RESEARCH ARTICLE

Telomerase Activity and Differential Expression of Telomerase Genes and c-myc in Chicken Cells In Vitro

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This study examined telomerase activity and gene expression profiles for three genes in *Gallus gallus domesticus*: telomerase reverse transcriptase (chTERT), telomerase RNA (chTR), and c-myc. Expression of these genes was studied in chicken embryonic stem (chES) cells, chicken embryo fibroblasts (CEFs), and DT40 cells using quantitative real-time polymerase chain reaction. Our results establish that, relative to transcription levels in telomerase-negative CEFs, chTERT and chTR are up-regulated in telomerase-positive chES cells. Transcription levels of chTERT, chTR, and c-myc are dramatically up-regulated in telomerase-positive DT40 cells, relative to CEFs and chES cells. These results are consistent with a model in which telomerase activity is up-regulated in proliferating embryonic stem cells requiring stable telomeres to endure multiple rounds of cell division; down-regulated in differentiated, lifespan-limited cells; and dramatically up-regulated in immortalized, transformed cells for which uncontrolled proliferation is correlated with c-myc dysregulation and telomerase activity. *Developmental Dynamics 231:14–21, 2004.*
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INTRODUCTION

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

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

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

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

Constitutive telomerase activity is a feature of mammalian ES cell systems (Thomson et al., 1998; Amit et al., 2000; Xu et al., 2001; Verfaillie et al., 2002; Carpenter et al., 2003). Telomerase is the enzyme responsible for replicating and maintaining the telomeres of proliferating cells (Blackburn and Gall, 1978). Telomere erosion due to a lack of telomerase is a well-studied mechanism that can promote cellular senescence or induce apoptosis (Forsyth et al., 2002; Swanberg and Delany, 2003, and references therein). Constitutive telomerase activity in ES cells may enable them to evade the telomere clock mechanism, which might otherwise impose a limitation on the proliferative potential of individual cells and lineages.

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

This study represents the first time these genes have been studied using a highly sensitive quantitative real-time polymerase chain reaction (PCR) assay and the first time chTERT and chTR expression have been studied in any context. Our results reveal distinct gene expression patterns among c-myc, chTERT, and chTR in three in vitro chicken systems: chES cells, CEFs, and DT40 cells, thereby providing insight into the mechanisms that may contribute to cellular immortalization in nontransformed and transformed cells in chicken.

RESULTS

Telomere Repeat Amplification Protocol Assay

Table 1 summarizes the telomere repeat amplification protocol (TRAP) results, and Figure 1 shows representative TRAP gels for the blastodermal embryos and chES cultures. Five of six pooled blastodermal embryo samples collected from unincubated, embryonated eggs, exhibited a positive telomerase activity profile as measured by the PCR-based TRAP (Kim et al., 1994). The remaining sample exhibited no telomerase activity. Of 21 samples of chES cells in culture for periods ranging from 7 to 181 days, 18 exhibited telomerase activity. A definitive conclusion regarding telomerase activity in the remaining three samples could not be drawn due to technical artifacts that could not be resolved. The results for these samples, therefore, were designated as inconclusive (Table 1). Representative TRAP results are illustrated in Figure 1. Of all the samples tested, 10 were from cultures older than 100 days. Nine of these samples were telomerase-positive, and the result for the remaining sample was inconclusive. Whether or not supplements such as chicken embryo extract (CEE) or turkey navel extract (TNE) were added to the media, chES samples were telomerase-positive (Table 1). As a check on antigenic status of the cells, a commonly used antigenic cell marker for ES cells, EMA-1, was examined in two long-term chES cultures. Cells from both samples were positive for EMA-1 (see Table 1; Fig. 2).

Because mouse STO cells are commonly used as feeder cell layers for ES systems, including chES cells and because mouse cells are typically telomerase-positive, irradiated and nonirradiated STO cells were examined for telomerase activity to determine the potential impact of this cell layer on telomerase assay results. In both cases, the STO samples were positive for telomerase activity and exhibited a TRAP ladder consistent with the nonprocessive nature of mouse telomerase (Prowse et al., 1993), see Figure 1.

Gene Transcript Analysis

Relative transcript levels of chTERT, chTR, and c-myc mRNAs were examined using quantitative real-time PCR in telomerase-positive chES cultures 24 and 25 (Table 1), cultured for 148 and 153 days, respectively. Gene-specific values from three replicate samples per culture were averaged, and an overall average for the two cultures was calculated for

TABLE 1. Summary of Telomerase Profiles of Blastodermal Embryos and Chicken Embryonic Stem Cells in Culture to 181 Day[a]

Sample	DIC	Source material	Telomerase activity	CEE	TNE	Other
1		BD embryos	+			Source for samples 7, 11–17, 21
2		"	+			
3		"	+			
4		"	+			
5		"	+			
6		"	−			
7	7	chES cells	+	No	Yes	
8	7	"	+	No	No	
9	7	"	+	Yes	No	
10	7	"	+	Yes	Yes	
11	7	"	+	No	No	
12	14	"	+	No	Yes	
13	14	"	+	No	No	
14	28	"	+	No	No	
15	28	"	IC	No	Yes	
16	56	"	+	Yes	No	
17	56	"	IC	No	No	
18	105	"	+	Yes	No	Membrane
19	105	"	+	Yes	No	
20	105	"	+	No	No	
21	122	"	IC	Yes	No	EMA-1 +
22	126	"	+	Yes	No	
23	126	"	+	No	No	
24	148	"	+	Yes	No	EMA-1 +
25	153	"	+	Yes	No	Samples 24 and 25 related
26	181	"	+	Yes	No	
27	181	"	+	No	No	

[a]DIC, days in culture; BD embryos, pooled blastodermal embryos; chES, cultured chicken embryonic stem cells. Telomerse activity: +, positive; −, negative; IC, inconclusive (see Results section); CEE, chicken embryo extract (see Experimental Procedures section); TNE, turkey navel extract (see Experimental Procedures section and U.S. Patent 6,140,118). Other, culture samples 7, 11–17, and 21 were related, that is, derived from the same group of blastodermal embryos (sample 1) but cultured to different time points to assay for telomerase activity; culture sample 18 cells were grown on a membrane insert (see Experimental Procedures section); culture samples 24 and 25 were also related but grown for different lengths of time, and these samples were used for transcript analysis (see text); EMA-1 +, cells were positive for the ES cell surface marker (from sister cultures to these samples, i.e., derived from the same source). Yes, extract added; No, extract not added.

each gene. The chES results were compared with those from early passage (~5 population doublings) CEFs and results from DT40 cells as described in the methods below.

Figure 3 illustrates the relative mRNA levels of chTERT, chTR, and c-myc among the three cell systems examined. Surprisingly, detectable levels of chTERT were observed in telomerase-negative CEFs. The chES cells exhibited moderately high levels of both chTR and chTERT (11-fold and 15-fold elevation, respectively) in comparison to early passage CEFs. In contrast, the mRNA levels of c-myc were similar in chES cells and CEFs. The DT40 mRNA levels for all three genes were dramatically higher when compared with the CEFs, 155-, 282-, and 324-fold higher for chTERT, c-myc, and chTR, respectively. When compared with chES cells, the DT40 values for c-myc, ch-TERT, and chTR were 10-, 30-, and 282-fold higher, respectively. Of interest, the chTERT/chTR ratios for chES cells and CEFs were the same, 6.4 to 1, whereas the chTERT/chTR ratio in DT40 cells was 3.1 to 1 (Fig. 4).

DISCUSSION

In this study, we examined the expression of telomerase and telomerase-related genes in chES cells in culture, comparing and contrasting chES expression patterns to patterns seen in CEFs and DT40 cells, thereby contributing new information regarding telomerase activity and the telomere clock mechanism to the field of chicken stem cell biology. A majority of the blastoderm embryo samples derived from unincubated eggs (in vivo samples, i.e., no culturing) exhibited telomerase activity that is consistent with previous reports (Taylor and Delany, 2000; Swanberg and Delany, 2003). A majority of cultured chES cells derived from the blastodermal embryos also exhibited telomerase activity. The chES cells exhibited telomerase ac-
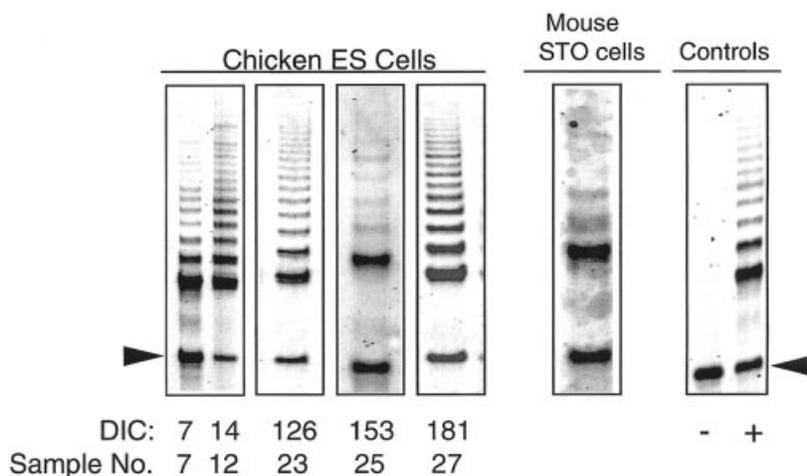
Fig. 1. Telomere repeat amplification protocol (TRAP) assay results illustrating telomerase activity profiles in cultured chicken embryonic stem (ES) cells (chES) and mouse STO cells. Representative TRAP assay results for chES cultures at 7, 14, 126, 153, and 181 days in culture. The positive telomerase activity of nonirradiated mouse STO cells is also shown. The polymerase chain reaction (PCR) -based TRAP assay detects the stepwise addition of TTAGGG repeats to an oligonucleotide primer by primer extension if telomerase is present in the sample extract. Controls include buffer only (negative control, −) and Hamburger and Hamilton (1951) stage 4 embryo (positive control, +). Arrowheads indicate the 36-bp PCR internal control amplicon. Presence of the control amplicon indicates that the PCR reaction was successful; thus, a "negative" TRAP assay result was not due to faulty PCR.
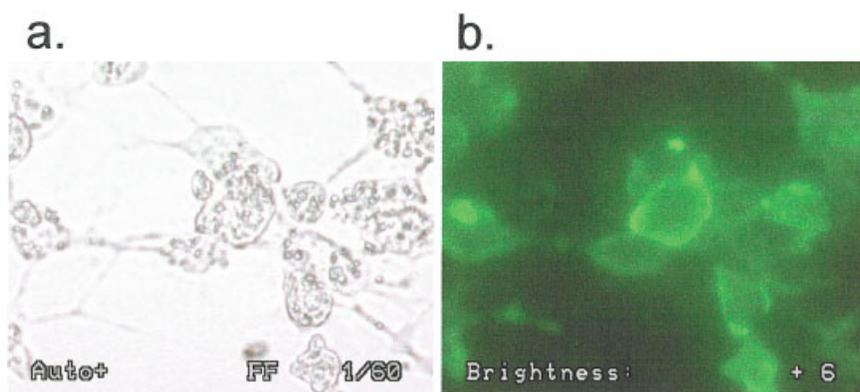

Fig. 2. EMA-1 status of chicken embryonic stem (chES) cells in culture. **a:** chES cells by phase optics illumination. **b:** The same cells with a positive staining pattern detected by indirect immunofluorescence using a monoclonal antibody against EMA-1. EMA-1 is a primordial germ cell surface marker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

tivity, regardless of which of several conditions were used to grow the cells. The results support the conclusion that telomerase activity is a general feature of chES cells in culture, as also observed for mammalian ES cells.

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

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

Levels of c-myc mRNA in DT40 cells were dramatically higher than in either chES cells or CEFs (282-fold higher). High expression of c-myc mRNA in DT40s was reported previously (Baba et al., 1985; Neiman et al., 2001) and is attributed to activation of the c-myc gene by the transforming agent, avian leucosis virus. Several studies suggest that c-myc re-activates telomerase in transformed cells by inducing expression of the catalytic subunit TERT (Wu et al., 1999). The c-myc protein mediates transcriptional activity of a target gene through binding to E-box elements with the core sequence CACGTG located in the promoter or enhancer region of the target (Kuramoto et al., 1999). Interestingly, Falchetti et al. (1999) found that telomerase activity emerged in v-myc-transformed avian cells that previously had been telomerase-negative.

Chicken TERT was only recently cloned, sequenced, and analyzed for regulatory features. The chTERT regulatory sequence was found to contain one E-box and chTR 3′ se-

quences contain c-myc binding sites, suggesting that TR may also be subject to regulation by c-myc (Delany and Daniels, in press; Delany and Daniels, 2003). DT40 chTR levels in this study were 324-fold higher than found in CEFs and the chTERT/chTR ratio in DT40 cells was 3.1 to 1 as opposed to the 6.4 to 1 ratio observed in CEFs and chES cells. These ratios are noteworthy as TR up-regulation is a hallmark of some human cancers (Avilion et al., 1996, Soder et al., 1997, 1998; Naito et al., 2001).

Chicken and human share many features of the telomere clock mechanism, including telomere shortening and down-regulation of telomerase in most somatic cells (Delany et al., 2003). Regulated expression of c-myc, chTERT and chTR is a feature of normally proliferating human cell populations, including embryonic stem cells. On the other hand, dys-regulation of c-myc accompanied by reactivation or over-expression of telomerase is a common attribute of the transformed phenotype in many human cancers. Substantial evidence in human suggests that c-myc can induce transcription of TERT, which is thought to be a rate-limiting determinant in the activation of telomerase. The results of this study are consistent with the human model in that telomerase activity is moderately up-regulated in proliferating embryonic stem cells (e.g., chES), which require stable telomeres to endure multiple rounds of cell division; down-regulated in differentiated, lifespan-limited cells (e.g., CEFs); and dramatically up-regulated in immortalized, transformed cells (e.g., DT40 cells) for which uncontrolled proliferation is associated with c-myc dysregulation and telomerase activity.

EXPERIMENTAL PROCEDURES

Chicken Embryonic (chES) Cells

Blastoderm embryos from unincubated SJ line chicken eggs (Stages IX–XI, Eyal-Giladi and Kochav, 1976) were harvested in unsupplemented LM medium (8.8 g/L Leibowitz L-15, 5.0 g/L McCoy 5A, 1.5 g/L NaHCO$_3$), gently agitated, and then spun at

$200 \times g$ for 5 min to remove yolk material. The supernatant was withdrawn and the blastodermal embryos were resuspended in 25 ml of LM in a 50-ml tube. After adding LM, the embryos were manually shaken for 30 sec to dissociate the tissue into individual cells or small clumps of cells, poured through a 100-μm cell strainer (Falcon), and plated at $\sim 2 \times 10^5$ cells/ml. Cell cultures were grown in 100-mm cell culture plates

at 39°C with 5% CO$_2$ in LM medium with 20% fetal bovine serum, 2.0 mM Glutamax (GIBCOBRL), 8.0 mM additional NaHCO$_3$, 1.0 mM sodium pyruvate, 5 μg/ml gentamicin, and 4 μg/ml Fungizone. In some cases, cells were grown on a Costar Transwell 75-mm polycarbonate membrane insert with 0.4-μm pores for maximum exposure to the medium.

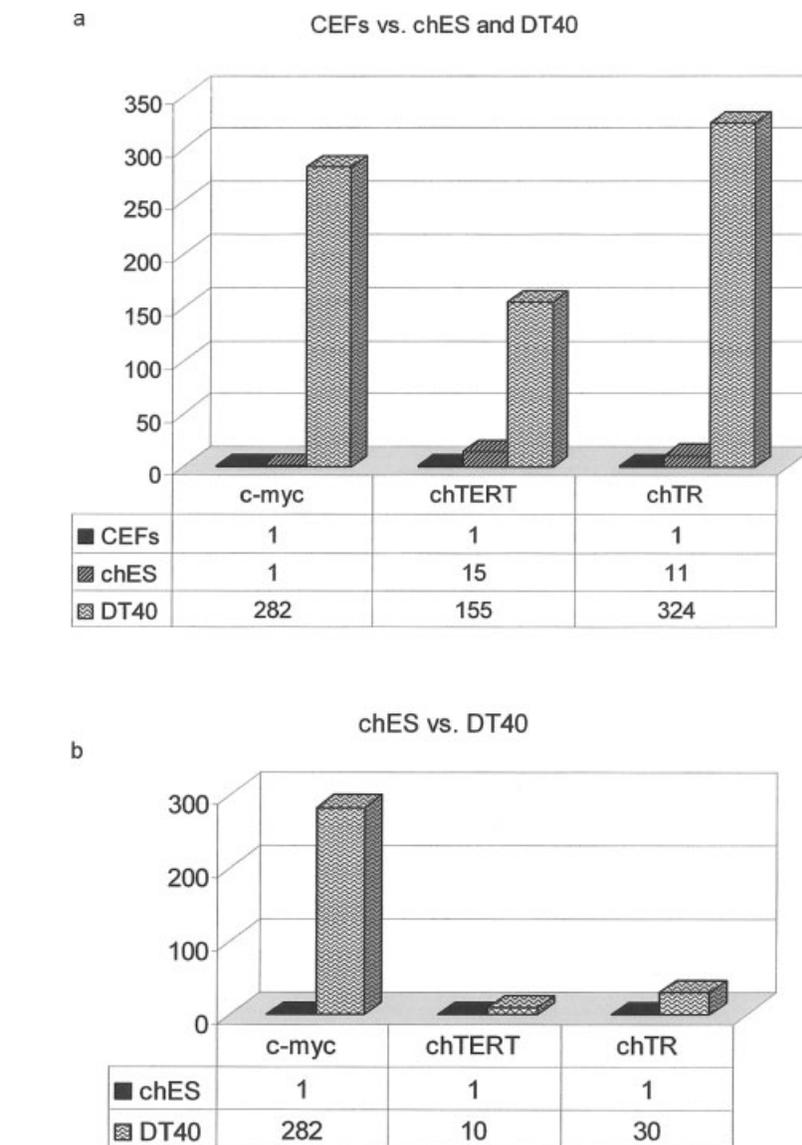In some culture systems, 5% CEE and/or TNE (Tsai et al., 1999 (U.S.

Fig. 3. Relative transcript levels of c-myc, chicken telomerase reverse transcriptase (chTERT), and chicken telomerase RNA (chTR) mRNA in chicken embryo fibroblasts (CEFs), chicken embryonic stem (chES) cells, and DT40 cells. Analysis was conducted by real-time TaqMan polymerase chain reaction using the comparative C$_t$ method. Results shown in **a** indicate fold differences in mRNA levels among the cell types. Six chES replicates (148 or 153 days in culture) were averaged. Values from six DT40 cultures were also averaged. These averages for chES and DT40 cultures were calibrated against early-passage CEFs, which were set to a value of 1. In **b**, the fold differences in mRNA levels for DT40 and chES cells are compared (chES values set to 1).
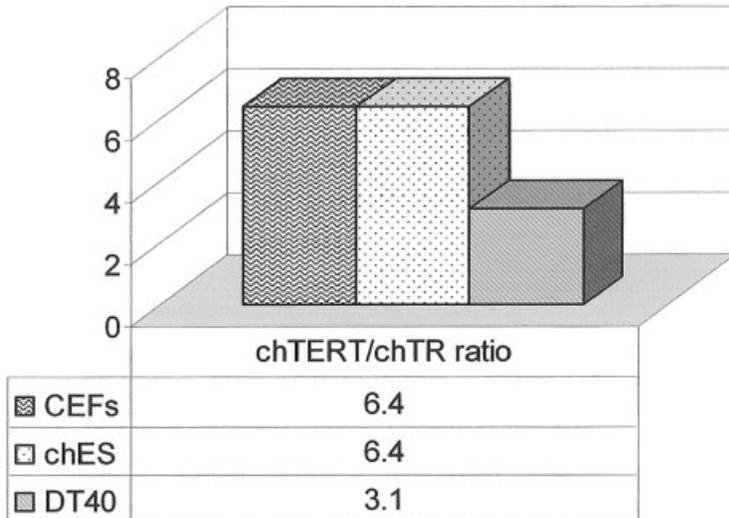
Fig. 4. Ratios of telomerase gene transcripts in chicken embryonic stem (chES) cells, chicken embryo fibroblasts (CEFs), and DT40 cells. Ratio of chicken telomerase reverse transcriptase (chTERT) to chicken telomerase RNA (chTR) transcripts is 6.4 to 1 in chES cells and CEFs. In contrast, the ratio in DT40 cells is 3.1 to 1. Both chTERT and chTR are up-regulated in DT40s, but this up-regulation is not proportional.

Patent 6,140,118)) were added to the culture medium to assess their effect on cell growth. Samples from chES cultures 24 and 25 (Table 1), cultured for 148 and 153 days, respectively, were used in the transcript analysis.

CEE preparation.

Three hundred embryonic day (E) 10 chicken embryos were added to 100 ml of unsupplemented LM medium. The medium was poured off, centrifuged briefly to remove red blood cells, and then added back to the tissue. This mixture was homogenized in a blender for 1 min, transferred to a plastic centrifuge bottle, incubated at 4°C for 1 hr, and frozen at −80°C. The extract was thawed and refrozen an additional two times. The extract was centrifuged at 7,000 rpm in a Sorvall GS3 rotor for 1 hr, dispensed into 50-ml aliquots, and frozen at −80°C. After adding CEE to the growth medium, the complete medium was sterilized by filtration.

TNE.

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

EMA-1 detection.

Status of the primordial germ cell surface marker EMA-1 was examined on chES culture by indirect immunofluorescence. A mouse anti–EMA-1 (IgM) was obtained from the Developmental Studies Hybridoma Bank (Department of Biological Sciences, University of Iowa, 007 Biology Building East, Iowa City, IA 522542). Biotinylated goat anti-mouse IgM (μ-chain specific) and fluorescein isothiocyanate (FITC) -conjugated Streptavidin were obtained from Sigma (St. Louis, MO). The EMA-1 antibody was used at 1:100 dilution. The remaining antibodies were used at the manufacturer's recommended dilution. The cells were stained and analyzed in the dish in which they were grown. Cells were incubated with the anti–EMA-1 monoclonal antibody for 20 min, washed with medium three times, incubated with the goat anti-IgM for 20 min, washed three times with medium, incubated with FITC-Strepavidin for 5 min, and washed three times with PBS. The cells were examined by using a Leica DM1RB (inverted ultraviolet microscope) fitted with an Optronics

DE1-750 CE digital output image capture system.

CEFs

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

DT40 Cells

DT40 cells were obtained from the American Type Culture Collection (ATTC CRL-2111) and from Dr. Jean-Marie Buerstedde (GSF-National Research Center for Environment and Health, Neuherberg, Germany). Culture conditions were followed as outlined previously (see Media I Formulation section in Chang and Delany, 2004). Three cultures from each DT40 source were used for transcript analysis; a final value was derived from the average of the six cultures, as the individual culture results were similar.

Telomerase Activity Detection

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

Gene Transcript Analysis by Quantitative Real-Time TaqMan PCR

Theoretical basis.

In traditional PCR, reactions are characterized by analysis of a product that has accumulated after a predetermined number of cycles. With real-time PCR, reactions are characterized by the point at which amplification of a target molecule is first detected. The greater the quantity of the target molecule in the re-

action mix, the earlier a significant (above background) increase in fluorescence is observed. The threshold cycle (C_t) is defined as the fractional cycle number at which fluorescence generated by cleavage of a probe for the target molecule exceeds a set threshold above the baseline (Bièche et al., 2000).

Primers and probes.

Primers and probes were developed for each of the three target genes (chTERT, chTR, and c-myc) as well as for three housekeeping genes for normalization purposes (housekeeping genes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank CB018343), chicken transferrin receptor (GenBank X55348.1), and chicken ribosomal protein L10 (GenBank CB271063)). Chicken GAPDH mRNA levels were found to show the least standard deviation for all samples examined; therefore, GAPDH values were used to normalize the target gene values. Primers and probes were selected with Primer Express software (Applied Biosystems, Foster City, CA) and are shown in Table 2.

Sample preparation.

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

Reverse transcription reaction and real-time PCR.

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

Each PCR reaction contained 400 nM primer and 80 nM TaqMan probe for the respective TaqMan system and commercially available PCR

TABLE 2. Primers and Probes for Quantitative Real-Time RT-PCR[a]

Gene	NCBI accession no.	Primers and probes (5'–3')
chTR	AF221938	Forward-CTCCGCTGTGCCTAACCCTAAT
		Reverse-TCGCCCGCTGAAAGTCAG
		Probe-AATTGATGGTGCTGTCGCCGCG
chTERT	AY505016	Forward-GTCAGAGCGAAGTCATCACAAGAAT
		Reverse-TGGCAAAACTCTGAAGTGACAAC
		Probe-ATGGATACTCCTTGCTGGATGAGAA
c-myc	X68073	Forward-AGCGACTCGGAAGAAGAACAAG
		Reverse-ATCGACTTCGCTTGCTCAGACT
		Probe-AGAAGATGAGGAAATCGATGTCGTTACA
GAPDH	CB018343	Forward-TTGTTTCCTGGTATGACAATGAGTTT
		Reverse-CTCACTCCTTGGATGCCATGT
		Probe-ATACAGCAACCGTGTTGTGGACTTGATGGT

[a]chTR, chicken telomerase RNA; chTERT, chicken telomerase reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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

Relative quantitation of gene transcription.

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

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