7
CEF 6 at 24 PD	0 *
Gastrula	4
DT40	3

*TERT transcripts were observed at culture arrest but TR Transcripts were not observed. Therefore the TERT:TR ratio could not be calculated.

Figure 3. Terminal telomere restrictrion fragment (TRF) analysis illustrating dramatic loss of telomeric DNA in a short-lived CEF culture. HaeIII-digested DNA is subjected to agarose gel electrophoresis, Southern-blotted, and hybridized with a labeled telomere probe $(5'\text{-TTAGGG-}3')_7$. A decrease in high molecular weight fragments along with an increase in low molecular weight fragments is indicative of telomere shortening (see open arrow). **(a)** TRF profile typical of long-lived and intermediate lifespan CEFs with a net telomeric DNA loss of 13% as measured by densitometry and an average telomere shortening rate of 22 bp per PD. **(b)** TRF analysis of a short-lived CEF culture #6 showing a net loss of 31% telomeric DNA and an average rate of telomere shortening of 148 bp per PD. Note also the complete lack of the high molecular weight TRF band (see closed arrow) typical of chicken TRF banding patterns.

Table 2. Telomerase activity, telomere

Figure 3.

Swanberg and Delany (2005)