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Endogenous and ectopic expression of telomere regulating genes in chicken embryonic fibroblasts

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Abstract

In this study, we compared the endogenous expression of genes encoding telomere regulating proteins in cultured chicken embryonic fibroblasts (CEFs) and 10-day-old chicken embryos. CEFs maintained in vitro senesced and senescence was accompanied by reduced telomere length, telomerase activity, and expression of the chicken (c) TRF1 gene. There was no change in TRF2 gene expression although the major TRF2 transcript identified in 10-day-old chicken embryos encoded a truncated TRF2 protein (TRF2′), containing an N-terminal dimerisation domain but lacking a myb-related DNA binding domain and nuclear localisation signal. Senescence of the CEFs in vitro was associated with the loss of the TRF2′ transcript, indicative of a novel function for the encoded protein. Senescence was also coupled with decreased expression of RAD51, but increased RAD52 expression. These data support that RAD51 independent recombination mechanisms do not function in vitro to maintain chicken telomeres. To attempt to rescue the CEFs from replicative senescence, we stably transfected passage 3 CEFs with the human telomerase reverse transcriptase (hTERT) catalytic subunit. While hTERT expression was detected in the stable transfectants neither telomerase activity nor the stabilisation of telomere length was observed, and the transfectant cells senesced at the same passage number as the untransfected cells. These data indicate that the human TERT is incompatible with the avian telomere maintenance apparatus and suggest the functioning of a species specific telomere system in the avian.

Keywords: Telomerase; Chicken embryonic fibroblast; Telomere regulating factor; Telomere

Chicken cells cultured in vitro undergo a limited number of cell divisions before they cease proliferation and enter replicative senescence [1]. The molecular mechanisms underlying senescence are poorly understood but the senescent state is often associated with dysfunctional or shortened telomeres which are specialised structures located at the ends of chromosomes. These function to protect chromosomal ends from degradation and fusion, and to facilitate chromosome replication (reviewed in [2]). The chicken genome is unique in that it contains 10 times more telomeric DNA than the human. Three overlapping classes of telomeric DNA designated Class I, II, and III have been identified ranging from 0.5 to 2 Mb in size [3]. Class I arrays span 0.5–10 kb and, as evidenced by their resistance to *Bal*31 exonuclease, are located within the chromosomes, and are often referred to as interstitial telomeric sequences. Class II arrays span 10–40 kb and Class III array span 40 kb to ~20 Mb, which is the longest reported for any vertebrate species to date. Class III arrays are rapidly digested by *Bal*31, indicating a terminal position [3].

Telomere maintenance is performed by telomerase, a ribonucleoprotein that is responsible for adding the telomeric repeat, TTAGGG, to the 3′ ends of chromosomes [4]. This G-rich strand generates a 3′ overhang of several

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hundred nucleotides which is protected by telomere binding proteins. Telomerase function is dependent on two major components, one being the telomerase RNA (TR), which contains the template to allow telomeric repeats to be added to the chromosome, and the second is the telomerase reverse transcriptase (TERT) catalytic subunit. In human cells the TR subunit is constitutively expressed, whereas hTERT is only expressed in embryonic stem cells, germ cells, and cancer cells [5,6]. The absence of telomerase activity and telomere shortening is associated with replicative senescence [7].

Chicken embryonic fibroblasts (CEF) maintained in culture are known to senesce but the fate of the telomeres during senescence is controversial. Senescence has been associated with either a decrease in telomere length, with the decline calculated as approximately 60 bp per population doubling [1], or no change in telomere length, with the telomere lengths of passage 19 senescent CEFs to be comparable to those of passage 3 and immortalised DF-1 chicken cells [8]. More recently the CEF telomere lengths have been shown to be quite dynamic with mean lengths increasing and decreasing throughout the lifespan of the in vitro cultures [9]. Despite these inconsistencies senescence was, in all studies, associated with low or undetectable telomerase activity.

In the absence of telomerase, telomere length can be maintained by the alternative lengthening of telomeres (ALT) mechanism, which is dependent on homologous recombination [10]. In general this pathway is associated with telomere length variability and includes the rapid lengthening or deletion of several kilobases. While this mechanism appears consistent with the variability in telomere length observed in CEFs [9], all ALT lines reported to date have been tumour cells.

Primary cells cultured in vitro can often be rescued from senescence by the activation of telomerase activity and many primary vertebrate cells, including human, sheep, rabbit, bovine, and deer, have been immortalised by the ectopic expression of human telomerase (hTERT) [11–15]. This exogenous enzyme activity leads to the stabilisation of telomere length, chromosome stability, and an extended cell lifespan, but without aberrant growth and malignant transformation. In this study, we were interested in investigating the telomerase activity, and expression profiles of genes encoding telomere regulating proteins in pre-senescent and senescent CEFs, and whether the stable ectopic expression of telomerase (hTERT) could rescue chicken embryonic fibroblasts (CEFs) from senescence.

Materials and methods

Cell culture, plasmids, and retroviral transfection. CEFs were isolated from 10-day-old chicken embryos (Gold Line) as described previously [16]. Cells were cultured and passaged in Dulbecco's modified Eagle's medium with high glucose, GlutaMaxI, and sodium pyruvate (Gibco), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Sigma), and maintained at 40 °C in a humidified atmosphere of 5% CO_2 in air.

The retroviral plasmid LPC-hTERT was provided by Geron Corporation. The vector control was constructed by excising the hTERT cDNA with *Eco*RI and religating. Plasmids BPIR8 and BPIR7 containing the full-length chicken telomeric repeat binding factors 1 and 2 (cTRF1 and cTRF2) cDNAs were kindly provided by Dr. Christine J. Farr, Department of Genetics, University of Cambridge. Chicken RAD51, RAD52, and GAPDH cDNA clones were obtained from MRC Geneservice, Cambridge.

Retroviral transfections were performed using the Pantropic Retroviral Expression System (BD Biosciences, Clontech), according to the manufacturer's instructions. GP2-293 cells were transiently transfected with the retroviral vectors using the Lipofectin reagent (Invitrogen). Passage 3 CEF cells were infected with retrovirus when they reached 60–80% confluence. The infected CEF cells were subjected to antibiotic selection (Puromycin 0.7 μg/ml, G-418 0.7 mg/ml) 24 h post retroviral infection.

Reverse transcriptase (RT)-PCR analysis. One microgram of total RNA was reverse transcribed to cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen). PCR amplifications were performed using the *Taq* PCR_x DNA Polymerase Recombinant kit (Invitrogen). A 282 bp fragment of the hTERT cDNA was amplified using the primers (5′-CTCTGTGCTGGGCCTGGACGATA-3′) and (5′-ACGGCTGGAGGTCTGTCAAGGTAG-3′). This primer pair was designed to cross intron sequences and resulted in the amplification of a 5.5 kbp sequence if the samples were contaminated with genomic DNA [17]. PCR conditions were 94 °C for 3 min, 30 cycles of 94 °C for 45 s, 61 °C for 30 s and 72 °C for 90 s, and a final extension period at 72 °C for 10 min. A 1.1 kbp fragment of the cTRF1 cDNA was amplified using the primers (5′-CCAGATTGAAAATGTCGGA AGC-3′) and (5′-AACTCATCATTCTGAGCAGC-3′) with an annealing temperature of 55 °C. A 570 bp fragment of the cTRF2 cDNA was amplified using the primers (5′-CAAGATGTGCAGAAA CCGCAAG-3′) and (5′-GG TTACAGTTCTAAAGGGCGTC-3′), and an annealing temperature of 60 °C. Amplification of 18s ribosomal DNA was performed using the Classic 18S Internal Standard primer pair (Ambion) and an annealing temperature of 64 °C.

Because of the high GC content of the cTR gene (76.8%), PCR amplification was performed using the Advantage-GC cDNA PCR kit and Polymerase mix (Clontech), and the following primers (5′-CGT GGCGGGTGGAAGGCTCC-3′) and (5′-GAATTCGCGTGTGGG AGCGACGCC-3′). PCR conditions were 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 64 °C for 90 s, and 72 °C for 2 min, and a final extension period at 72 °C for 10 min. This resulted in the amplification of a 462 bp product which contains the whole transcribed region of the gene.

All cDNA products were identified by DNA sequencing or restriction analyses.

Senescence-associated-β-galactosidase staining. Senescence of the CEF cells was observed using a senescence-associated β-galactosidase (SA-β-Gal) staining method [18]. The CEFs were designated senescent when approximately 80% of the cells stained positive for SA-β-Gal and displayed morphological changes such as increased vacuolisation and enlarged cell size.

Telomerase activity assay. Telomerase activity of cell extracts was analysed using the telomeric repeat amplification protocol (TRAP) assay using the Telomerase PCR ELISA kit (Roche) and according to the manufacturer's instructions. Samples were measured at 450 nm within 30 min of the addition of stop solution.

Telomere length assay. To discriminate between true telomeric DNA and interstitial genomic fragments with telomeric repeat sequences, the length of the distal telomeric fragments was determined using their unique property of having a single strand overhang at their 3′ end. The length of single-stranded terminal overhangs in telomeres

was measured by in-gel hybridisation of telomeric probes onto non-denatured DNA. For preparation of highest quality DNA, cells were embedded in low-melting agarose plugs and treated with proteinase. The DNA was digested until completion with *Hin*fI (60 U per plug; Boehringer–Mannheim) at 37 °C and electrophoresed in a 1% agarose gel using pulsed field electrophoresis (CHEF-DRIII-SYSTEM, Bio-rad) at 5.5 V/cm for 17 h with a switching time of 2–10 s in 0.5× TBE. The gel was dried at room temperature and the non-denatured gel hybridized with [γ-^{32}P]ATP end-labeled $(CCCTAA)_4$ at 35 °C. After washing in 2× SSC and 0.2× SSC the gel was exposed overnight in a phosphoimager (Storm 820, Molecular Dynamics, Amersham). Telomere length was determined using profile analysis of the native gel and correlated to the molecular weight marker. Average telomere length was evaluated by AIDA software (Raytek) as weighted mean of optical density.

Protein quantification and immunoblotting. Protein concentrations were determined using the Bradford assay. For Western blots analyses 50 μg protein was separated on a SDS–7.5% polyacrylamide gel and transferred onto a PDVF transfer membrane (Hybond P, Amersham). Membranes were incubated overnight at 4 °C in a blocking solution (100 ml PBS, 0.1% v/v Tween 20 (Sigma), and 5% w/v milk powder using 1:10,000 dilution of rabbit anti-hTERT monoclonal antibody (Alpha Diagnostic International) raised against a 21 amino acid peptide from the human EST2 protein). Membranes were washed in PBS–Tween 20 (0.1%) and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:5000 in blocking solution. After washing the secondary antibody was detected using an ECL Plus kit (Amersham).

Northern blotting. Total RNA was isolated from chicken embryos or CEF cells cultured in vitro using RNAzol B (Biogenesis). Isolation of mRNA from total RNA was performed using an NucleoTrap mRNA kit (Clontech). Two micrograms mRNA was separated by electrophoresis on a 1.2% agarose gel containing formaldehyde and blotted onto a nylon membrane (Genescreen, Perkin-Elmer). The membrane was hybridised with a ^{32}P-labelled dCTP chicken specific probe. Signal intensities were quantitated using a Packard Instant Imager.

Results

Telomere length and telomerase activity in CEFs

In view of the conflicting data surrounding the length of telomeres in senescent CEFs, we analysed the telomere lengths of fibroblasts isolated from CEFs cultured in vitro by measuring the G overhang length. In our study, we observed definite telomere shortening, approximately 300 bp per cell passage, and a net decrease in telomere length (Fig. 1A).

Telomerase gene expression and activity, the latter equivalent to that of the telomerase positive human cell line, MRC-5hTERT, was observed in the cell extracts prepared from the chick embryos and reduced, but detectable, activity was identified in P3 CEFs (Fig. 1B). Western blot analyses of the embryonic and

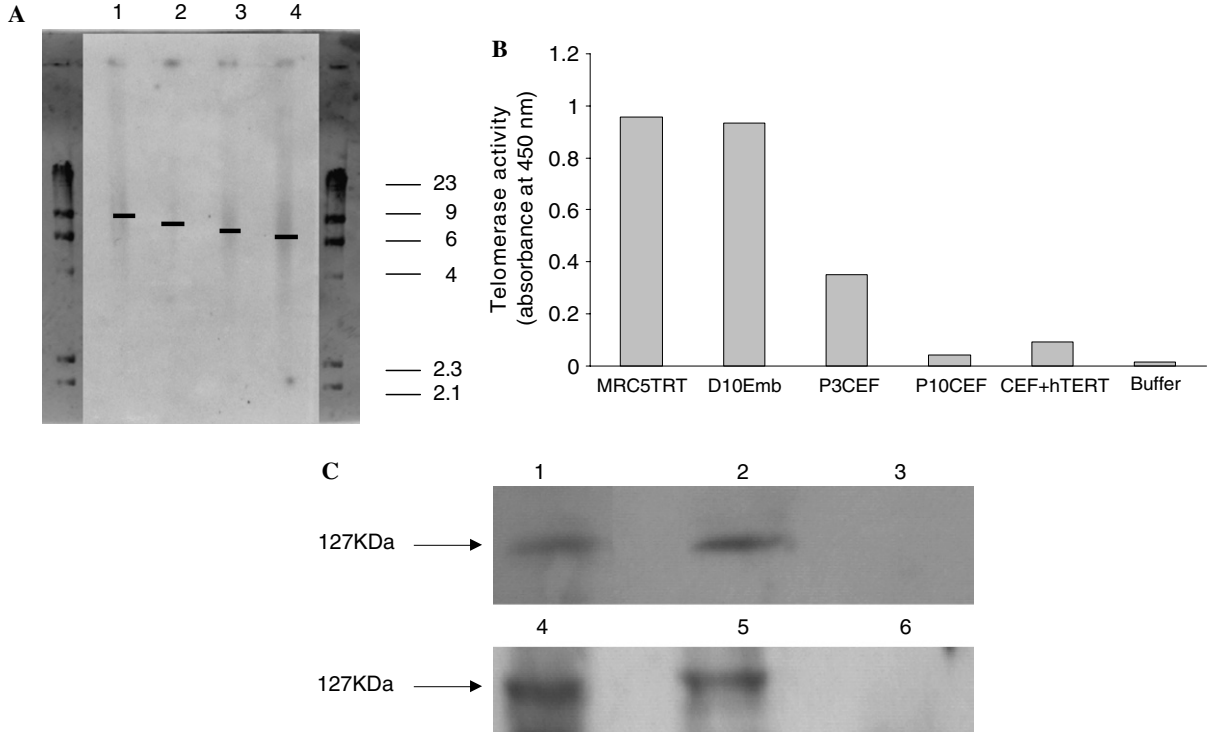

Fig. 1. Telomere length profiles and telomerase activities of CEFs isolated from 10-day-old embryos and CEFs maintained in vitro. (A) Telomere lengths of passage P5 (1), P6 (2), P8 (3), and P12 (4) CEFs; (B) Telomerase activity of CEFs isolated from day 10 chick embryos (D10Emb), P3 and P10 CEFs, and CEFs stably transfected with cDNA encoding hTERT (CEFhTERT). Controls include buffer only (−ve control) and MRC5-hTERT (MRC5TRT) cells (+ve control); (C) Western blot analysis of protein extracts prepared from human BJ5TA fibroblasts (1,4); P3 CEFs (2); P10 CEFs (3,6); 10-day-old chicken embryos (5) using an anti-hTERT monoclonal antibody raised against a 21 amino acid peptide from the human EST2 protein. (The human BJ5TA fibroblasts were a gift of Geron Corporation.)

P3 cells using a monoclonal antibody to human telomerase indicated both preparations to contain a protein of ~130 kDa, which suggested that the observed telomerase activity was due to the endogenous chicken enzyme (Fig. 1C). Analyses of CEFs beyond passage 3 failed to detect either telomerase gene expression, activity or protein and passaging was associated with increased β-galactosidase (SA-β-gal) staining (Fig. 4B). These data therefore indicated that CEF senescence in vitro was associated with reduced telomerase gene expression, activity, and telomere length.

TRF1 and TRF2 expression in CEFs

A number of investigations in mice and human cells have provided evidence that telomerase activity and telomeric maintenance are dependent on specialised proteins, including the telomeric-regulating factors TRF1 and TRF2. In vivo TRF1 functions as a negative regulator of telomere length, while TRF2 serves to protect the chromosome terminus by preventing the fusion of chromosome ends (reviewed in [2]). To investigate the roles of these molecules in the maintenance of chicken telomeres' we determined the expression of the chicken (c)TRF1 and cTRF2 genes in RNA extracted from embryos, P3 CEFs (telomerase positive) and P10 senescent CEFs (telomerase negative), respectively.

Northern blot and RT-PCR analyses of RNA extracted from 10-day-old chick embryos identified TRF1 gene expression. In contrast, no signal was observed when the RNA from P3 and P10 CEFs was subjected to Northern blot analysis although RT-PCR analyses revealed, in each case, faint bands relating to cDNA products of 1.1 kbp (Fig. 2A). These data showed that telomere shortening in vitro was related to downregulation of TRF1 gene expression.

RT-PCR analysis, with primers designed to amplify a 570 bp cTRF2 product, was used to identify cTRF2 gene expression in RNA prepared from 10-day-old chick embryo fibroblasts, P3 and P10 CEFs, respectively. Comparison of gene expression in vivo and in vitro did not provide evidence for alterations in the expression of the 570 bp cTRF2 gene product as the CEFs senesced and the telomeres shortened (Fig. 2B). However, the major cTRF cDNA product detected in vivo (10-day-old chicken embryo) samples was 800 bp rather than 570 bp. Analysis of the 800 bp cDNA sequence indicated >99% similarity to exon 6 of the published cTRF2 cDNA sequence, a consensus splice site sequence (AAG/GT) followed by novel sequence encoding 10

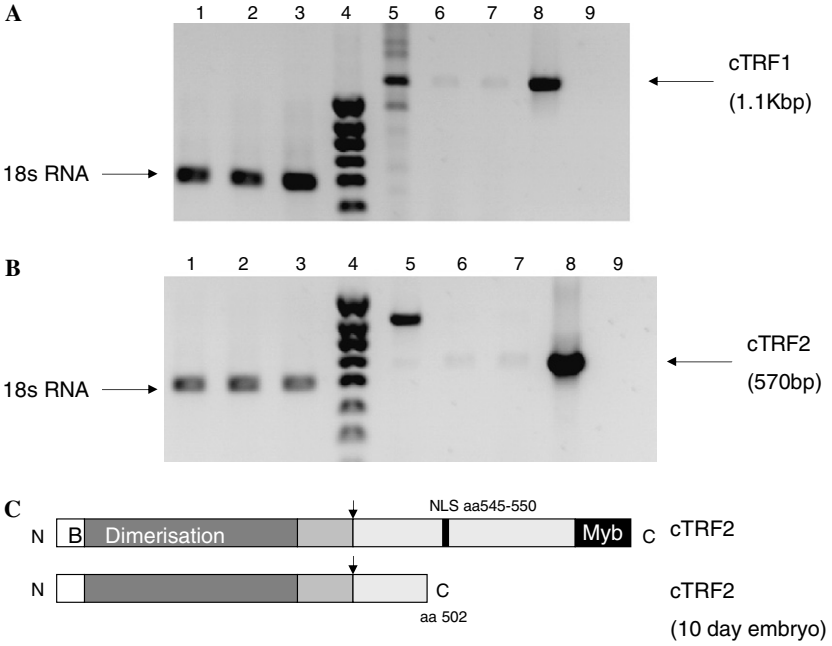

Fig. 2. cTRF1 gene expression in 10-day-old chicken embryos and CEFs maintained in vitro. (A) 18s RNA controls of 10-day-old embryos (1), P3 CEFs (2), and P10 CEFs (3) RNA; 100 bp ladder (4); TRF1 RT-PCR products of the 10-day-old embryo (5), P3 CEFs (6), and P10 CEFs (7) RNA; pBPIR8 +ve control; (8); 10-day-old embryo RNA, no reverse transcriptase (9). The number in brackets represents the predicted size of the amplified gene. (B) cTRF2 gene expression in 10-day-old chicken embryos and CEFs maintained in vitro. (A) 18s RNA controls of 10-day-old embryos (1), P3 CEFs (2), and P10 CEFs (3) RNA; 100 bp ladder (4); TRF2 RT-PCR products of the 10-day-old embryo (5), P3 CEFs (6), and P10 CEFs (7) RNA; pBPIR7 +ve control; (8); 10-day-old embryo RNA, no reverse transcriptase (9). The number in brackets represents the predicted size of the amplified gene. (C) Schematic of the chicken and chicken embryo TRF2 protein domains. The domains shown are the N-terminal 'basic' domain (B), the dimerisation domain, an intervening region in which the putative nuclear localisation signals (NLS) are localised and the myb-type DNA binding domain in the C-terminus. The position of the chicken specific 'repeat' domain is denoted by the arrow (adapted from [33]).

amino acids before a TAG stop codon. This suggested the synthesis of a truncated TRF2 protein (TRF2′) in the 10- day-old chicken embryo. Further analysis of the encoded amino acid sequence suggests this protein to lack the TRF2 myb-related DNA binding domain and putative nuclear localisation signal, but to contain an N-terminal dimerisation domain and an unique domain consisting of multiple copies of a degenerate amino acid repeat exclusive to the avian protein (Fig. 2C). This novel 800 bp TRF2 transcript was not detected in the CEFs maintained in vitro indicative of downregulation. These data indicated that the reduction in telomere length and telomerase activity associated with CEF senescence was accompanied by the reduced expression of genes encoding the telomeric-regulating factors TRF1 and TRF2′.

RAD51 and RAD52 expression

Telomeres are not recognised generally as double-strand breaks (DSB) but some DSB repair proteins are present at telomeres and are required for telomere maintenance [19,20]. In the chicken DT40 cell line the DSB repair protein, RAD 51, has been proposed to play a key role in telomere maintenance with deficient cells showing an increased telomeric G-strand overhang [21]. We investigated the patterns of expression of the DSB repair proteins cRAD51 and cRAD52 in fibroblasts prepared from day 10 embryos and senescent (P10) primary CEFs. The reduction in telomere length associated with senescence was associated with decreased cRAD51 expression but upregulation of cRAD52 expression (Fig. 3).

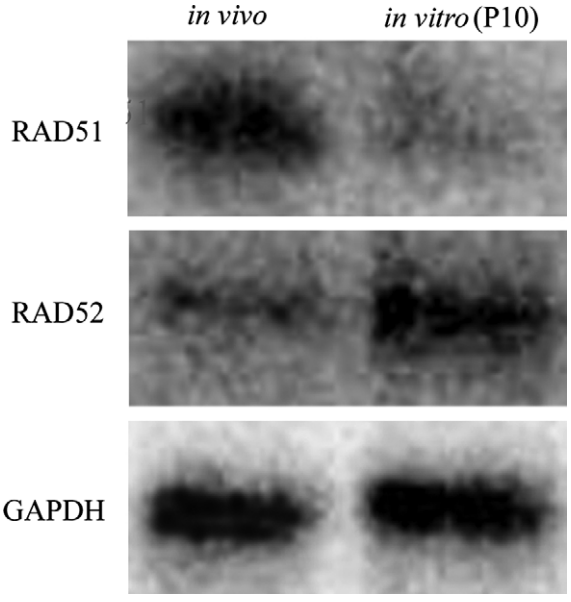

Fig. 3. Northern blot analyses of RNA isolated from 10-day-old chicken embryos and P10 CEFs maintained in vitro using cRAD51, cRAD52, and cGAPDH probes.

Ectopic expression of TERT

Many human tumour cells acquire immortality through expression of telomerase [1,22]. To investigate if the ectopic expression of TERT could rescue the CEFs from senescence passage 3 (pre-senescent), CEFs were transfected with the human telomerase catalytic subunit (hTERT) cDNA and stable transfectants selected using puromycin. The CEFhTERT clones (approximately 10) from each retroviral transfection were pooled, cultured in vitro, and shown by RT-PCR to express the hTERT gene (Fig. 4A). The transfected CEFs were serially passaged and at each passage was stained for SA-β-gal activity. More than 80% of the passage 12 cells stained positive for SA-β-gal activity indicative of senescence. Taking into account the fact that the cells were hTERT transfected at passage 3 this was comparable to the senescence patterns observed in the untransfected cells (Fig. 4B).

Western blot analyses of the CEFhTERT cells at passages 2 and 6 posttransfection did not detect hTERT protein (Fig. 4C). Furthermore, TRAP analyses indicated that the levels of telomerase catalytic activity were comparable to those of the untransfected P10 CEFs (Fig. 1B). This suggested that hTERT expression in

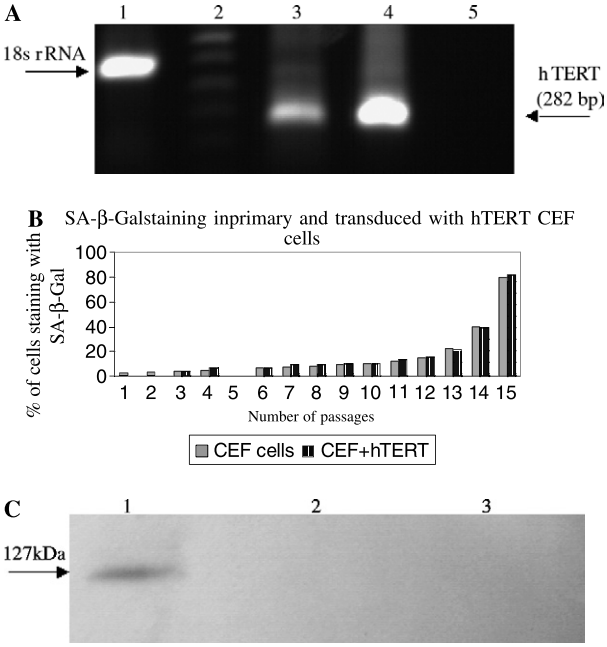

Fig. 4. hTERT expression in stably transfected CEFs. (A) 18s RNA control of CEF-hTERT cells (1); 100 bp marker (2); P3 CEFhTERT transfected cells (3); pLPC-hTERT +ve control (4); P3 CEFhTERT transfected cells, no reverse transcriptase (5). The number in brackets represents the predicted size of the amplified gene. (B) SA-β-Gal staining in CEF and CEFhTERT cells maintained in vitro. (C) Western blot analysis of protein extracts prepared from human BJ5TA fibroblasts (1); P2 CEFhTERT cells (2); P6 CEFhTERT cells (3) using an anti-hTERT monoclonal antibody raised against a 21 amino acid peptide from the human EST2 protein.

the CEFs did not result in a functional telomerase. There was no rescue of the telomeres (data not shown). Thus ectopic expression of hTERT did not appear to rescue or extend the in vitro lifespan of the CEFs.

Discussion

We have shown in support of others [1,23] that primary CEFs maintained in culture have a limited proliferative lifespan and undergo a finite number of divisions before entering an irreversible nondividing state called replicative senescence. In our model the loss of replicative capacity was characterised by the loss of telomerase activity, Class II telomere shortening, a reduction in TRF1 and RAD51 gene expression, but the upregulation of RAD52 gene expression.

The protection and maintenance of the ends of human chromosomes require the function of a number of telomere regulating factors, including TRF1 and TRF2. The loss of TRF1 in mammalian cells is known to induce chromosome fusion, genome instability, and growth defects [24]. Our observations of cTRF1 down-regulation during CEF senescence are consistent with others [25], and suggest that this factor plays a similar role in the avian telomeric apparatus and the maintenance of the avian telomeres. The identification in 10-day-old chick embryos of a novel major truncated cTRF2 transcript in which the encoded protein is predicted to lack a myb-related DNA binding domain and nuclear localisation signal has not been observed previously. The human TRF2 protein has been shown to facilitate the organisation of telomeric ends into T loops preventing their recognition as double-strand breaks [26]. More recently, TRF-2 has been reported to interact with chromatin containing DSBs and interestingly, this latter response requires the TRF2 N-terminal basic domain, but not the Myb domain [27]. The identification of TRF2 in the cytoplasm of neural cells, where it functions in neurite formation, also indicates a non-telomeric function for this protein and neither the N-terminal basic domain nor myb-like C terminus of the protein is required for such activity [28]. The actual role of the truncated cTRF2 protein is not known, but neither a role in DSB repair nor neural differentiation can be excluded.

The telomere lengths of CEFs maintained in vitro have been reported to be unchanged [8] or to exhibit shortening and lengthening during senescence [9]. This has led to the suggestion that an alternative lengthening of telomere (ALT) mechanism involving homologous recombination (HR) may operate in avian cells [9]. In such studies the analyses of chicken telomere length was performed using standard Southern hybridisation techniques and telomeric probes. However, this system is flawed as the characteristic smear of telomeric restriction fragments is obscured by DNA bands arising from the abundant interstitial telomeric sequence found in avian chromosomes. The telomeric G-strand overhang assay, as used in this study, avoids this problem as hybridisation to the G-strand overhang is performed under denaturing conditions, and because interstitial telomeric sequence is double stranded this ensures that interference from such sequence is eliminated [1]. Using this non-denaturing system, our data show that senescence is clearly associated with a reduction in telomere length.

HR generally involves the double-strand break proteins RAD51 and RAD52. Vertebrate cells deficient in key proteins such as Rad51 are generally non-viable, but in yeast, the Rad52 epistasis group proteins can maintain telomeres by a recombination-based pathway [20]. Although, we observed the up-regulation of Rad52 expression in the CEFs maintained in vitro this was accompanied by the loss of Rad51 expression, reduced telomere length, and senescence. Thus our data suggest that homologous recombination mechanisms are unlikely to function to maintain chicken telomeres in vitro.

The ectopic expression of telomerase has been shown to allow somatic cells the ability to by-pass replicative senescence and attain immortality. It has been suggested that human and avian telomere biology are similar and the chicken may provide model for studies of human cellular senescence and transformation [9]. Studies have shown that the human telomerase hTERT is compatible with and can immortalise the cells of a number of different mammalian species. However, in our study, the ectopic expression of hTERT in primary CEFs did not result in telomerase activity and an extension of their in vitro lifespan. In fact, the stably transfected CEFs followed a pattern of senescence comparable to the untransformed cells. This result was consistent with previous observations [29], although the mechanisms by which stable transfectants were selected in this earlier study are unclear.

The reasons for the inability of hTERT to function in CEFs are not known. The chicken TERT sequence has recently been identified, and shows significant homology to the human and other vertebrate TERTs encoding the highly conserved N-terminal and telomerase specific domains and seven reverse transcriptase motifs [30]. Its only major distinctive feature is an unique flexible linker sequence, 144 amino acids longer than in human, but this region has been shown to have little effect on either ribonucleoprotein assembly or TERT activity [31]. However, it cannot be excluded that the lack of telomerase activity in the hTERT-CEFs was the result of its incorrect assembly due to inappropriate chaperone proteins. One further explanation includes the inability of the hTERT protein to function with the chicken RNA component. It is interesting that the DNA sequences

of the human, rabbit, and bovine TR genes share high homology while that of the chicken is less conserved [32]. Since telomerase needs the correct template to function this may explain why hTERT immortalised the rabbit and bovine cells but failed to immortalise the chicken fibroblasts.

The chicken has been proposed as a potential model for studies of human senescence and cellular transformation [9]. However, our data indicate that differences do exist between the human and avian telomere maintenance apparatus suggestive of a species specific telomerase system in the avian.

Acknowledgments

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