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Differential expression of genes associated with telomere length homeostasis and oncogenesis in an avian model

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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 two-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.

Keywords: Chicken; Telomere; Telomerase; TRF; Tankyrase; c-myc

1. 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 (Levy et al., 1992). 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). Telomerase RNA, TR, contains the template for addition of telomeric repeats (Greider and Blackburn, 1989) and is generally believed to be constitutively expressed (Yi et al., 2001). Telomerase reverse transcriptase, 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).

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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; Smogorzewska 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 6.6 × 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.

2. Materials and methods

2.1. 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:

$$\text{population doubling} = \frac{\log N_t - \log N}{\log 2}$$

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 to 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.

2.2. Embryo samples

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

2.3. 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.

2.4. 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).

2.5. 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 (Bio-Rad) 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 h at 55 V.

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).

2.6. Gene transcript analysis by quantitative real-time TaqMan PCR

2.6.1. 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) 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/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.

2.6.2. RNA isolation and reverse transcription

Media was removed from the flask and the CEFs were washed with 1 × PBS (without $MgCl_2$ and $CaCl_2$). Cells were scraped from the surface of the flask and placed in 800 μl 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 min at 37 °C followed by inactivation of the DNase at 85 °C for 5 min.

2.6.3. Real-time TaqMan PCR

Reactions were conducted in 96-well plates with 1 × 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 μl of H_2O per reaction. PCR cycles were

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

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
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-GGGTCTTATGACCACTGTCCA Reverse-TGGACGCTGGGATGATGTT Probe-AGACGGTGGATGGCCCCTCTGG
Transferrin receptor	X55348	Forward-CATGCCCACCTTGGAACTG Reverse-GGTGGAAACTGGTGTGGTT Probe-AGACCCTTACACCCCAGGCTTCCCTT
Ribosomal protein L10	CB271063	Forward-CCGGCGCGGTGTTACA Reverse-CACCCCCCGGCAGAAG Probe-CTGCAAAAATAAACCCTACCCCAAATCGC

Primers and probes were designed using sequences from the accession numbers provided. Except for TERT, for which intron/exon boundaries had not been ascertained, and TR, which has no introns, one of the two primers for each gene spans an exon/exon boundary and will therefore not align with genomic sequence preventing the amplification of a product from any residual genomic DNA.

as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. 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.

2.6.4. 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:

$$\frac{2^{-\Delta C_t}}{2^{-\Delta C_t'}}$$

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).

3. 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
Transcript profiling of seven target genes over in vitro lifespan of six telomerase-negative chicken embryo fibroblast (CEF) cultures

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

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. ↔ = less than a two-fold difference in mRNA expression, ↑ = greater than or equal to a two-fold increase in mRNA expression, ↓ = greater than or equal to a two-fold decrease in mRNA expression, PD = culture lifespan.

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

Table 3
Transcript profile comparisons among the gastrula-stage embryo, intermediate lifespan CEFs[a], and transformed DT40 cells

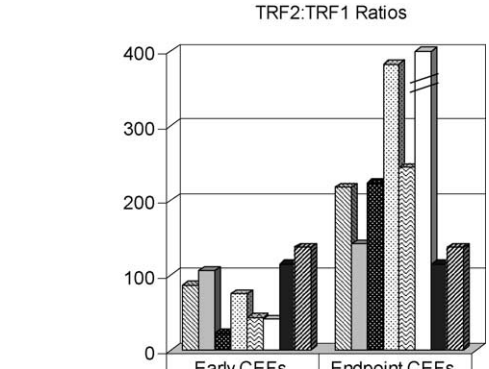
Cell type	Tank 1	Tank 2	TRF1	TRF2	c-myc	TERT	TR
Gastrula[b]	1	1	1	1	1	1	1
DT40	↔	3 × ↑	4 × ↑	4 × ↑	184 × ↑	4 × ↑	4 × ↑
CEFs: Early passage	3 × ↓	↔	↔	3 × ↓	2 × ↓	41 × ↓	54 × ↓
CEFs: Middle passage	↔	↔	↔	5 × ↓	5 × ↓	19 × ↓	61 × ↓
CEFs: Endpoint	3 × ↓	↔	8 × ↓	4 × ↓	2 × ↓	55 × ↓	164 × ↓

Genes involved in telomere length regulation are down-regulated in telomerase-negative CEFs relative to the gastrula. Six of these genes are up-regulated in telomerase-positive DT40 cells compared to the gastrula. ↔ = less than a two-fold difference in mRNA expression; ↑ = greater than or equal to a two-fold increase in mRNA expression; ↓ = less than or equal to a two-fold decrease in mRNA expression.

[a] Mean values for the four intermediate lifespan cultures were used in this analysis.

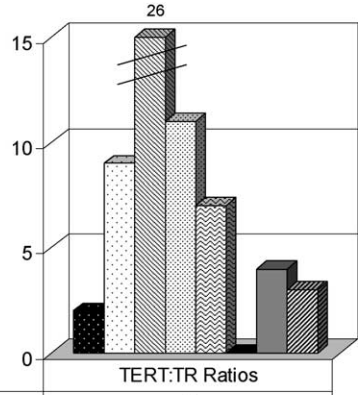
[b] The gastrula sample was used as the calibrator and is therefore set at 1.

(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 (Figs. 1 and 2). A gene was



	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

Fig. 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. Note that early and endpoint CEFs are compared to the same gastrula and DT40 values.



	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

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

considered to be up- or down-regulated if it showed at least a two-fold increase or decrease in expression, respectively.

3.1. 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 two-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 two- to three-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 two-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 (Fig. 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 four-fold down-regulation to a four-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 nine-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 (Fig. 2).

3.2. 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 eight-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 (Fig. 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 (Fig. 2).

3.3. 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.

3.4. 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; Swanberg and Delany, 2003; Swanberg et al., 2004).

3.5. 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 Fig. 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 (Fig. 3b).

4. 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; Black-

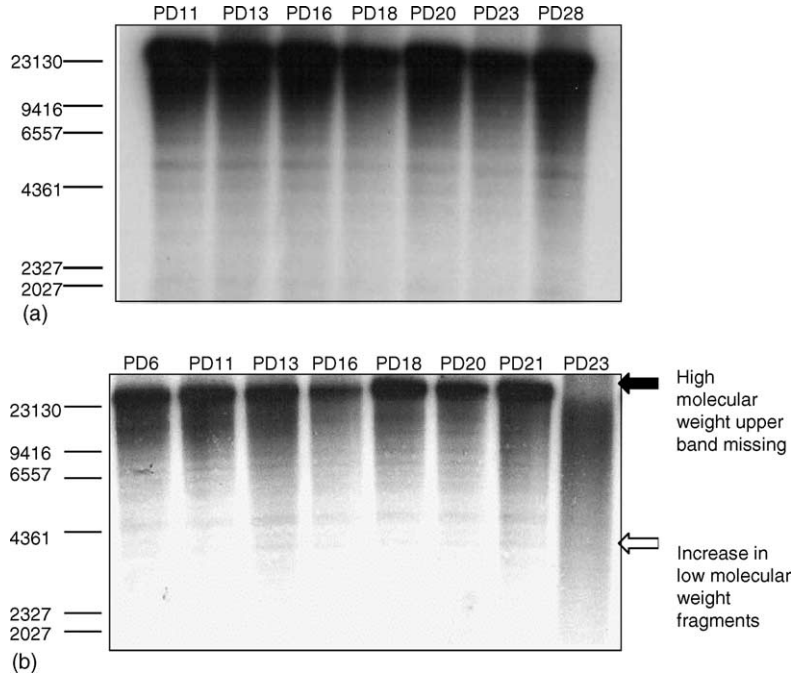


Fig. 3. Terminal telomere restrictrion fragment (TRF) analysis illustrating dramatic loss of telomeric DNA in a short-lived CEF culture. HaeIII-digested DNA is subjected to agarose gel electrophoresis, Southern-blotted, and hybridized with a labeled telomere probe $(5'\text{-TTAGGG-}3')_7$. A decrease 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.

burn, 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 Woodcock, 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).

4.1. 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.

4.2. 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., 2002). 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 two-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.

4.3. 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., 2002).

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 (Neiman 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 four-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).

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

Cell Type	Differentiation status	Phenotype			Transcript profiling		
		T-ase[a]	Transformed	Immortalized	c-myc	TERT	TR
chES cells	Totipotent	+	No	Yes	1	1	1
Gastrula	Pluripotent	+	No	No	↔	2 × ↑	6 × ↑
CEFs: Early passage	Differentiated	−	No	No	2 × ↓	19 × ↓	9 × ↓
CEFs: Middle passage	Differentiated	−	No	No	5 × ↓	8 × ↓	10 × ↓
CEFs: Endpoint	Differentiated	−	No	No	↔	26 × ↓	27 × ↓
DT40[b]	Differentiated bursal stem cell	+	Yes	Yes	282 × ↑	10 × ↑	30 × ↑

Source: Swanberg et al. (2004), Taylor and Delany (2000), Swanberg and Delany (2003).
[a] Telomerase activity.
[b] 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).

4.4. 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 versus non-transformed, telomerase negative versus telomerase positive, early passage versus senescent and differentiated versus 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.

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