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Dynamics of telomere erosion in transformed and non-transformed avian cells *in vitro*

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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. Telomere length 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 telomere length profiles of several transformed cell lines resembled telomere length profiles of senescent CEFs in that they exhibited little of the typical smear of terminal restriction fragments (TRFs) 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.

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, 2001b). In this model, short telomeres are recognized as damaged, signaling a G_2/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 (Karlseder et al., 2002; Chan et al., 2003). 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. Reactivation 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.

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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 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, 2003; Taylor and Delany, 2000).

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 end replication due to a lack of telomerase. The telomere profiles of telomerase-positive transformed avian cell lines examined here 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.

Materials and methods

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 (Lima and Macieira-Coehlo, 1972). For the pooled cell culture (CEF2), E11 commercial layer-type embryos (n = 12) were utilized. For individual-embryo cell cultures (CEF3 1–6), 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.

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-infected 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.

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). 50 ng of DNA per lane were separated by electrophoresis in 0.6 % agarose gels for 4 h at 55 volts. Using this protocol, high molecular weight Class III 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 $\Sigma(OD_i \times Li)/(\Sigma\ OD_i)$ (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 ($\Sigma\ OD_i$) 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).

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.

Results

Non-transformed cells

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 Fig. 1A). Exceptions included low activity detected in CEF3 cultures 3 and 4 (the first 50 bp band of the telomerase oligonucleotide ladder was faintly

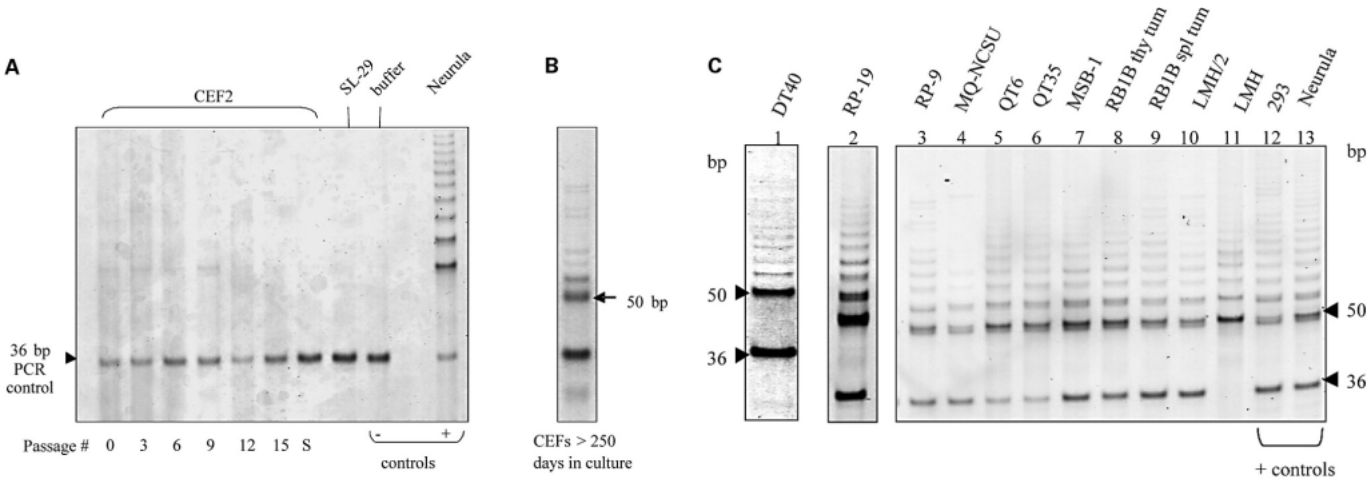


Fig. 1. Telomerase activity is lacking in primary CEFs and present in transformed avian cell lines and tumors. (**A**) Representative TRAP assay results showing lack of telomerase activity in the CEF2 culture in passages 0–15, senescent CEF2 cells (S) and in ATCC CEFs (SL-29). (**B**) Positive TRAP assay results of CEF2 cells >250 days in culture. (**C**) TRAP assays of transformed avian cell lines and tumors. Lane 12: transformed 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.

Table 1. Telomerase profiles of avian cells and cell lines

Designation[a]	Telomerase	Description[b]	Agent of transformation[c]
Non-transformed			
SL-29	-	ATCC, CEF, PD_{18}	
CEF2	-	$P_o - P_{15}$	
CEF2[d]	+	> 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[e]	+	turkey B cell	MDV
DT40[f]	+	B cell (bursal lymphoma)	ALV
RP-9[g]	+	B cell (lymphoblastoid)	ALV
MSB-1[h]	+	T cell (spleen tumor cells in vitro)	MDV
MQ-NCSU[i]	+	macrophage (spleen phagocyte)	MDV
QT6[j]	+	quail fibroblast (fibrosarcoma)	MC
QT35[j]	+	quail fibroblast (fibrosarcoma)	MC
LMH and LMH/2A[k]	+	hepatocyte (hepatocellular carcinoma)	DEN
293 cells	+	human embryonic kidney	HAdV-5
Transformed *in vivo*			
spleen	+	tumor cells[l]	MDV (RBIB)[m]
thymus	+	tumor cells[l]	MDV (RBIB)[m]

[a] All of the above are chicken cells except where indicated.
[b] ATCC = American Type Culture Collection; CEF = chicken embryo fibroblast; PD_n = Population doubling; P_n = passage number.
[c] MDV = Marek's disease virus; ALV = Avian leukosis virus; MC = Methylcholanthrene; Den = Diethylnitrosamine; HAdV5 = Human adenovirus type 5.
[d] Non-transformed at culture initiation, earlier passages were telomerase negative.
[e] Nazerian et al. (1982); Nazerian (1987).
[f] Baba et al. (1985).
[g] Okazaki et al. (1980).
[h] Akiyama and Kato (1974).
[i] Qureshi et al. (1990).
[j] Moscovici et al. (1977).
[k] Kawaguchi et al. (1987).
[l] Personal communication from Dr. Carol Cardona of the UC Davis School of Veterinary Medicine.
[m] Schat et al. (1982).

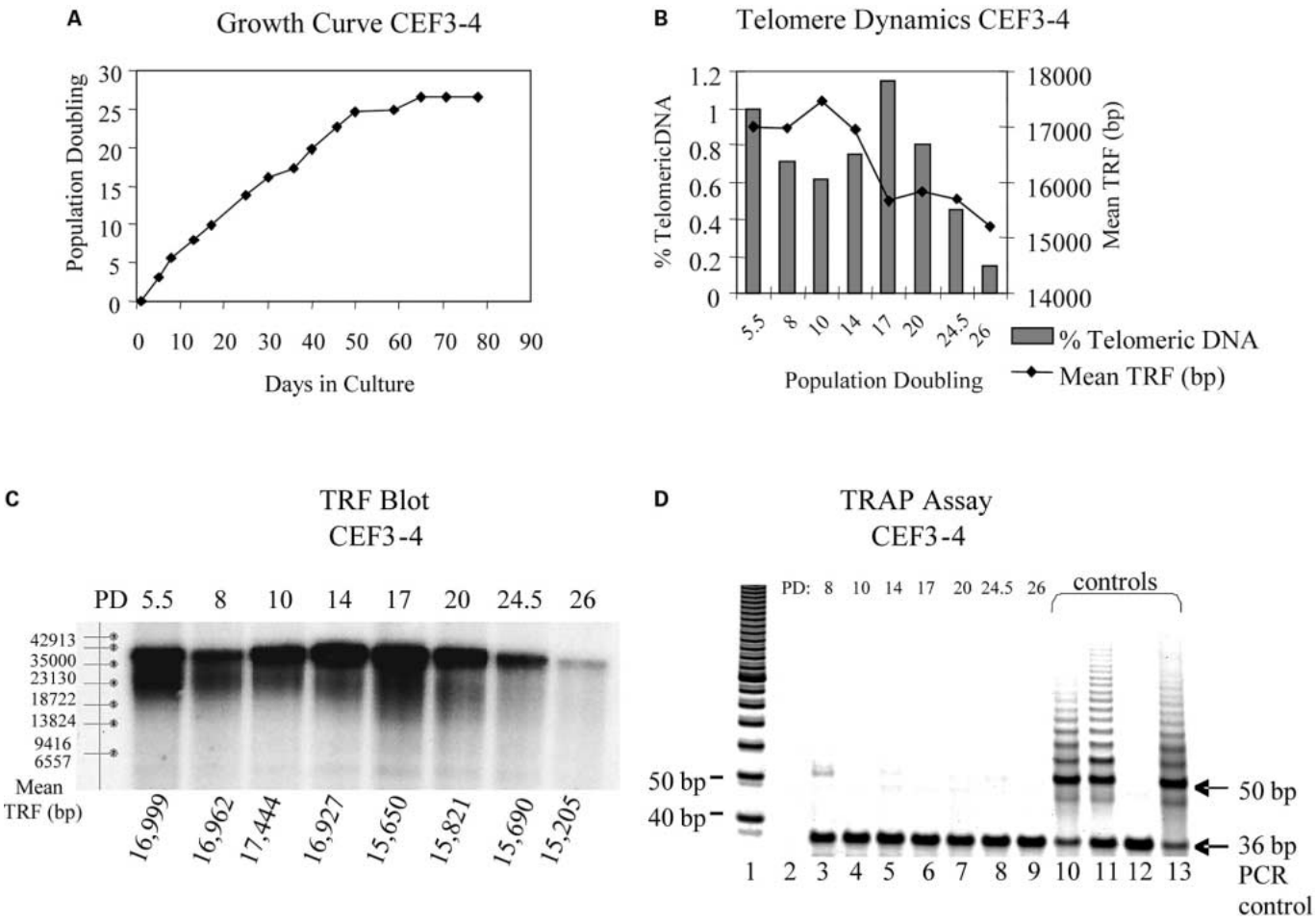
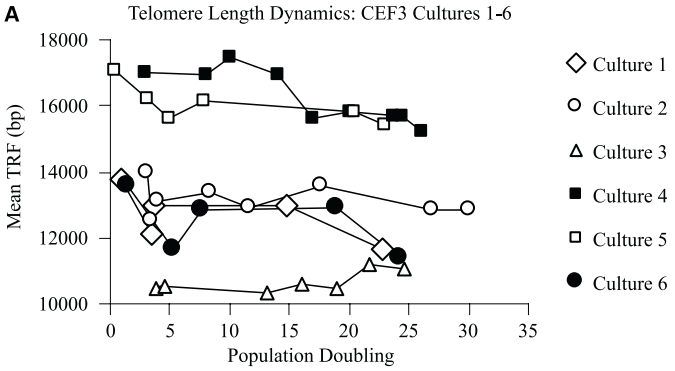


Fig. 2. Telomere dynamics, telomerase profiles and growth data for a representative CEF culture. (**A**) Growth curve for CEF3-4 culture showing cumulative increase in population doubling over the lifetime of the culture and plateau as population doublings decrease (see Materials and methods). (**B**) 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} 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).

evident) at PD_4 and PD_8 respectively, the earliest timepoints for which protein extract samples were prepared for these cultures (see Fig. 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 (Fig. 1B). CEFs (PD_{18}) which were obtained from ATCC exhibited no telomerase activity (Fig. 1A). Non-transformed adult chicken kidney fibroblasts and early stage chicken embryos were positive for telomerase (Table 1).

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 Figs. 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 2,191 bp. However, as shown in Fig. 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, Figs. 2B and 3).



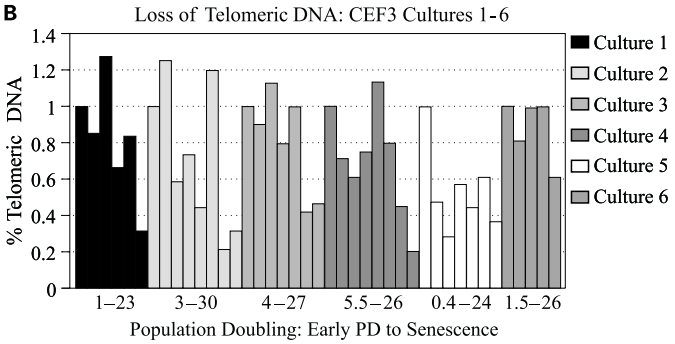


Fig. 3. Telomere Dynamics of CEF cultures 1–6. (**A**) Mean terminal restriction fragment (TRF) length was measured in six CEF cultures from early passages to senescence. TRF analysis showed an increase in lower molecular weight fragments in five of the six cultures by senescence, an indication of telomere shortening. One of the six cultures showed an increase in mean TRF (CEF3-3). (**B**) In a second measure of telomere shortening, telomeric DNA loss (Percent of Telomeric DNA = $PD_{initial}$:PD_n), all six cultures showed dynamic shifts culminating in eventual reductions in telomeric DNA, including CEF3-3.

Transformed cells

Telomerase activity in transformed avian cells and cell lines. As indicated in Table 1 and Fig. 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.

Telomere length profiles in transformed avian 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 (Fig. 4). In some cases, the smear was nearly indiscernible by eye and only detectable by densitometry. In fact the profiles of transformed

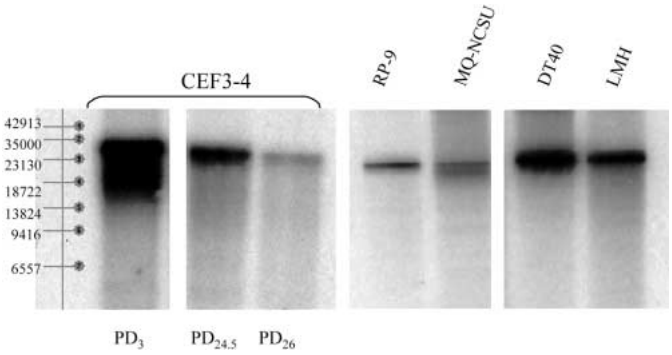


Fig. 4. TRF profiles of transformed cells resemble those of senescent CEFs lacking the typical smear of overlapping TRF fragments. Representative TRF blots of samples from CEF3-4 cell culture and transformed cell lines. By senescence, much of the telomeric DNA smear in the CEF culture has disappeared. 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.

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)		Mean TRF length (bp)	Mean TRF/ population doubling (bp)[a]	Loss of telomeric DNA[b]
	Early	Senescent			
CEF3-1	13,832 (PD_1)	13,211 (PD_{23})	-621 (PD_1 : PD_{23})	-28.0	69% (PD_1 : PD_{23})
CEF3-2	13,934 (PD_3)	12,840 (PD_{30})	-1094 (PD_3 : PD_{30})	-40.5	69% (PD_3 : PD_{30})
CEF3-3	10,468 (PD_4)	11,032 (PD_{27})	+564 ($PD_{8.5}$: PD_{27})	+ 24.5	54% (PD_4 : PD_{27})
CEF3-4	16,999 (PD_8)	15,205 (PD_{26})	-1794 (PD_{10} : PD_{26})	-87.5	85% ($PD_{5.5}$: PD_{24})
CEF3-5	17,097 ($PD_{0.4}$)	15,655 (PD_{24})	-1442 ($PD_{0.5}$: PD_{24})	-61.0	63% ($PD_{0.4}$: PD_{24})
CEF3-6	13,569 ($PD_{1.5}$)	11,378 (PD_{26})	-2191 ($PD_{1.5}$: PD_{26})	-88.0	40% (PD_1 : PD_{26})

[a] Based on TRF smear analysis.
[b] 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).

cells resembled the profiles of senescent CEFs in this respect (see Fig. 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).

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 downregulation *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 Fig. 2). Most notably, all six cultures exhibited dramatic and potentially catastrophic loss of telomeric DNA by senescence (Table 2 and Fig. 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.

Table 3. Mean TRF lengths for selected transformed cell lines and senescent CEFS

Cell type	Mean TRF
RP-9	18,235
MQ-NCSU	16,530
DT40	18,731
LMH	18,945
CEF3-1 (PD_{23})	11,945
CEF3-2 (PD_{30})	12,840
CEF3-3 (PD_{27})	11,032
CEF3-4 (PD_{26})	15,205
CEF3-5 (PD_{24})	15,655
CEF3-6 (PD_{26})	11,378

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 produced 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-repli-

cation 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, 1939). 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.

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.

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