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The Regulation of Telomerase by Alternative Splicing of TERT

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1. Introduction

1.1 Telomerase canonical function

Telomeric DNA consists of tandem oligonucleotide repeats that serve to protect ends of linear eukaryotic chromosomes against chromosome end-to-end fusion. The sequence of the repeat differs among distinct groups of eukaryotes and, with the exception of several Fungi species, is usually 5 to 8 nucleotides-long (Gomes et al., 2010; Watson & Riha, 2010). Since DNA polymerase cannot replicate the ends of linear chromosomes, chromosomal end shorten after each cell cycle in the absence of telomerase (Olovnikov, 1971; Watson, 1972). This gradual loss of telomeric repeats limits the potential lifespan of cells since cells with critically short telomeres undergo senescence (Bodnar et al., 1998). Rapidly proliferating cells activate the telomerase to synthesize telomeric DNA repeats in order to protect telomeres from shortening as a result of DNA replication and oxidative damage (Cech, 2004; Osterhage & Friedman, 2009; von Zglinicki, 2002). Telomerase is a multisubunit enzymatic ribonucleoprotein complex consisting of the telomerase reverse transcriptase (TERT), the telomerase RNA subunit (TR or TERC) which acts as the template, as well as other associated proteins (Blackburn & Collins, 2011).

1.1.1 Domain structure of TERT and TR

Ciliate, yeast, plant and vertebrate TERT protein contains four conserved domains, the telomerase essential N-terminal (TEN) domain, the telomerase RNA binding domain (TRBD), the reverse transcriptase (RT), and the C-terminal extension (CTE) domains (for reviews see (Blackburn & Collins, 2011; Mason et al., 2011; Podlevsky & Chen, 2012; Sekaran et al., 2010; Wyatt et al., 2010)). In vertebrates and plants the TEN and TRBD domains are connected by a linker of variable length which may have a conformational function as a hinge. Certain species apparently lost some of the TERT domains during their evolution. Insect species lack the TEN domain, and nematodes the CTE domain. Each of the TERT domains contain several important conserved motifs which are engaged in the formation of tertiary structures that permit the binding of the telomeric template and telomerase RNA subunit. The TEN domain contains 'anchor' sites which bind single-stranded DNA and also the RNA interacting domain 1 (RID1) which binds to TR with low affinity. The TRBD domain contains RNA interacting domain 2 (RID2) which has a high affinity for TR. The RT

domain contains seven motifs conserved between all conventional RTs (1, 2, A, B, C, D, and E). The TERT polymerase active sites are formed by three conserved aspartic acids, with one located in motif A and two in motif C. The CTE domain binds telomeric DNA and contributes to stabilization of the RNA-DNA heteroduplex in the active site of the enzyme. Crystallographic studies reveal a tertiary structure of core TERT protein which resembles a right hand (Gillis et al., 2008). The protein is folded into a ring shaped structure by interactions between the N- and C-terminal domains. The telomerase primer (telomeric DNA) and template are positioned in the center of this ring.

TR is the RNA component of the telomerase holoenzyme and serves as the template for extension. In contrast to the TERT gene, TR is much less conserved during evolution and its size ranges from 300 to 2200 nucleotides. However, several TR domains are essential for its function and their general plan is conserved. The TR molecules of most species contain the pseudoknot/template core domain and the trans-activating domain (CR4/CR5) which are necessary and sufficient for telomerase enzymatic activity *in vitro* and *in vivo*. Moreover, vertebrate TR contains a box H/ACA domain at the 3′ end, which binds two copies of the protein complexes formed from dyskerin, NOP10, NHP2 and GAR1 proteins that are important for biogenesis, localization, transport and stability of the functional RNA molecule. In the 3′ stem-loop of the H/ACA domain is the Cajal body localization sequence (CAB) for binding the Cajal body protein 1 (TCAB1).

Telomerase biogenesis begins with TERT mRNA transcription, maturation and translation. The TERT protein is trafficked from the cytoplasm to nucleoli and then to Cajal bodies for assembly with TR and other proteins of the telomerase complex. The TR precursor is transcribed, bound in the nucleus by accessory proteins which perform the processing and internal modifications of the RNA molecule. The binding of the TCAB1 protein facilitates transport of the mature TR to Cajal bodies where it is assembled with TERT protein into the functional telomerase ribonucleoprotein complex. At least two chaperone proteins (hsp90 and p23) are added before the telomerase complex can be localized to telomere ends.

1.1.2 Telomerase enzymatic function

The telomerase complex is a specialized reverse transcriptase with the ability to produce a long track of telomeric DNA repeats using a short RNA template provided by the TR subunit. This repetitive addition processivity contrasts with the prototypical RT which copies large RNA genomes into DNA. The telomerase catalytic cycle has two phases: nucleotide addition to the 3' end of the telomeric DNA primer and the template translocation for the synthesis of additional repeats. The translocation involves realignment of the RNA template to the new DNA 3′ end (Lewis & Wuttke, 2012; Podlevsky & Chen, 2012). Vertebrate telomerases add six nucleotides (5′-GGTTAG-3′) at the end of the DNA primer. The base-pair binding between the 3′ region of the TR template and 3′ end of the DNA primer is dependent on template length and the presence of several DNA-binding motifs in the TERT molecules. Mutations in these TR and TERT motifs (in motifs 3, 'insertion in fingers' domain (IFD) in the RT domain as well as TEN and CTE domains) alter the rate and processivity of the telomerase reaction (Autexier & Lue, 2006; Christodoulou et al., 2010; Podlevsky & Chen, 2012; Xie et al., 2010). Moreover, POT1 (protection of telomeres 1) and TPP1 (TIN2 and POT1-interacting protein 1), which are components of the telomere DNA-

binding protein complex, shelterin, also enhance repeat addition processivity (Latrick & Cech, 2010; Wang, 2007).

1.1.3 Diseases associated with telomere shortening

The regulation of telomere length is the sum of two balanced processes – telomere erosion and telomere synthesis. Therefore, both accelerated telomere shortening and insufficient telomerase function can erode telomeres. Insufficient telomerase function has been implicated in several diseases that are referred to as syndromes of telomere shortening (for reviews (Armanios, 2009; 2012)). Mutations responsible for these conditions were mapped to several components of the telomerase complex (hTERT, hTR, dyskerin, NOP10, NHP2, and TCAB1). Moreover, it was recently described that mutations in the shelterin component, TINF2, and and in conserved telomere maintenance component 1, CTC1, are also implicated in the pathogenesis of one of these diseases (Alter et al., 2012; Keller et al., 2012). Most of these mutations compromise catalytic activity and alter processivity of the telomerase complex that eventually result in telomere shortening. However, the specific mechanism of some remains unknown (Robart & Collins, 2010).

Telomere shortening may manifest in several different diseases depending on the severity of the defect. Dyskeratosis congenita (DC) disease has the X-linked form known to be associated with mutations in dyskerin (Nelson & Bertuch, 2012). Several autosomal forms of DC are associated with mutations in hTERT, hTR and other components of the telomerase ribonucleoprotein complex as well as in TINF2 and CTC1 (Armanios, 2009; Keller et al., 2012; Nelson & Bertuch, 2012; Walne et al., 2012). Patients with DC suffer from abnormal skin pigmentation, nail dystrophy, and oral mucosal leukoplakia. The disease causes premature death as a result of aplastic anemia due to bone marrow failure. Idiopathic pulmonary fibrosis (IPS) is another disease which strongly correlates with telomere shortening due to mutations in telomerase components (Armanios, 2012). This disease has a much higher prevalence than DC and causes a progressive, severe degeneration of lung tissue that leads to respiratory failure. Additionally, several other syndromes were linked to short telomeres, including liver disease and type 2 diabetes (Armanios, 2012; Salpea et al., 2010; Zee et al., 2010). Many of these syndromes occur in different combinations, dependent on the type of mutations, time of disease onset and interaction with environmental factors. DC and other patients with telomere shortening syndromes are more prone to develop three forms of cancer: myelodysplastic syndrome, acute myeloid leukemia and chronic lymphocytic leukemia. Shortened telomeres are implicated in the development of other neoplasias, including lung, pancreatic, and glioma cancers (Baird, 2010). The shortening of telomeres to critical levels increases the risk of chromosomal instability which, in turn, leads to re-arrangements and the acquisition of new oncogenic mutations.

The length of telomeres is in part inherited (Chiang et al., 2010; Jeanclos et al., 2000; Kappei & Londoño-Vallejo, 2008; Nawrot et al., 2004; Njajou et al., 2007; Nordfjäll et al., 2005; Slagboom et al., 1994). However, many environmental factors, especially those related to lifestyle, also have a strong impact (Lin et al., 2012). These include chronic psychological stress, diet, uptake of food, vitamins, aerobic exercise, and obesity. All these factors strongly correlate with telomere length. Other environmental factors, such as smoking, were shown to interact with genetic predispositions in determining the age of onset of diseases of shortened telomeres as has been demonstrated for emphysema (Armanios, 2012).

1.2 Telomerase non-canonical function

In addition to telomere DNA synthesis, there is an accumulating body of evidence which indicates that telomerase has additional activities (Majerská et al., 2011; Parkinson et al., 2008). These non-canonical activities include stimulation of cell proliferation, protection against oxidative damage, inhibition of apoptosis, modulation of global gene expression, activation of stem cells, and tumor promotion (Bollmann, 2008; Cong & Shay, 2008). Some of these functions, such as the protection of mitochondria against oxidative stress, and modulation of the DNA damage response, require the enzymatic activity of TERT. On the other hand, stimulation of proliferation, inhibition of apoptosis and the regulation of gene expression are independent of TERT enzymatic activity. The cell proliferation and anti-apoptotic functions may be mediated by TERT's ability to induce the expression of genes that promote cell proliferation while simultaneously suppressing pro-apoptotic genes (Smith et al., 2003). TERT promotes the proliferation of hair follicle stem cells in the absence of the TR subunit, providing compelling evidence that this function is independent of telomerase canonical function (Sarin et al., 2005). Interestingly, TERT interacts with the chromatin remodeling factor, BRG1, and as a component of a TCF/β-catenin transcription complex, binds to promoters of Wnt target genes and activates their transcription (Park et al., 2009). The catalytic activity of TERT for Wnt activation is also not required.

1.3 Telomerase transcriptional regulation

Telomerase is downregulated during development in most somatic tissues. Telomerase activity may be reactivated during the immune response and also during tumorigenesis. Several experiments in which the expression profile of a reporter gene under control of the hTERT promoter in transgenic mice simulated the specific expression profile of telomerase activity in human tissues demonstrated that transcriptional regulation of TERT plays a key role in the regulation of telomerase activity (Horikawa et al., 2005; Ritz et al., 2005). The TERT promoter is regulated by several transcription factors (for reviews see (Cifuentes-Rojas & Shippen, 2012; Zhu et al., 2010)). Human as well as the TERT promoter of many other vertebrates contains E-boxes, which are bound by the transcription factor c-Myc which activates TERT transcription. In the proximal human promoter there are also binding sites for other activators such as AP-1, HIF-1 (hypoxia-inducible factor 1), SP1, Ets, E2Fs, NF-κB, PAX-5, and IRF-4 (Interferon regulatory factor 4). Distal promoter elements contain estrogen receptor binding sites which are responsible for the activation of TERT transcription by sex hormones. The repression of the TERT promoter is primarily mediated by competition of USF1 (upstream stimulatory factor 1) and Mad1 with c-Myc for E-boxes. Other repressors of TERT activation are WT1 (Wilms tumor protein 1), Smad3, p53 and MZF2 (myeloid zinc finger protein 2).

Epigenetic regulation of the human *TERT* locus through chromatin modifications may preclude binding of transcriptional activators and repressors to their respective cis sites (Gladych et al., 2011; Zhu et al., 2010). The *hTERT* locus is located in a chromosomal domain (at least 100 kb) which normally has a condensed chromatin structure in most somatic cells (Wang & Zhu, 2004). Only after acetyltransferases modify histone proteins to facilitate the relaxation of the *hTERT* locus may transcription factors actively bind the *TERT* locus and regulate TERT transcription. Histone methylation also plays a role in the activation of TERT since one histone methyltransferase, SMYD3, has been shown to activate TERT transcription

in tumor cells by methylation of histone H3 at lysine 4 (Liu et al., 2007). In addition to histone modifications, CpG methylation and demethylation also play an important role in the transcriptional regulation of TERT. Demethylation of the *hTERT* locus in telomerase-positive cell lines results in a decrease in telomerase activity. CpG methylation in these cell lines is thought to block telomerase repressors from accessing the TERT promoter (Guilleret & Benhattar, 2003). On the other hand, methylation in other cell lines results in transcriptional silencing suggesting that the effect of CpG methylation on hTERT expression is cell type-specific (Devereux et al., 1999).

1.4 Alternative splicing of TERT

Alternative splicing is a common mechanism that increases the transcriptome complexity in higher eukaryotes. Alternatively spliced variants of TERT are abundant and have been cloned from many vertebrate and plant species (Sýkorová & Fajkus, 2009). However, in depth analysis of their expression, evolution and function is limited. The human TERT gene is expressed principally as alternatively spliced (AS) forms in both normal cells and tumor cells (Hisatomi et al., 2003; Hrdličková et al., 2012a; Saebøe-Larssen et al., 2006; Ulaner et al., 1998; Ulaner et al., 2001; Ulaner et al., 2000; Wick et al., 1999). The chicken is the only other species in which alternative splicing of TERT has been extensively characterized and a large number of AS variants have been identified (Amor et al., 2010; Chang & Delany, 2006; Hrdličková et al., 2012a; Hrdličková et al., 2006).

AS TERT variants can be divided into two groups – the first group retains the original open reading frame (ORF), while the second group of transcripts contains premature termination codons (PTCs). The AS variants which maintain the original ORF have the potential to be translated into functional proteins and contribute to proteome diversification (Nilsen & Graveley, 2010). A frequent human AS variant with a small deletion in the reverse transcriptase (RT) domain retains the original TERT ORF, lacks telomerase activity and has been proposed to function as a dominant-negative mutant (Colgin et al., 2000; Yi et al., 2000). Recently, we have described another abundantly expressed human AS variant, which maintains the original TERT ORF, but has deleted exons 4 through 13 (Hrdličková et al., 2012a). This variant also lacks telomerase activity, but in contrast to the dominant-negative variant retains the ability to stimulate cell proliferation. Similarly, two frequently expressed chicken variants that maintain an original TERT ORF stimulated cell proliferation in the absence or reduction of telomerase activity. We also isolated an AS TERT variant similar to one of these chicken variants from platypus, suggesting that this specific splicing event has been conserved during evolution (Hrdličková et al., 2012b). In contrast to the AS variants which can be translated into protein, the AS variants with PTCs are predicted to be degraded by nonsense mediated decay (NMD) during the first round of translation (Lewis et al., 2003). Nevertheless, two-thirds of the human and chicken AS TERT variants are spliced out-of frame and expressed at levels similar to AS variants that retain an original ORF. These observations suggest that at least some of these TERT AS variants escape NMD and may provide an important though unrecognized function. In conclusion, the alternative splicing of TERT mRNA transcripts is a mechanism which decreases the level of telomerase activity, however, AS TERT variants may retain at least some of the non-canonical functions of TERT.

1.5 Telomerase regulation in different species

Telomerase activity is downregulated in adult tissues of most vertebrates. However, the levels of repression differ among vertebrate species. There are large differences in telomerase activity between cold-blooded fish and amphibians, and warm-blooded mammals and birds. The expression of TERT in all adult organs of fish and frogs remains at high levels through their lifespan (Bousman et al., 2003; Hartmann et al., 2009; Pfennig et al., 2008; Yap et al., 2005). By contrast, in most mammalian and avian adult organs, the expression of TERT is severely repressed (Greenberg et al., 1998; Kim et al., 1994; Prowse & Greider, 1995; Taylor & Delany, 2000). Early studies suggested that even among mammals there are significant differences in telomerase levels in adult tissues (Gomes et al., 2010; Greenberg et al., 1998; Prowse & Greider, 1995). In rodents telomerase activity inversely correlates with body mass (Gorbunova & Seluanov, 2009). Recently, an exhaustive analysis of telomerase activity and telomere length in mammals has been reported (Gomes et al., 2011). This analysis revealed striking differences among major mammalian groups. High telomerase activity tends to correlate with low body mass extending the previous observation in rodents. However, whether similar differences in telomerase activity are present in avian species and the mechanism leading to these differences remains unknown.

1.6 Telomerase regulation in chicken and quail

The goal of this analysis was to determine whether differences in telomerase activity also exist in birds, an independently evolved warm-blooded branch of vertebrates, and to explore if TERT transcription or alternative splicing of TERT may account for these differences. We took advantage of two closely related avian species, the quail (*Coturnix japonica*) and chicken (*Gallus gallus*) from the Galliformes order, which differ substantially in their body mass, in their lifespan, and time when they reach reproductive maturity. Quail is a small bird (0.09 kg) with a lifespan between 2-3 years. By contrast, the closely related chicken is on average 10 times larger and can reach 15 and 20 years (30 years maximum). We expected that the TERT genes of these species would encode highly related proteins, but that quail and chicken cells would express different levels of telomerase activity. We cloned both the quail and chicken TERT genes and their AS variants, determined the steady-state levels of TERT transcripts, the patterns of alternative splicing, and correlated it with telomerase activity in various tissues in these two species. We also isolated a partial TERT clone of a more distantly related species, the duck, and performed a similar analysis. Body mass and lifespan (1-1.4 kg, 10-15 years, 29 maximum) of ducks resemble chicken more closely than quail. Therefore, including this species in analysis allows the correlations possibly drawn from comparison of quail and chicken to be extended.

The results of this analysis revealed that despite a high degree of similarity between the quail and chicken TERT gene (94% of identity), these genes differ greatly in their expression of alternatively spliced forms. In contrast to chicken and duck TERT, where 37 and 10 AS variants were identified, respectively, only three quail AS variants were detected. All three of the quail TERT variants were also present in chicken tissues, consistent with their close evolutionary origin. While the total levels of all TERT transcripts in quail organs were similar to levels in chicken or duck cells, quail tissues principally express full-length TERT transcripts. By contrast, in the chicken and duck, most of the TERT transcripts are alternatively spliced. At least some of the differences in the frequency of the AS variants

between chicken and quail were determined by different splicing sequences in the TERT locus. The frequency and complexity of AS variants of TERT correlated with telomerase activity in quail and chicken cells. The lower frequency of TERT AS variants in quail tissues relative to the frequency in chicken and duck tissues was associated with a significantly (5×) higher levels of telomerase activity. Interestingly, these differences in telomerase activity were also discernible in early embryonic tissues. Telomerase activity is downregulated approximately 1,000 times in adult organs of all three species but remains significantly higher in quail. In addition, *in vitro* analysis of embryonic fibroblasts demonstrated that while quail retain high levels of telomerase activity and telomere length, chicken and duck cells rapidly repressed telomerase levels and their telomeres shorten during cell replication. Quail embryonic fibroblasts are at least three times more sensitive to oxidative stress than chicken and duck cells suggesting that relatively high telomerase levels may compensate for this deficiency. In conclusion, the results of these studies suggest a role for the regulation of TERT through alternative splicing as one of mechanisms for determining interspecies differences in telomerase activity.

2. Material and methods

2.1 Cloning of quail TERT and its variants

The chicken TERT (chTERT) gene was cloned previously (Delany & Daniels, 2004; Hrdličková et al., 2006). The quail and duck TERT genes (qTERT and dTERT) were cloned using cDNA obtained from 8 and 12 day-old quail and duck embryos using sets of primers against chicken TERT. Quail genomic sequences were cloned by PCR from genomic DNA of QT6 cells.

2.2 GenBank accession numbers

Newly determined nucleotide sequences were deposited in GenBank (DQ681292 [quail TERT intronic sequences], DQ681293 [duck TERT cDNA], DQ681294 [quail TERT cDNA], JF896279, DQ681295 to DQ681313 [alternatively spliced forms of quail, chicken and duck TERT]).

2.3 Sequence analysis

The full ORF sequence of quail (*Coturnix japonica*) TERT and partial sequence of duck (*Anas platyrhynchos*) TERT were determined in the course of these studies. The TERT protein sequences of chicken (*Gallus gallus* - NP_001026178.1) and Muscovy duck (*Cairina moschata* - ABO65149.1) were retrieved from GenBank. TERT sequences of turkey (*Meleagris gallopavo* - ENSMGAP00000007237), zebra finch *(Taeniopygia guttata* - ENSTGUP00000008676), and green anole (*Anolis carolinensis* - ENSACAP00000001407) were obtained from the Ensembl database (www.ensembl.org). Ensembl models were checked against genomic DNA sequence and minor modifications were introduced where the models did not conform to the evolutionary conserved exon-intron structure of the vertebrate TERT genes. Sequence homology (percentage of identical and similar amino acids) was determined using GeneDoc computer program (http://www.nrbsc.org/gfx/genedoc/). Amino acid similarity was calculated based on BLOSUM62 matrix. Protein sequence alignments were constructed by the ClustalX program (Larkin et al., 2007). All columns containing either gap or unidentified

amino acid were removed using Gapstreeze tool (Los Alamos HIV Sequence Database; http://www.hiv.lanl.gov/content/sequence/GAPSTREEZE/gap.html). Evolutionary trees were constructed by Bayesian inference phylogenetic method performed by MrBayes 3.1.2 program using fixed-rate amino acid substitution model Jones (Ronquist & Huelsenbeck, 2003). Two Bayesian analyses each consisting of four Metropolis-coupled Markov chains Monte Carlo were run in parallel for 200,000 generations and sampled every 100th generation. Convergence of both analyses was assessed using a plot of the generations versus the log probability of the data. The consensus tree was created with burn-in value set to 500. The tree was plotted by the tree-drawing program Dendroscope (Huson et al., 2007).

2.4 Animals, cell lines, and tissue culture

Quail (*Coturnix japonica*) embryonated eggs and birds were obtained from University of Texas, Austin. Embryonated duck eggs (Khaki Campbell) were obtained from McMurray hatchery (Webster City, IA). Embryonated eggs from pathogen-free White Leghorn chickens (the SPF-SC strain) were obtained from Charles River SPAFAS, North Franklin, CT. Adult chickens and a Peking duck were obtained from a local vendor. Quail, chicken, and duck embryonic fibroblasts (QEF, CEF, and DEF) were prepared from 8, 10, and 12 day-old embryos, respectively. Tissue culture procedures were carried out as described previously (Hrdličková et al., 2006). QT6 is a quail sarcoma cell line (Moscovici et al., 1977). The duck embryonic cell line (DCL) was obtained from ATCC (ATCC Number: CCL-141) (Marcovici & Prier, 1968).

2.5 Telomere Repeat Amplification Protocol (TRAP)

The level of telomerase activity was evaluated using the TRAP assay as described previously (Hrdličková et al., 2006). Briefly, cells were extracted with CHAPS buffer and protein concentrations were determined by the Bradford method with Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Protein extracts (20 µg of total protein or less) were first incubated with 0.5 µg of the TS primer and all four dNTPs (1 mM each) in TRAP reaction buffer, 0.8 mM spermidine, 5 mM β-mercaptoethanol in a total reaction volume of 50 µl for 30 minutes at 37^{o}C. The reaction was stopped by incubation at 94^{o}C for 2 minutes. Aliquots of synthesis (2.5 µl) were then PCR amplified as described (Hrdličková et al., 2006). The TRAP PCR products were separated on 7.5% acrylamide gels (ratio of acrylamide to bis-acrylamide 19:1) in 0.5 × TBE (TBE is 0.09 M Tris-borate, 2 mM EDTA pH 8.0) and gels were stained with VISTRA Green (GE Healthcare, Piscataway, NJ). For molecular weight determination a 10 bp ladder (Invitrogen, Carlsbad, CA) was used.

2.6 Terminal restriction fragment (TRF) length analysis

TRF analysis was performed as described previously (Hrdličková et al., 2006). High-molecular-weight genomic DNA (0.2 µg) was digested with a cocktail of restriction enzymes (HinfI, HaeIII, MspI, and RsaI) and separated in TBE in a 0.6% agarose gel. Undigested λ phage DNA mixed with λ digested with EcoRI and HindIII was used as marker. DNA was Southern transferred to a Hybond-N+ membrane (GE Healthcare) and hybridized to the telomeric probe $(CCCTAA)_6$ end-labeled with [γ-^{32}P]-ATP at 42^{o}C using Ultrahyb solution (Ambion, Austin, TX). Blots were washed under stringent conditions. Subsequently, the blots were rehybridized with a λ probe to visualize the position of the markers.

2.7 Identification of TERT and its alternatively spliced variants by semiquantitative RT-PCR

Total RNA was isolated by RNAwiz (Ambion) and cDNA synthesis and RT-PCR were carried out as described previously (Hrdličková et al., 2006). The expression of avian TERT, its AS variants, and GAPDH was detected by RT-PCR using primers specific for TERT and GAPDH genes (Table 1).

Name[a]	Gene	Sequence
Q14F	qTERT	5′-CTGATACTGCTTCATGCTGCTATTATATCC-3′
Q16B	qTERT	5′-GATGGTTCCGTCACTGTCTTCAGCAGTTC-3′
C14F	chTERT	5′-CTGATACTGCTTCATGCTGCTATTTTATCC-3′
C16B	chTERT	5′-GATGGTTCCGTCACCGTCTTCAGCAGTTC-3′
D14F	dTERT	5′-CTGAGAATGCATCGTGCTGCTATTCTATGC-3′
D16B	dTERT	5′-GATGGTTCTGTCACTGTCTTCAGTAGTGC-3′
qGAPDH1	qGAPDH	5' -ATTATCTCAGCCCCCTCAGCTGATGC-3'
qGAPDH2	qGAPDH	5' -CACAACTTCCCAGAGGGGCCGTCCAC-3'
chGAPDH1	chGAPDH	5'-ATCATCTCAGCTCCCTCAGCTGATGC-3'
chGAPDH2	ch/dGAPDH	5'-CACAGCTTCCCAGAGGGGCCATCCAC-3'
dGAPDH1	dGAPDH	5′-ATCATCTCCGCCCCCTCAGCTGATGC-3′
QKbSpWT	qTERT	5′-AGTGAATGACTGCGTATGGCTTCGTCTAG-3′
QKbSpA	qTERT	5′-CATGCTGGTTCCCCCAAGTAACTGTTACCAGGTAATC-3′
T6.2	qTERT	5'-CCACACCATGAGATCAAACGACAATCTGG-3'
KbSpWT	chTERT	5'-AGTGAATGACTGCGTATGGCTTCGTCTGG-3'
KbSpA	chTERT	5'-CAGCCTTCTGCAAAAGTGAACTTTCAAGCAGGTAATC-3'
T6	chTERT	5'-CCACACCATGAGATCAAGCGACAATCTGG-3'
D3F	dTERT	5′- GCTGATGGATACGTATGTTGTTCAGTTGCTCAGATC -3′

[a] Primer pairs Q14F and Q16B (quail), C14F and C16B (chicken), D14F and D16B (duck) were used for RT-PCR determination of TERT mRNA levels. Primers pairs qGAPDH1 and qGAPDH2, chGAPDH1 and chGAPDH2, dGADPH1 and chGADPH2 were used for determination of the levels of quail, chicken, and duck GAPDH, respectively. Primers T6.2, QKbSpWT, and QKbSpA were used for cloning the quail TERT isoforms, primers T6, KbSpWT, and KbSpA were used for cloning the chicken TERT isoforms. KbSpWT/QKbSpWT and T6/T6.2 primers detect only TERT transcripts lacking alternative splicing A/qA2 because the KbSpWT/QKbSpWT primer is located in the region which is deleted by this splicing event. KbSpA/QKbSpA and T6/T6.2 primers detect only the alternative splicing A/qA2 form of TERT because the KbSpA/QKbSpA primer binds to the junction created by alternative splicing A/qA2 and is, therefore, incapable of priming the synthesis of wild type TERT. Finally, primers D3F and D16B were used for cloning duck TERT mRNAs from different tissues.

Table 1. Oligonucleotide primers used for RT-PCR analyses

2.8 Determination of cell resistance to oxidative challenge

Exponentially growing cells (0.5×10^6) were seeded into 60 mm plates one day before the experiment. Freshly diluted H_2O_2 (Sigma, St. Louis, MO) was added and cells were counted after 24 hours. The dead cells were excluded by trypan-blue staining.

3. Results

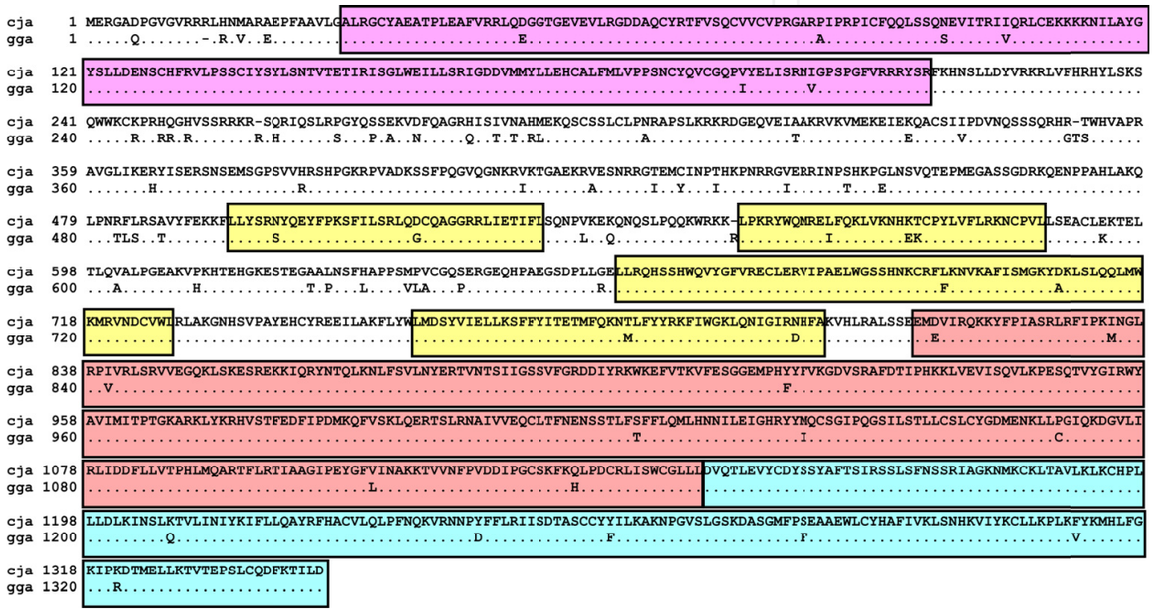
To perform comparative analysis of TERT proteins of three avian species, quail, chicken, and duck, we cloned quail and duck TERT, compared their sequences with the chicken TERT sequence and defined their evolutionary distances. We also cloned alternatively spliced TERT variants from these species and determined their expression in different tissues. We defined the telomere length in embryos and telomerase activity in tissues of these species and correlated them with the frequency and complexity of alternatively spliced variants. Analysis of *in vitro*-cultivated embryonic fibroblasts established the differences in cell growth, telomerase activity, and telomere length during continuous cell passage and compared them with the sensitivity of these cells to oxidative challenge. Finally, to evaluate the role of transcription in the regulation of telomerase activity, we measured the steady-state levels of total TERT transcripts in tissues of these species.

3.1 Quail and chicken TERT have a very similar protein primary structure

Quail and chicken *TERT* genes encode very similar proteins (Fig. 1). The quail reverse transcriptase gene is 1344 amino acids, which is two amino acids shorter than the chicken protein as a result of four single amino acid indel differences. The proteins of both species contain the same conserved regions which encode the structural domains of TERT and represent 70% of the molecule. The amino acid sequences are 94% identical and percentage of identity reaches 97% (100% if similar amino acids are considered) in some of the conserved regions. The majority (65%) of the differences between the quail and chicken TERT protein are concentrated in the less conserved regions of the N-terminus and three linker regions (L1-L3).

To further characterize the similarities of quail and chicken TERT and compare them with the TERT proteins of related species, the best characterized TERT sequences of the sauropsid amniots were aligned. The group Sauropsida includes all living reptiles and birds and their extinct reptilian predecessors and represents one of the two evolutionary branches of amniotes (Benton, 2005). The other amniotic branch, Synapsida, includes mammals and their extinct reptilian grade ancestors. The amino acid homologies of seven sauropsid proteins were then compared in each functional domain/motif (Table 2). Quail TERT exhibited the highest sequence homology with chicken and turkey TERT in all regions. Even in the three linker regions (L1-L3), quail and chicken proteins were 85-91% identical which contrast with the lower conservation over longer evolutionary distances (e.g., 9-19% for quail and anole). The TERT proteins of ducks (both Mallard and Muscovy) are significantly more dissimilar to quail TERT, with the levels of similarities not much different from zebra finch TERT.

The evolutionary tree of these seven sauropsid TERT proteins confirmed the close relationship among the three proteins of the galliform birds - quail, chicken, and turkey (Fig. 2). Apparently TERTs of quail and chicken retained high level of similarity despite that the last common ancestor of the two species lived 30-40 million years ago (MYA) (van Tuinen & Dyke, 2004). The TERT proteins of anseriform birds, ducks, are much more distant even



Regions broadly conserved among vertebrate TERTs are color-coded: TEN domain (purple), TRBD motifs v-II/VSR, v-III/CP, QFP, and T (yellow), RT domain (red), CTE-terminal domain (blue). Three linkers of less conserved sequence are situated: L1 between TEN and v-II, L2 between v-II and v-III, and L3 between v-III and QFP. Alignment gaps are indicated by dashes. In chicken TERT, only amino acids that are different from quail TERT are shown.

Fig. 1. Comparison of quail (*Coturnix japonica* - cja) and chicken (*Gallus gallus* - gga) TERT protein sequences

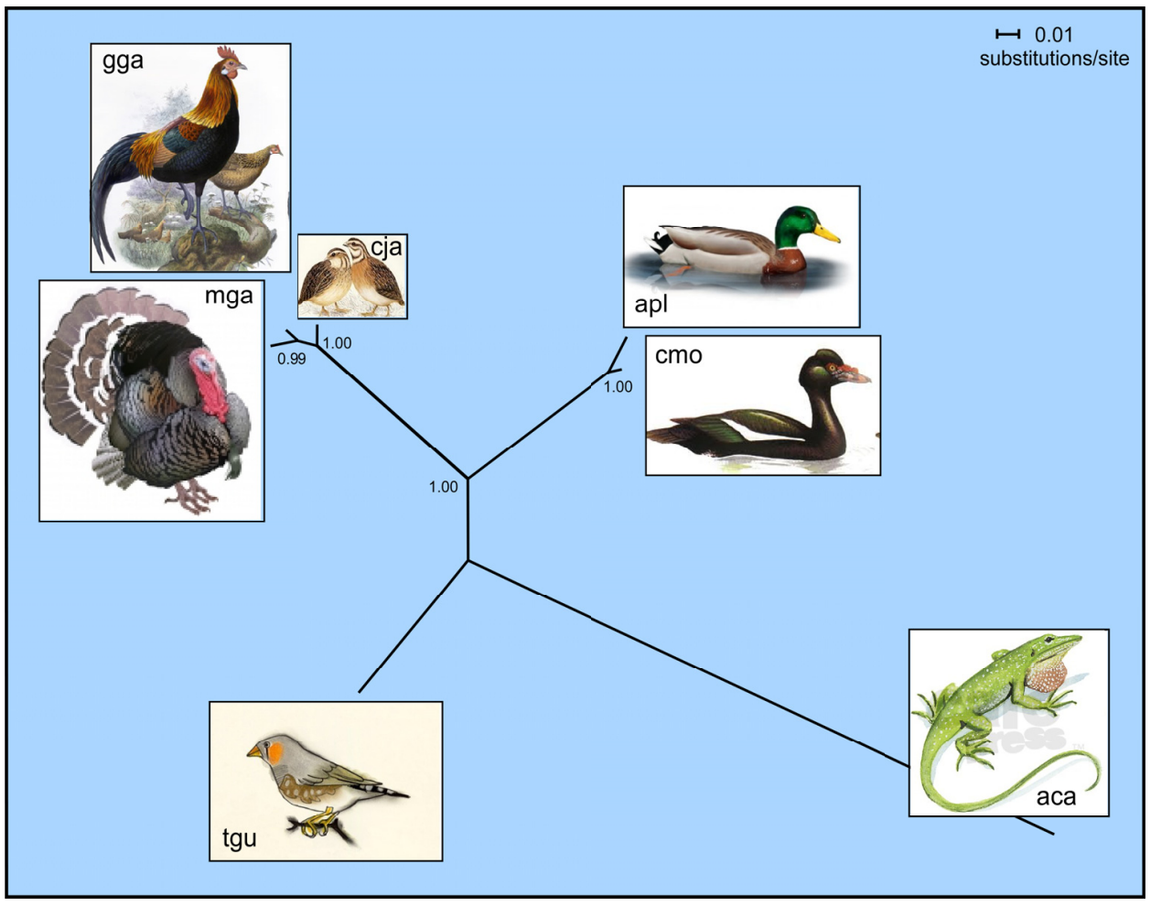
though the tree agrees with the joining of both Galliformes and Anseriformes in a one monophyletic group of Galloanserae (Chubb, 2004). Interestingly, the TERT gene of Neoaves (zebra finch) is equally evolutionarily distant from Galliformes and duck TERT. The TERT of Lepidosaurs (green anole) is significantly more distant from two basal avian branches (Neoaves and Galloanserae) than these branches are from each other.

In conclusion, the three avian TERT genes of quail, chicken, and duck exhibit different level of sequence similarity of their protein products. The primary structures of quail and chicken TERT proteins are closely related suggesting high degree of similarity in function and regulation at the protein level while the duck TERT protein is significantly more distant.

Domain/	Percentage of identical amino acids[a]						Percentage of similar amino acids					
motif	gga	mga	cmo	apl	tgu	aca	gga	mga	cmo	apl	tgu	aca
TEN	96.8	ND	73.3	ND	74.9	52.9	99.5	ND	85.6	ND	84.0	72.7
L1	87.8	83.5	45.3	ND	41.7	17.6	92.4	89.9	60.8	ND	60.1	36.7
v-II	94.4	91.7	66.7	ND	72.2	41.7	97.2	97.2	83.3	ND	88.9	72.2
L2	90.5	90.5	71.4	ND	61.9	19.0	100.0	95.2	85.7	ND	90.5	47.6
v-III	91.4	88.6	62.9	ND	65.7	62.9	97.1	97.1	80.0	ND	77.1	80.0
L3	84.5	80.3	39.4	ND	38.0	8.5	87.3	88.7	53.5	ND	56.3	26.8
QFP	97.1	97.1	82.9	ND	80.0	78.6	98.6	98.6	92.9	ND	94.3	90.0
T	95.7	97.9	83.0	78.7	87.2	74.5	97.9	97.9	91.5	93.6	93.6	89.4
RT	97.3	97.0	78.6	78.0	78.3	62.2	99.7	99.4	90.2	89.0	89.6	78.6
CTE	97.0	ND	81.2	81.7	75.6	64.5	98.5	ND	90.4	91.4	84.3	79.7
TERT	93.9	ND	67.9	ND	66.5	48.2	96.8	ND	80.0	ND	79.2	65.8

[a] Animals: gga (*Gallus gallus*) - domestic chicken, mga (*Meleagris gallopavo*) - turkey, cmo (*Cairina moschata*) - Muscovy duck, apl (*Anas platyrhynchos*) - domestic (Mallard) duck, tgu (*Taeniopygia guttata*) - zebra finch, aca (*Anolis carolinensis*) - green anole. Domains and motifs are described in the Fig. 1. TERT indicates the entire protein. ND - not determined, because the complete sequence was not available. The highest numbers for each region are shown in red.

Table 2. Comparison of the functional domains and motifs of quail TERT with six other sauropsid TERT proteins

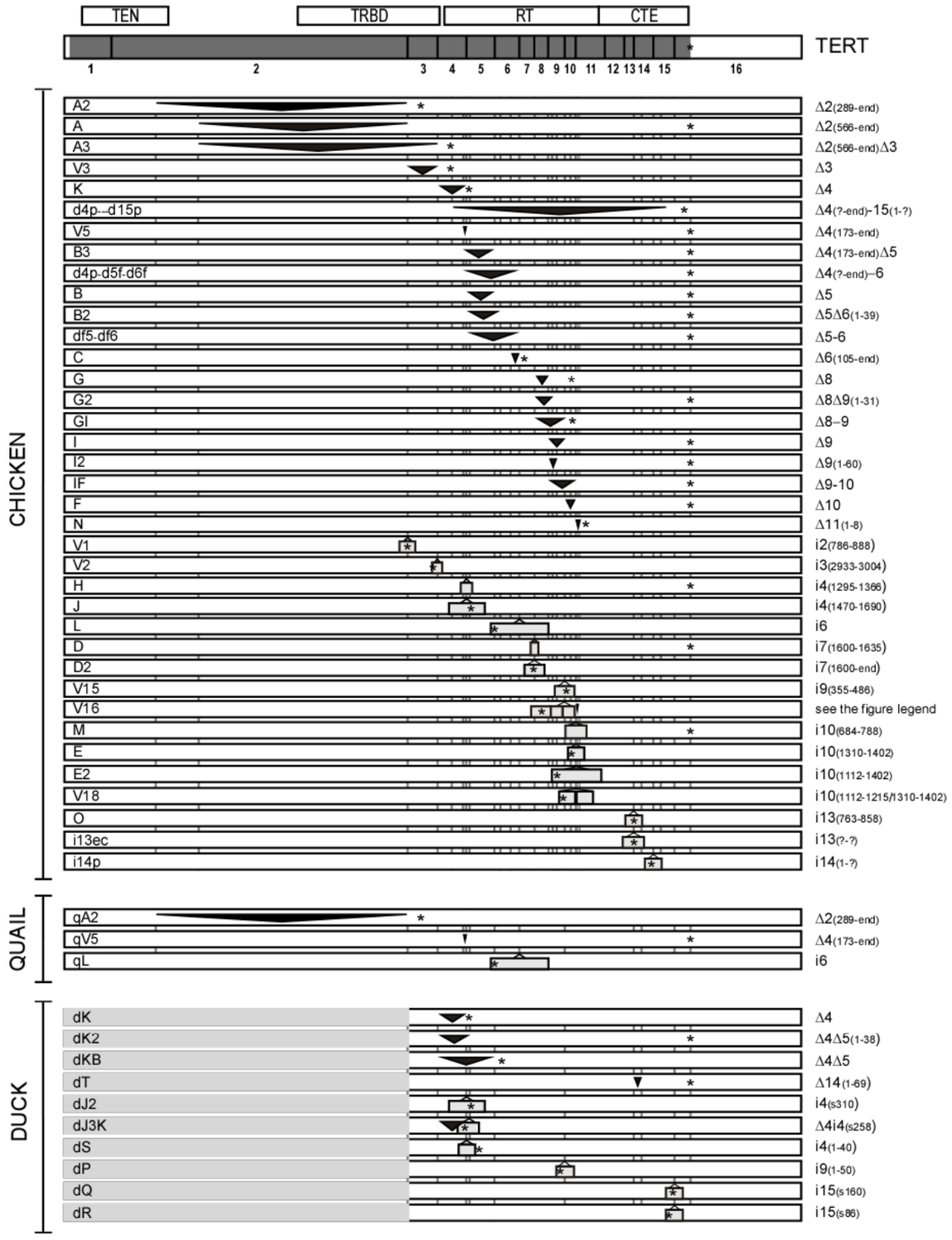


The tree is built on the alignment consisting of 592 columns of amino acid sequences where all seven proteins were aligned without any gaps. The bar represents 0.01 substitutions per site. Species abbreviations are shown in the legend to Table 1. Posterior probabilities are indicated at each node except the node that connects the selected outgroup species - anole.

Fig. 2. Bayesian phylogenetic tree of sauropsid TERT proteins.

3.2 Quail and chicken differ greatly in the number of alternatively spliced variants

Fifty alternatively spliced variants of chicken, duck, and quail TERT were cloned (Fig. 3). Sixty percent of the alternative splicing events of avian TERT introduced novel stop codons which would lead to prematurely terminated proteins during translation and their RNAs would likely be subject to nonsense-mediated mRNA decay (Green et al., 2003). The remaining forty percent were spliced in-frame. In most of these cases, alternative splicing eliminated or modified functional domains or motifs important for telomerase activity. The highest number of alternatively spliced variants was identified in chicken (37), followed by 10 AS variants identified in duck. This number is likely an underestimate since we didn't obtain the N-terminal region of the duck sequence where several alternative splicing events occur in chicken, human, platypus, and one in quail. Also, a variety of TERT mRNA molecules which contain multiple AS events in many combinations were detected in chicken and duck, thereby creating even higher diversity of AS isoforms in these species. In contrast, only three alternatively spliced variants were identified in quail despite screening various



A large number of single AS event variants of avian TERT were identified. Many of the chicken variants were described previously (Amor et al., 2010; Chang & Delany, 2006; Hrdličková et al., 2006). The structure of wild-type (WT) TERT mRNA is shown at the top with the TERT ORF shaded. The positions of exons and the functional domains (TEN - telomerase essential N-terminal domain, TRBD - telomerase

RNA binding domain, RT - reverse transcriptase domain, CTE - C-terminal domain) of avian TERT are indicated. In a set of variants, deletions (triangle) and insertions (gray rectangles) resulting from splicing events are indicated. Asterisks identify the location of stop codons. The trivial names of the TERT AS variants are shown at the left. The descriptive names ("Δ" indicates deletion of exon-derived and "i" insertion of intron-derived sequences) are shown in the right margin. The numbers in parentheses indicate positions within exon or intron. Some of the duck AS variants are described only by the size of the insertion indicated by the preceding letter s. Two complex chicken variants, V16 and V18, likely arose through combinations of several single AS events. The descriptive name of the V16 variant is i9 (355-486/926-988/1091-1162)/Δ11(1-3). The sequence of the first two exons of duck TERT was not determined (gray shading).

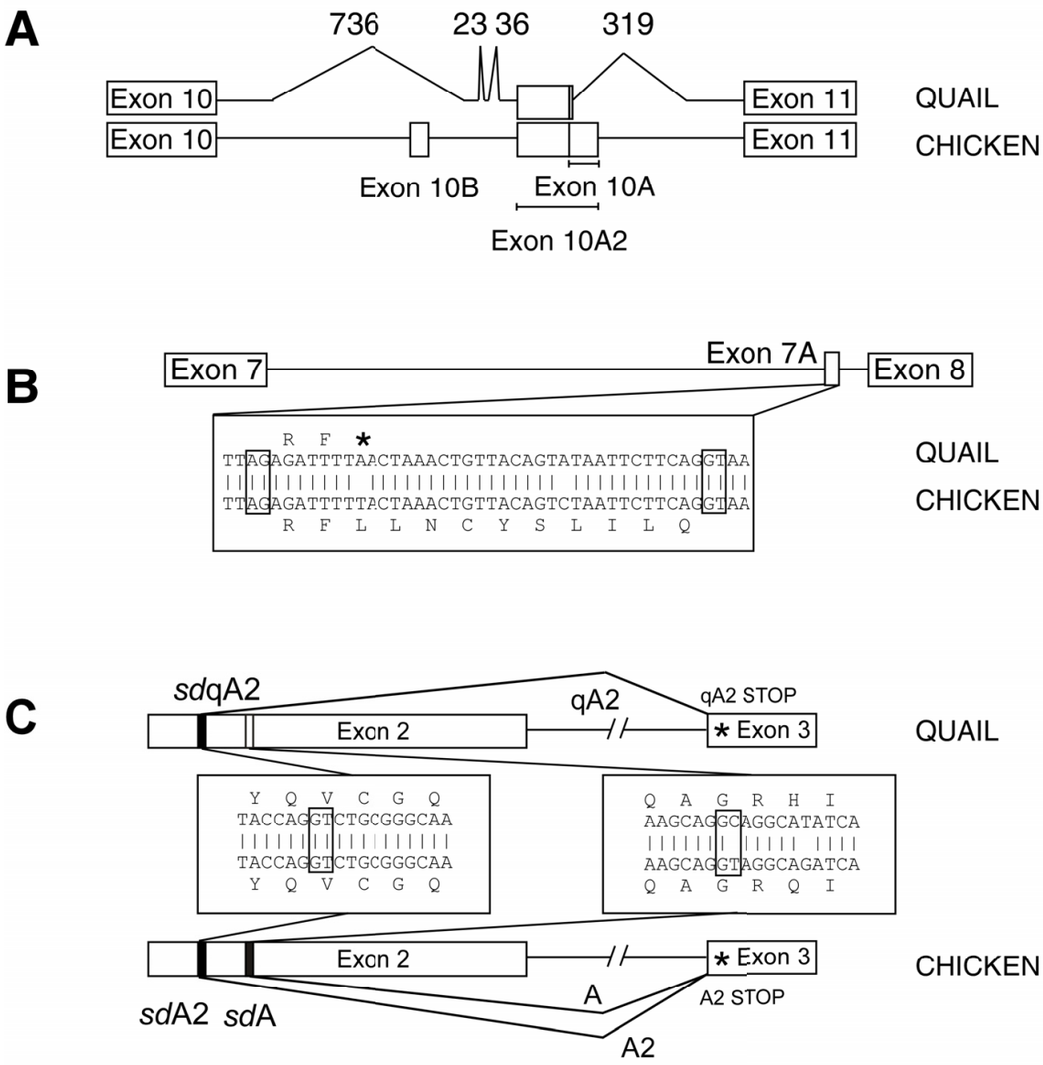
Fig. 3. Alternative splicing of quail, chicken, and duck TERT

tissues and obtaining over 60 independent clones of qTERT. All three quail AS variants have identical orthologs among chicken variants consistent with their close evolutionary relation. On the other hand, the more distantly related duck share only variant K with chicken, but five other duck variants have certain structural similarity to chicken AS variants (KB, J2, J3K, S, and P). KB is fusion of chicken AS variants K and B, and J3K is fusion of chicken J with K. The AS variants J2, S, and P introduced novel intron cassette sequences from the same intron as the chicken variants.

In conclusion, despite the close sequence homology, quail expresses ten times less alternatively spliced variants than chicken and three times less than the more distantly related duck. While three quail AS variants identified are also expressed in chicken, the duck AS variants have only limited structural similarly with the chicken AS variants.

3.3 Differences in *cis* regulation of species-specific alternative splicing

Comparison of quail (GenBank, DQ681292) and chicken genomic DNA (GenBank, AADN00000000) suggests that many changes in alternative splicing may have evolved as a result of differences within the TERT gene (*cis*) (Fig. 4). These differences include absence of intron-based alternative cassette exons, presence of premature stop codons, and alteration in alternative splice donor sequences. The most obvious difference is the absence of exon 10B and large regions of exons 10A, 10A2 (cassette exons located in chicken intron 10) (Fig. 4A). Consequently quail cannot produce alternative splicing variants E, E2, and M. Further, although the sequence of exon 7A is conserved in quail, it contains a stop codon (Fig. 4B). This stop codon may prevent the production of TERT isoform D by nonsense-mediated suppression of splicing or decrease its level by nonsense-mediated decay (Wachtel et al., 2004). Finally, chicken exon 2 contains two alternative splice donors for the isoforms A and A2 (Fig. 4C). While quail TERT also produces the isoform A2 (qA2), the quail isoform A was not detected. The quail sequence found in the location of chicken splice donor site for variant A does not contain the critical core dinucleotide (GT) normally used in the removal of more than 98% of all introns, but a much weaker sequence (GC), employed in the removal of less than 1% of introns (Abril et al., 2005). This difference between sequences of the two species may favor use of the upstream located splice donor site (s*d*qA2) in quail TERT. Collectively, these results demonstrate that at least some differences in the alternative splicing pattern of the TERT gene between quail and chicken are regulated in *cis*.



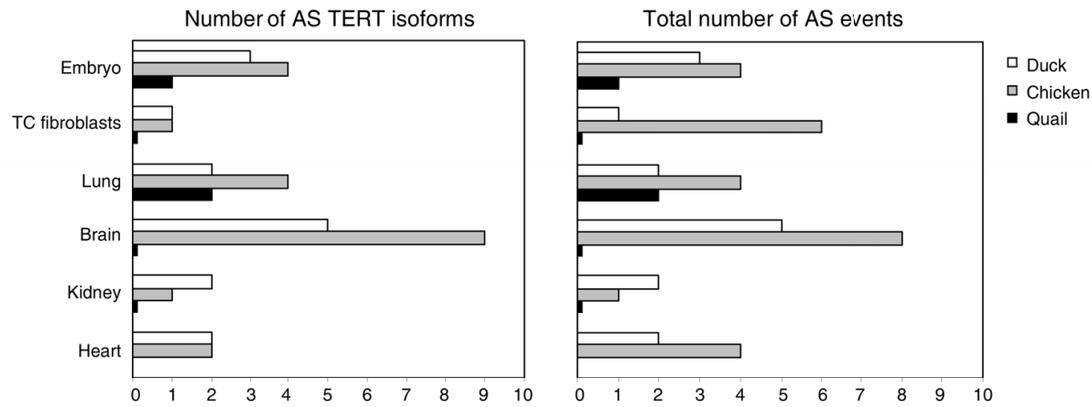
A. Key differences in the sequences of quail and chicken intron 10. Four large regions of DNA present in chicken TERT gene are not found in the corresponding quail intron (736, 23, 36, and 319 nt). B. A stop codon (indicated by asterisk), absent from the chicken cassette exon 7A, is present in the corresponding quail sequence. C. Comparison of splice donor A site in chicken exon 2 (*sd*A) with its quail sequence homolog, and splice donor A2 (*sd*A2) site in the same exon with quail qA2 splice donor site (*sd*qA2). In contrast to the chicken isoform A, chicken isoform A2 and quail isoform qA2 contain stop codons in exon 3 sequences (asterisks).

Fig. 4. *Cis* regulation of species specific alternative splicing

3.4 Quail cells have a reduced frequency and lower complexity of TERT AS variants than do chicken and duck cells

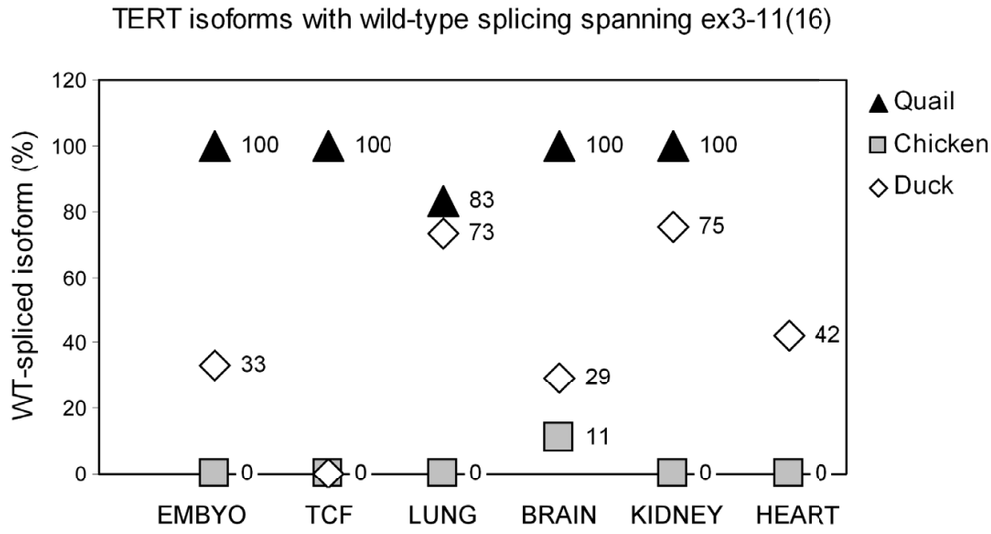
To determine whether the differences in the numbers of AS variants are maintained in different cells and tissues of quail and chicken, large regions of the TERT genes were cloned from each tissue source and 5-20 clones were analyzed by sequencing or by restriction enzyme analysis (Figs. 5 and 6). Differences in alternative splicing were quantified either as

a number of distinct isoforms (containing one or more alternative splicing events per molecule) or as a total number of AS events detected in all isoforms (Fig. 5). Analysis of quail cDNAs revealed that TERT AS forms are relatively rare. Only two quail TERT AS variants, qA2 and qV3, were detected in this experiment. The most frequent quail AS variant, qA2, was isolated only from tissues, like embryo and lung, with high proliferation activity. In cells where the qA2 isoform was detected, the isoform was present at much lower levels than were wild-type (WT) TERT transcripts (data not shown). An additional spliced variant (qV3) was detected only once, in lung tissue. In contrast, the analysis of clones from chicken and duck cells revealed the abundance of AS variants with different combinations of AS splicing events. In relatively homogenous cell populations (in embryonic fibroblasts and heart), only one or two spliced variants were detected. In contrast, a great variety of different AS variants were present in cells derived from the brain (5-9 different variants), most likely due to the heterogeneity of cell populations in this tissue. Chicken and duck AS variants contained from one to six splicing events on a single molecule. Generally, measuring AS complexity as either the number of isoforms or the total number of AS events gave very similar results, except in chicken fibroblasts, where one isoform carrying six AS events was detected.



Complexity of TERT alternative splicing is shown as a number of distinct isoforms as well as a total number of alternative splicing events detected in tissues and cells of three galloanserine birds. TC (tissue culture) fibroblasts are embryonic fibroblasts cultivated *in vitro* for 6 days after the establishment of primary cultures. The WT TERT isoform is not included in the number of AS variants. The short columns for quail AS isoforms/AS events in fibroblasts, brain, and kidney indicate that no AS variants (only WT-spliced transcripts) were detected in these cells and tissues. We failed to isolate any TERT clones from quail heart. Identity of AS variants was established by the RT-PCR amplification of several TERT mRNA regions using the primer pairs described in section 2.7. For each tissue (except for chicken fibroblasts) the sequences of 5-18 quail or chicken clones or 3-16 duck clones were determined and the identity of additional 3-11 duck clones was established by restriction analysis. Only two AS chicken TERT clones were obtained from chicken fibroblasts.

Fig. 5. Complexity of TERT alternative splicing



Isolation frequency of WT-spliced clones spanning exons 3-11 (3-16 in duck) cloned from embryo, tissue culture fibroblasts (TCF), lung, brain, kidney, and heart of three galloanserine birds. The RT-PCR amplification used 5' primers complementary to the 3' end of exon 2 (beginning of exon 3 in duck), and 3' primers complementary to the middle of exon 11 (beginning of exon 16 in duck). We failed to isolate any TERT clones from quail heart in any PCR reactions in this study.

Fig. 6. Isolation frequency of WT-spliced clones

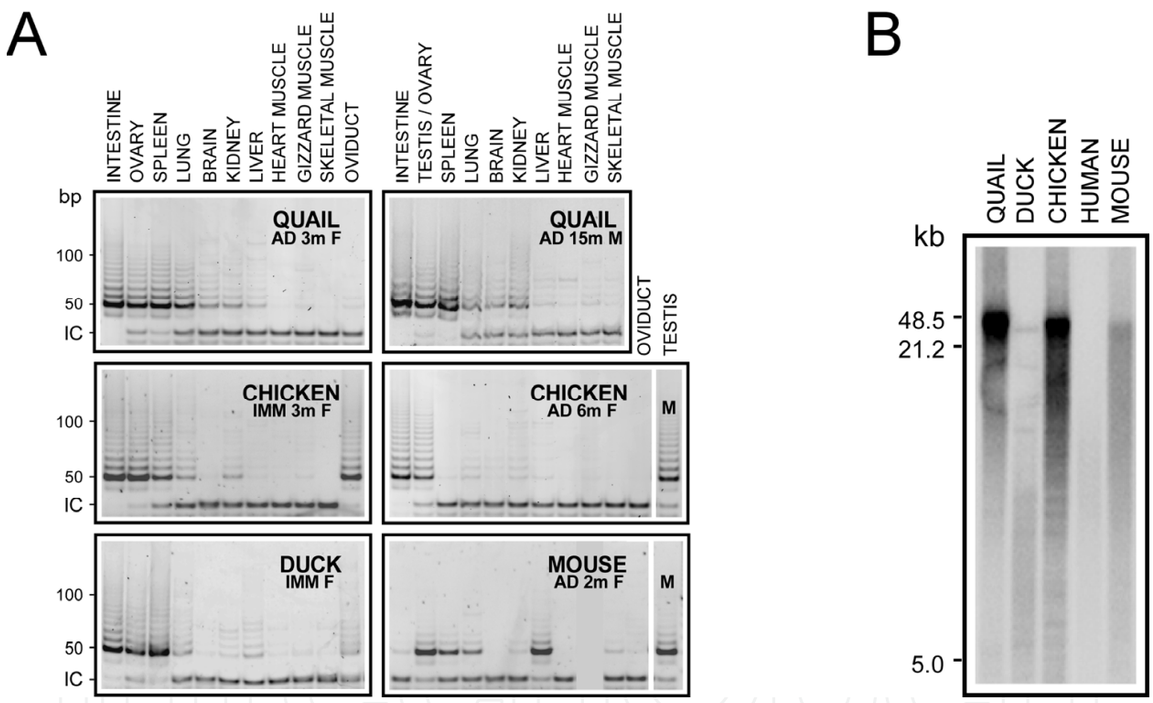
The abundance of WT isoforms was estimated by RT-PCR amplification of the specific regions of quail, chicken, and duck TERT (Fig. 6). All AS events identified in this study are located in these regions (except the chTERT AS event O). PCR products were cloned and sequenced. The final percentage was calculated from forms with AS events located between exons 3-11 (3-16 in duck) except the clones from chicken fibroblasts (TCF), lung, and kidney. We failed to isolate any clones from chicken fibroblasts, lung, and kidney with this PCR strategy, suggesting that the both WT and AS isoforms are below the threshold of detection. All clones isolated from these sources contained an A splicing event in the second exon. The analysis revealed that WT-spliced isoforms are frequently expressed in quail tissues, while in chicken tissues WT clones were obtained only from the brain. The frequency of isolation of the WT isoform in duck was intermediate between those in quail and in chicken.

In conclusion, chicken and duck TERT genes produce multiple and abundant AS isoforms in several tissues while the complexity and frequency of AS forms in quail tissues are severely limited. In a complementary manner, the WT-spliced isoform (potentially encoding an enzymatically active protein) is more prevalent in quail tissues than in the tissues of the other two galloanserine birds.

3.5 Quail expresses higher levels of telomerase activity than chicken and duck in all tissues

To investigate whether there are differences in the telomerase activity in quail, chicken, and duck, various organs of these birds were isolated and telomerase activity was determined in tissue extracts by a telomerase repeat amplification protocol (TRAP) assay (Fig. 7A).

Telomerase activity was determined in adult quail (3 month-old), senescent quail (15 month-old), immature chicken (3 month-old), adult chicken (6 month-old), and an immature duck (approximately 5-6 month-old). Telomerase activity was also measured in the corresponding organs from an adult 2 month-old mouse, the prototype of a small mammal with high telomerase activity. Telomerase activity was high in the intestine, ovary, and testes in birds of all ages. In other organs, including the spleen, lung, brain, kidney, and liver, telomerase activity was significantly higher in adult quail (3 month-old) than in the chicken at a comparable developmental stage (6 month-old) and remained high even in the 15 month-old quail. Telomerase activity in duck organs was also lower than in adult quail even though the duck analyzed was not yet sexually mature. The distribution of telomerase activity in the different tissues was similar in all birds with the exception of the duck liver, which had higher activity compared to chicken. Mouse, like quail, had high telomerase activity in several organs but the tissue distribution was strikingly different and telomerase had a lower processivity.



A. Telomerase activity in extracts from different tissues of quail and chicken was determined by the TRAP assay. Sexual maturity (IMMature or ADult), age in months (where known), and sex (F or M) of the experimental animals are indicated. Telomerase activity in the testes from an age matched 6 month-old chicken and 2 month-old mouse are shown on the right. Each lane represents the activity in whole cell extract containing 1 µg of total protein. The position of the PCR internal control (IC) bands is indicated. B. Telomere length was determined by terminal restriction fragment (TRF) length analysis using a 0.6% agarose gel. This analysis included DNA isolated from quail, duck, and chicken embryos, human (BD Biosciences Clontech, Cat. No. S0950), and spleen from a 2 month-old mouse (strain C57BL/6).

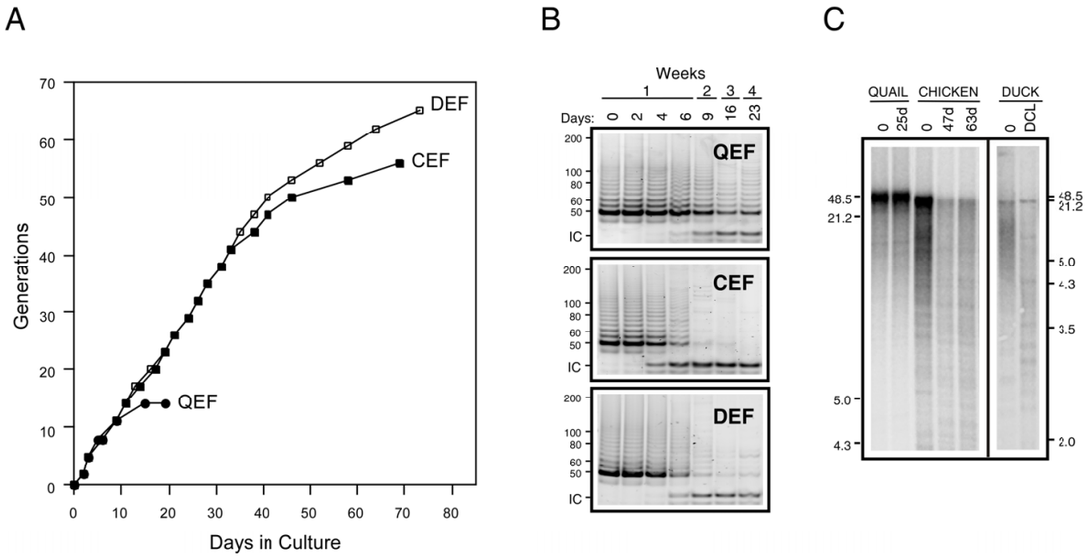
Fig. 7. Species-specific differences in telomerase activity and telomere length in quail, chicken, and duck

The detailed study of telomere length of mammals demonstrated that the average length of telomeres ranges from very long (up to 50 kb in some rodents and lagomorphs) to very short (below 10 kb in primates). However, species can be divided into two groups according to their telomere length, one group with the average telomere length below and the second above 20 kb (Gomes et al., 2011). The average length of telomeres in quail, chicken, and duck was determined and compared to human and mouse by terminal restriction fragment (TRF) analysis (Fig. 7B). While quail has slightly longer telomeres than chicken, this difference is only subtle and telomeres of both chicken and quail were above 20 kb (approximately 30-40 kb long) similarly to mouse. In contrast, human and duck had much shorter telomeres (4-10 kb). These results indicate that the length of telomeres only weakly correlates with telomerase activity.

3.6 Quail embryonic fibroblasts express higher levels of telomerase activity than chicken and duck fibroblasts

To further investigate the regulation of telomerase activity in avian cells, telomerase activity was measured in fibroblast cultures of all three avian species (Fig. 8). Quail embryonic fibroblasts (QEFs) underwent senescence after 14 doublings, as determined by β-galactosidase staining, a marker of cellular senescence. In contrast, chicken and duck embryonic fibroblasts (CEFs and DEFs) became senescent after 50 doublings (Fig. 8A).

Telomerase activity was high at the time the cultures were initiated (Fig. 8B). In QEFs, levels of telomerase remained high through the 9th day and then declined slightly, but substantial



A. The cumulative increase in cell generations with time in culture was determined. Each culture was split 1:8 when it reached confluence. Cells were cultivated until they reached senescence and stopped dividing. This experiment was repeated twice with similar results. B. Telomerase activity was measured by a TRAP assay at various times after establishment of the culture. C. Telomere length in serially passaged quail and chicken fibroblasts at various times after establishment of the cell culture was determined by TRF in a 0.6% agarose gel. Telomere length of freshly prepared DEF (0) was compared to duck embryonic cells cultivated 45 passages (DCL).

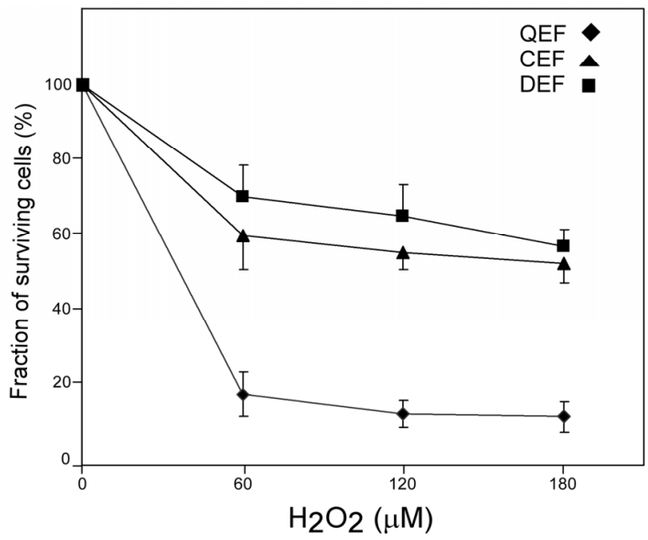
Fig. 8. Growth of quail (QEF), chicken (CEF), and duck (DEF) embryonic fibroblasts

levels of activity were detected in the senescent cells. By contrast, the telomerase activity in CEFs and DEFs decreased after 6 days in culture and was completely diminished by the 9th day and remained undetectable for additional four weeks. Telomere length did not change significantly in QEFs, while telomeres of CEFs gradually shortened (Fig. 8C). Similarly, telomere length in DEF cells after approximately 45 passages (DCL) is significantly shorter than from freshly prepared DEFs.

These results indicated that telomerase activity is not strongly repressed in quail cells, and telomeres do not shorten, however, these cells underwent early senescence during *in vitro* cultivation. By contrast, in both CEFs and DEFs telomerase activity was strongly repressed, followed by the shortening of telomeres. In conclusion, these results are consistent with the findings that telomerase activity is higher *in vivo* in quail than chicken or duck tissues and suggest that, in contrast to chicken or duck, quail cells do not undergo replicative aging, the process, in which aging is dependent on shortening of telomeres during cell proliferation.

3.7 Telomerase levels inversely correlate with H_2O_2 resistance

Quail embryonic fibroblasts underwent senescence after a relatively short time in tissue culture regardless of high levels of telomerase activity and long telomeres. Previous studies demonstrated that primary embryonic fibroblasts from the short-lived quail are more sensitive to oxidative stress than fibroblasts from long-lived birds such as budgerigar (common pet parakeet), *Melopsittacus undulatus* (Ogburn et al., 2001). It is possible that quail is also more sensitive to oxidative stress than chicken or duck, accounting for its relatively accelerated senescence *in vitro*. To determine whether differences also exist between quail, chicken, and duck cells in their sensitivity to oxidative stress, QEF, CEF, and DEF cultures were exposed to H_2O_2 and their viability determined (Fig. 9). QEFs were at least 3 times more sensitive to H_2O_2 treatment than chicken or duck cells.



Quail, chicken, and duck fibroblasts were treated with various concentration of hydrogen peroxide for 24 hours and cell number determined. Means and standard errors were calculated from three independent experiments for each cells. Statistically significant differences relative to negative controls are indicated.

Fig. 9. Sensitivity of avian fibroblasts to oxygen radicals

3.8 Telomerase activity is intrinsically higher in quail cells and inversely correlates with frequency and complexity of AS TERT variants

The previous results indicate that the lower frequency and complexity of AS TERT variants correlate with higher telomerase activity in quail cells. However, in samples with high telomerase activity the levels could not be quantitatively measured, because the assay may be saturated. Therefore, to obtain more accurate results, the relative levels of telomerase activity were determined by end-point titration in selected tissues as well as in cultured cells (Fig. 10A). Telomerase activity was approximately 5 times higher in the quail embryo than chicken embryos. In all cases, the activity in adult organs was approximately 1000-fold lower than in the embryonic tissues analyzed. Telomerase activity in adult quail tissues was 2-8 fold higher than chicken or duck tissues (except in heart where telomerase activity is at the threshold of detection). These results indicate that the relative differences in the levels of telomerase activity between quail and chicken or duck are already present in the embryo and are retained in adulthood.

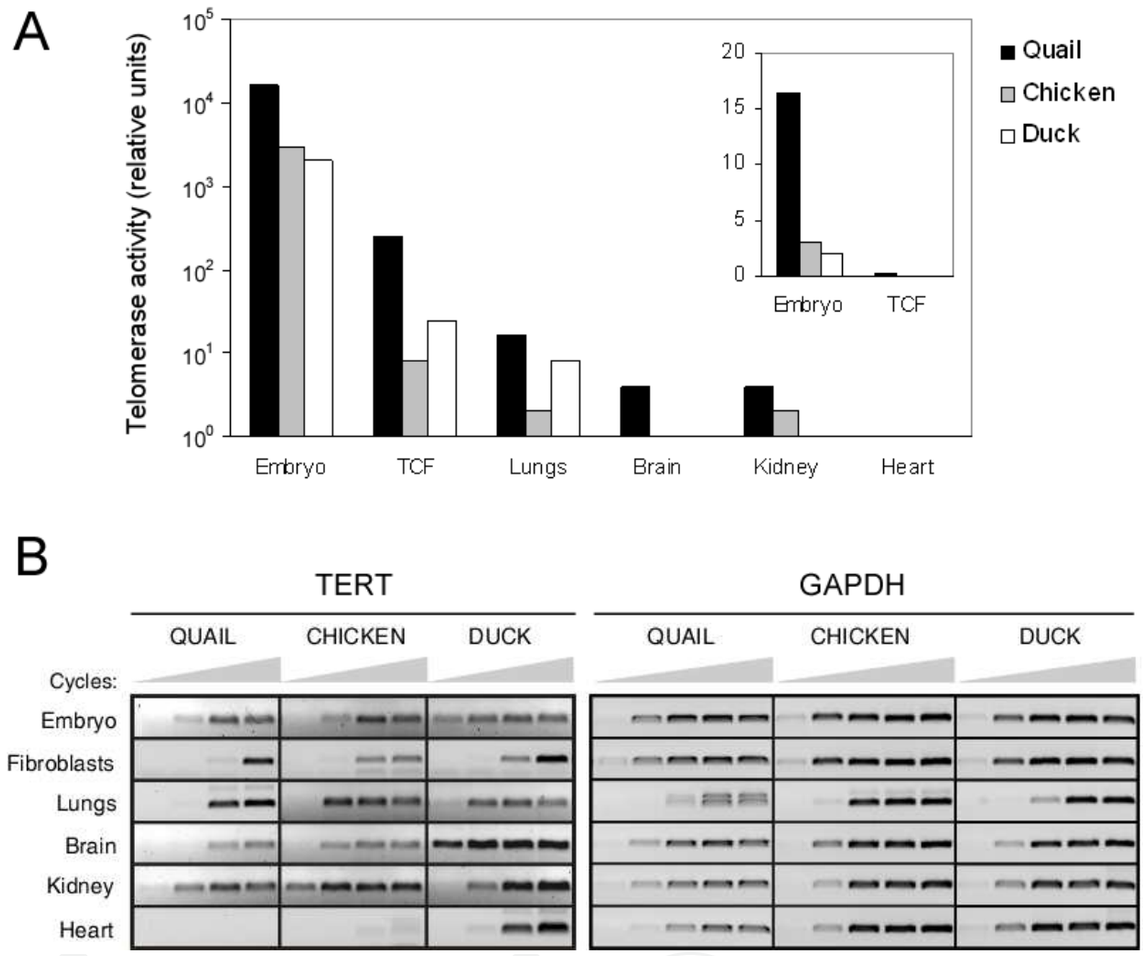
Differences in the levels of telomerase activity between mouse and human cells have been attributed to differential transcription of the TERT gene (Horikawa et al., 2005; Takakura et al., 2005). To determine the contribution of differential transcription of TERT to differences in telomerase activity between quail, chicken, and duck, semiquantitative RT-PCR was performed against the regions of the TERT gene which are not involved in alternative splicing (Fig. 10B). This assay detects the levels of all TERT transcripts, including wild-type TERT as well as the AS TERT variants. The levels of TERT mRNA in some cases mimic the differences in the levels of telomerase activity among the analyzed organs. The cells with the lowest telomerase activity such as heart, and brain had mostly lower amounts of TERT transcripts. However, the levels of TERT mRNA in the corresponding organs of different species did not correlate with the differences in telomerase activity between the species. For example, telomerase activity was approximately five times higher in embryonic cells obtained from quail than in cells from chicken embryo but the levels of TERT mRNA were the same. The levels of TERT mRNA in all analyzed quail tissues were same or lower than in chicken or duck tissues while telomerase activity was always the highest in quail tissues.

In conclusion, these results indicate that there are intrinsic differences in the levels of telomerase activity between quail and chicken or duck, which are already present in embryonic cells and are retained at the same ratio in adult cells. These differences correlate with the frequency and complexity of AS TERT variants expressed in these tissues and not with the steady-state levels of TERT transcripts.

4. Discussion

4.1 The regulation of telomerase activity during ontogenesis and phylogenesis

Telomerase activity is downregulated during ontogenesis in vertebrates. However, poikilothermic animals retain high telomerase activity in all adult organs since their continuous growth throughout their lifespan requires the maintenance of telomere length (Bousman et al., 2003). Homoeothermic animals (mammals and birds) downregulate telomerase activity to a greater extent than poikilotermic animals presumably because they do not grow after they reach sexual maturity and also to decrease the risk of cancer resulting from a higher metabolic rate. However, mammals differ in the extent to which telomerase is



A. Telomerase activity in extracts from different tissues of quail, chicken, and duck was determined by the TRAP assay. CHAPS protein extracts were prepared from quail (4 day-old), chicken (5 day-old), and duck (14 day-old) embryos, embryonic fibroblasts passaged for six days in tissue culture (TCF) and from lung, brain, kidney, and heart of 3 month-old quail, 6 month-old chicken and a sexually immature duck of adult size. Telomerase activity was determined by a TRAP assay in a series of up to 16 two-fold dilutions of the CHAPS extracts. Quail embryo extract where the telomerase activity was detected at the highest dilution (14^{th} dilution) was arbitrarily considered to have 2^{14} relative units per µg of extract and activity in other samples were compared to this standard. B. Steady-state levels of TERT mRNA were determined by RT-PCR. The expression of GAPDH, was analyzed by RT-PCR using primers specific for quail, chicken, and duck GAPDH. Aliquots from the PCR reaction were taken every 5^{th} cycle beginning with cycle 30 for TERT and 20 for GAPDH, PCR products were resolved on agarose gels, and visualized with ethidium bromide. RNA was prepared from the same cells and tissues as at Fig. 10A.

Fig. 10. Interspecies differences in telomerase activity do not correlate with TERT mRNA steady-state levels

downregulated. Initial studies indicated that mice maintain higher telomerase activity in a broader spectrum of tissues than humans (Kim et al., 1994; Prowse & Greider, 1995; Taylor & Delany, 2000). The following study of 15 rodent species demonstrated remarkable differences in telomerase activity (Seluanov et al., 2007). In general, the higher levels of telomerase activity correlated with the smaller body mass of the animal. Lastly, the comparative study of more than 60 mammalian species representing all the important mammalian evolutionary groups supported this conclusion (Gomes et al., 2011). Our report demonstrates that the differences in telomerase activity exist not only in mammals but also in other warm-blooded animals - birds. Quail and chicken are closely related species with a significantly different body mass and life expectancy (Pereira & Baker, 2006). Though sequences of quail and chicken TERT proteins are 94% identical, the levels of telomerase activity in quail and chicken cells and organs are strikingly different. Quail embryonic cells express 5 times the level of telomerase activity than chicken embryonic cells. Telomerase activity is downregulated to the same extent (approximately 1000 times) in adult organs of both species but substantially higher levels (about 5 fold) remain in quail relative to chicken tissues. This demonstrates that differences between telomerase activity in adult organs of different species are intrinsic and result of different levels of telomerase activity in their respective embryos.

The downregulation of telomerase activity in the organs of adult homoeothermic vertebrates is believed to provide protection against cancer (Blasco & Hahn, 2003; Shay & Wright, 2005). Quail and mice are short-lived species because they are small, principally terrestrial animals, with limited ability to escape predation (Kirkwood, 2005). Due to the short lifespan of quail and mice, suppression of telomerase activity is not required to protect against tumor development (Prowse & Greider, 1995). The increased sensitivity of quail and mouse cells to spontaneous, carcinogen- or oncogenic virus-induced immortalization is consistent with this hypothesis (Hartl et al., 1995; Moscovici et al., 1977; Prowse & Greider, 1995). However, the question remains whether the high levels of telomerase activity expressed in species with small body mass provides a survival advantage. Quail cells are more sensitive to oxidative stress than chicken or duck cells, and in contrast to chicken or duck cells, they undergo senescence when cultured *in vitro.* Cells from other short-lived mammals are also more sensitive to oxidative stress than cells from long-lived species, and undergo accelerated telomere-length-independent senescence when exposed to higher levels of oxygen during *in vitro* cultivation (Kim et al., 2002; Ogburn et al., 2001; Parrinello et al., 2003). The quail cells stop proliferation and undergo senescence in cell culture in two weeks even though they retain high telomerase activity and long telomeres indicating that the shortening of telomeres is not responsible for cell cycle arrest. Avian TERT, like its mammalian homologs, is able to partially protect cells against apoptosis induced by exposure to oxidative agents (Hrdličková et al., 2006). In conclusion, it is likely that the major role of the higher telomerase activity in cells from quail and other species with small body mass is to partially compensate for their reduced antioxidative defense.

4.2 The mechanism of regulation of telomerase activity during ontogenesis

Transcription rate and alternative splicing of TERT has been proposed as the key mechanisms involved in the developmental regulation of telomerase activity in mammals (Kaneko et al., 2006; Ulaner et al., 2001; Ulaner et al., 1998). Our results suggest that other

mechanisms are also likely to be involved in the downregulation of telomerase in the quail, chicken, and duck. Differences in telomerase levels in the embryo relative to adult organs did not strongly correlate with differences in TERT transcription or alternative splicing. Embryos expressed approximately 1000 times higher levels of telomerase activity than adult tissues in all species and expressed similar mRNA levels and numbers of AS variants. In some tissues general transcription of TERT was significantly decreased, however, other non-transcriptional mechanisms likely contribute to the robust downregulation of telomerase activity during differentiation. Likewise, telomerase activity in the quail and mouse are downregulated in tissues where only a limited amount of alternative splicing of TERT transcripts takes place. Other studies also do not support a general role for splicing of TERT during developmental regulation. Like avian species, alternatively spliced TERT forms are present in human gametes and preimplantation embryos (Brenner et al., 1999). If alternative splicing negatively regulates telomerase activity during development one would not expect spliced forms in germinal cells in which telomerase activity is highest.

4.3 The role of alternative splicing in interspecies differences in the regulation of telomerase activity

TERT is the key regulatory component responsible for the regulation of telomerase levels. The most important mechanisms responsible for interspecies differences in the regulation of TERT are probably the structural differences in the regulatory domains encoded by the TERT gene, the level of transcription and alternative splicing. Changes in protein-protein interaction-based regulatory pathways would evolve slowly since only a limited number of mutations in the coding regions of TERT would affect its regulation without complete elimination of the telomerase activity. The downregulation of telomerase by terminating transcription could lead to the methylation of the TERT promoter limiting the possible reactivation of TERT transcription (Devereux et al., 1999). In contrast, changes in alternative splicing sites can be introduced into the genome without interfering with the complex promoter regulation of TERT or its protein structure. Moreover, alternative splicing preserves the transcription of the TERT locus preventing promoter methylation and, thereby, allowing for the rapid reactivation of telomerase when necessary in adult tissues (during immune reaction or healing processes). All these mechanisms of regulation of interspecies differences in the levels of telomerase are used, however, their relative contribution still remains to be evaluated.

The differences in the levels of telomerase activity between the closely related quail and chicken species strongly correlated with differences in alternative splicing. In quail the majority of the TERT transcripts expressed in adult tissue and cells are wild type and telomerase activity levels are high. In contrast, alternatively spliced variants which fail to encode functional TERT proteins, represent nearly all of the TERT transcripts expressed in adult chicken tissue and cells which have low levels of telomerase activity. Our results suggest that alternative splicing of TERT contributes to the establishment of the different levels of telomerase between these two species. Importantly, we demonstrated that these differences are intrinsically present already in embryonic cells. This is consistent with the suggestion that the many changes in the alternative splicing repertoire between quail and chicken are encoded in genomic sequences in the TERT locus.

Recently, differences in the regulation between the human and mouse TERT promoters have been suggested to be responsible for the differences in the levels of telomerase activity in

these species (Horikawa et al., 2005; Jia et al., 2011; Takakura et al., 2005; Wang et al., 2009). In our comparison of quail, chicken, and duck, the regulation of transcription did not appear to play a significant role because we did not find differences in the steady-state levels of TERT transcripts (as an approximate measure of TERT transcription) among these species. In contrast, differences in alternative splicing best correlated with differences in levels of telomerase activity between quail and chicken. The probable reason for the preferential use of alternative splicing in quail and chicken is that the evolutionary distance is much smaller between quail and chicken than mouse and human (30 MYA against 75 MYA). Therefore, it is likely that alternative splicing plays the primary role in defining differences in levels of telomerase in these species because the short time window in which quail and chicken diverged. The evolution of new splice donor and acceptor sites for expression of novel AS variants is likely to be more rapid since this does not interfere with existing transcriptional regulation or protein structure. In contrast, more evolutionary distant species, such a duck, which expresses similarly low levels of telomerase like the chicken, has an intermediate number of alternative splice variants, suggesting that other mechanisms are also involved in regulation of telomerase levels among species.

On the other hand, the longer and extremely rapid evolution of the rodent branch of mammals allowed high divergence not only in promoter region, but also resulted in greater structural differences between the mouse and human TERT proteins (mouse/human - 62/74%, quail/chicken - 94/97% identity/similarity of TERT protein sequence). Divergences at the protein level resulted in differences for at least some enzymatic characteristic of telomerase since these differences contribute to their different processivity (Fakhoury et al., 2010). However, it is likely that even between mouse and human the difference in frequency and complexity of TERT alternative splicing plays an important role in differential levels of telomerase in addition to in the promoter and protein structure. Twenty-one AS variants of human TERT have been identified and most of the TERT transcripts in adult human tissue are alternatively spliced (Ulaner & Giudice, 1997). In contrast, mice express only a few AS forms (Sýkorová & Fajkus, 2009) suggesting that alternative splicing of TERT also correlates with levels of telomerase in these two species. In conclusion, alternative splicing of the TERT genes appears to be an important factor in the establishment of the interspecies differences in telomerase activity.

4.4 The co-evolution of alternative splicing of TERT with levels of telomerase in birds and mammals

In many metazoan species TERT transcripts are alternatively spliced. Several AS TERT variants have been reported in a number of species including human, rat, dog, chicken, zebrafish, and plants (Sýkorová & Fajkus, 2009). In the human and chicken, which have been extensively studied, 21 and 37 single-event AS TERT variants have been identified (Amor et al., 2010; Chang & Delany, 2006; Hisatomi et al., 2003; Hrdličková et al., 2012a; Hrdličková et al., 2006; Saebøe-Larssen et al., 2006; Wick et al., 1999). Interestingly, none of the human and chicken AS variants are identical, suggesting that AS TERT variants evolved independently in placental mammals and birds. Results presented in this study offer the explanation for this finding. The evolution of AS splicing may serve as a mechanism in part for the determination of different levels of telomerase activity in homoeothermic species. Apparently, the levels of telomerase activity changed several times in evolution of mammals closely following the respective differences in body mass (Gomes et al., 2011; Seluanov et al.,

2007). We propose that alternative splicing contributes to these changes by reducing the number of AS TERT forms in species with high levels of telomerase activity as a result of the elimination or modifications of splice sites as occurred in quail. In new species that originate from such predecessors, novel AS variants would have evolved by the introduction of new splice sites and enhancers creating structurally different AS variants. Repeated changes in the intrinsic levels of telomerase during evolution of mammals, therefore, resulted in the repeated elimination of AS TERT variants followed by the evolution of new variants, ultimately creating different repertoires of AS variants in birds and mammals. Interestingly, we have exhaustively analyzed alternative splicing of TERT in the platypus and found that this species from the basal branch of the mammalian lineage expresses a limited number of AS variants, which are very closely related to chicken AS variants (Hrdličková et al., 2012b). This suggests that the evolution of levels of telomerase activity and alternative splicing was less dynamic in bird species than in placental mammals. In mammals, the predecessor of placental animals probably evolved after a prolonged period when different species with a small body mass and high levels of telomerase had a reduced number of alternatively spliced TERT variants. This was later followed by the evolution of new repertoire of alternatively spliced forms in placental mammals with a greater body mass.

5. Conclusion

There are great differences in telomerase activity among several groups of mammals indicating that several changes in the regulation of telomerase activity occurred during mammalian evolution (Gomes et al., 2011). However, differences in telomerase activity in birds, an independently evolved branch of homeothermic animals have not been reported. Lastly, the mechanism responsible for these differences in telomerase activity in homeothermic animals is largely unknown. Our comparative analysis of quail, chicken, and duck demonstrated differences in telomerase activity in avian species. Moreover, analysis of the closely related quail and chicken suggested that alternative splicing may play a direct role in determining levels of telomerase expressed in both embryonic and adult tissues of these two species. Elevated telomerase activity in quail tissues correlates with ten times less AS variants. The analysis of the more distantly related duck supports the general conclusion that alternative splicing plays an important role in determining interspecies differences of telomerase activity, but also suggests that other mechanisms are probably involved.

These results suggest that alternative splicing is the key mechanism for differences in telomerase activity in homeothermic animals. We propose that alternative splicing of TERT is a mechanism which contributes to fluctuations in telomerase canonical activity during evolution without losing the non-canonical functions of telomerase and reflects interspecies differences in aging.

Future research will focus on defining the function of alternatively spliced variants, with the emphasis on variants with premature termination codons. Additionally, the identification of AS TERT variants in key mammalian species would further evaluate the role of alternative splicing in evolution of differences in levels in telomerase and will create the precedent for analysis of the role of alternative splicing in evolution of other genes. These studies of alternative splicing from a functional angle would help us understand its role beyond the "stochastic noise" (Melamud & Moult, 2009).

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