

Regulatory mechanisms of human and mouse telomerase reverse transcriptase gene transcription: distinct dependency on c-Myc

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Abstract Telomerase—a complex ribonucleoprotein enzyme—synthesizes telomeric repeats to avoid telomere loss that accompanies cell division and chromosomal replication. Expression of telomerase is detectable in embryonic cells and cancer cells, but not in normal human cells. On the other hand, in mice, substantial expression of telomerase is detected in normal cells and tissues as well as in immortalized cells. These results suggest that the regulatory mechanisms of telomerase activity in humans and mice differ. Considering these results along with the fact that the expression of the telomerase reverse transcriptase (*TERT*) gene is a rate-limiting step for telomerase activity, we compared transcriptional regulatory mechanisms of both the species. A series of luciferase assays and RT-PCR analyses demonstrated that c-Myc, a dominant transactivator for human *TERT* (*hTERT*), is not involved in the regulation of mouse *TERT* (*mTERT*). These results suggest that distinct molecules and pathways are involved in the process of immortalization and tumorigenesis in human and mouse cells.

Keywords Telomerase · *hTERT* · *mTERT* · c-Myc · Transcription

Introduction

Telomeres are repetitive DNA sequences at the ends of eukaryotic chromosomes with a specialized structure that stabilizes the chromosomes and prevents their fusion during mitosis. Telomerase, a ribonucleoprotein complex, is capable of de novo synthesis of telomeric DNA. The most important component that is responsible for the catalytic activity of telomerase is telomerase reverse transcriptase (*TERT*) (Martin-Rivera et al. 1998; Nakamura et al. 1997). Numerous studies have shown that human *TERT* (*hTERT*) is expressed in most malignant tumors, but not in normal human tissues, and that expression of *hTERT* is closely correlated with telomerase activity (Kilian et al. 1997; Meyerson et al. 1997; Takakura et al. 1998). These findings indicate that *hTERT* is a rate-limiting determinant of the catalytic activity of telomerase. Recently, the promoter region of the *hTERT* gene was cloned, and initial analyses indicated that c-Myc is involved in the transcriptional activation of the *hTERT* gene in tumor cells (Wang et al. 1998; Xu et al. 2001). In addition, several researchers have demonstrated that Mad1 acts in an antagonistic manner in response to c-Myc-induced transactivation, thereby resulting in a potent repression of the *hTERT* gene via binding to

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the E-box elements located in the proximal core promoter (Gunes et al. 2000; Oh et al. 2000; Xu et al. 2001). As compared to the accumulated evidences regarding the regulatory mechanisms of *hTERT* transcription, knowledge on the regulatory mechanisms of mouse *TERT* (*mTERT*) transcription is limited. In the present study, we attempted to investigate the regulatory mechanisms of the *mTERT* promoter and clarify the differences in the regulatory mechanisms and competence of c-Myc between the *hTERT* and *mTERT* core promoters.

Materials and methods

Cell lines and culture conditions

A human lung adenocarcinoma cell line (A549) was cultured in eRDF medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Invitrogen). TIG-1 cells (Institute of Development, Aging and Cancer (IDAC), Tohoku University, Miyagi, Japan) and mouse embryonic fibroblasts (MEFs) derived from embryonic day 13.5 mice were cultured in MEM medium (Nissui, Tokyo, Japan) supplemented with 10% FBS at 37 °C in 5% CO₂. A mouse fibroblast cell line (NIH3T3) was obtained from IDAC and cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 10% FBS.

Quantitative real-time PCR

RNA was prepared using the FastPure RNA kit (TaKaRa, Shiga, Japan). cDNA was prepared using M-MLV RT RNase H⁻ (Promega, Madison, WI) according to the manufacturer's protocol. Quantitative PCR was performed using SYBR Premix Ex Taq (TaKaRa) and Thermal Cycler Dice Real Time System TP-800 instrument (TaKaRa). PCR amplification began with a 10-s denaturation step at 95 °C and then 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s. The samples were analysed in triplicate, and the *hTERT* and *mTERT* levels were normalized to the corresponding β -actin levels. The PCR primer sequences used were as follows: *hTERT* top primer CGTACAGGTTTCACGC ATGTG and bottom primer ATGACGCGCAGGAA AA ATG; human β -actin top primer TGGCACCCAG-CACAATGAA and bottom primer CTAAGTCATAG

TCCGCCTAGAAGCA; *mTERT* top primer CAG-CCATACATGGGCCAGTTC and bottom primer ACAGGCTGCTGCTGCTCTCA; mouse β -actin top primer GGCCAGGTCATCACTATTG and bottom primer GAGGTCTTTACGGATGTCAAC.

hTERT and *mTERT* promoter-reporter constructs

We amplified the 5'-flanking region of the *hTERT* and *mTERT* genes by using genomic DNA prepared from A549 cells and NIH3T3 cells, respectively. Templates and primers were designed based on the deposited sequences (*hTERT*, AB016767; *mTERT*, AF121949). DNA fragments of various lengths that were located upstream of the initiation ATG codon were amplified using PCR and were inserted into the firefly luciferase reporter plasmid pGL3-Basic (Promega). E-box elements in the isolated *hTERT* and *mTERT* promoter regions (*hTERT*, -242 to -237 and -34 to -29; *mTERT*, -230 to -225 and -32 to -27) were changed from CACG/CTG to TTTGTG using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega).

Transfection and luciferase assay

All transfections were carried out in triplicate in 24-well plates (Becton–Dickinson Labware, Franklin Lakes, NJ). The cells were seeded at subconfluence and were cultured overnight. In order to standardize transcription efficiency, the luciferase reporter plasmid and effector plasmids were cotransfected along with the Renilla luciferase plasmid pRL-TK (Promega) into the cells by using the LipofectAMINE PLUS reagent (Invitrogen) in accordance with the manufacturer's instructions. The cells were exposed to the transfection mix for 3 h and harvested for analysis after 48–72 h. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated by dividing the firefly luciferase activity by the Renilla luciferase activity. The experiments were repeated at least three times; standard deviations are shown in the figures.

Plasmids

Full length human c-myc cDNA was prepared from pSPT-myc (JCRB GeneBank, Japan) and was inserted

into pcDNA3 (pcDNA3-c-Myc). The mad expression plasmid (pcDNA3-Mad) was a generous gift from R. Robert (Fred Hutchinson Cancer Research Center), and MadMyc and MadMyc Δ C expression plasmids were generous gifts from R. Bernards (The Netherlands Cancer Institute).

Results and discussion

Identification of *cis*-elements in *hTERT* and *mTERT* promoter region

Previous studies have shown that the *hTERT* gene is expressed in most tumors cells, but not in normal cells, where the c-Myc oncogene plays a critical role in regulating the expression of this gene (Ramakrishnan et al. 1998). In this study, we attempted to clarify differences in the transcriptional regulatory mechanisms of the *TERT* gene between human and mice. We analyzed the expression of TERT gene between normal and tumor/immortalized cells by quantitative real time PCR (Fig. 1). As shown in Fig. 1, TERT gene expression was not detected in human normal cells, but in mouse normal cells. To analyze the transcriptional regulatory mechanisms of the *hTERT* and *mTERT* gene, we isolated their promoter regions. The promoter fragment for *hTERT* (from -286 to -25), *mTERT* (from -281 to -25), and their 5'-truncations were amplified by PCR and cloned into pGL3-basic (Fig. 2a, b). The region from -286 to

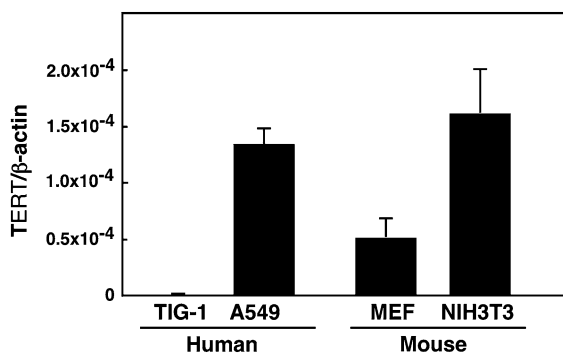
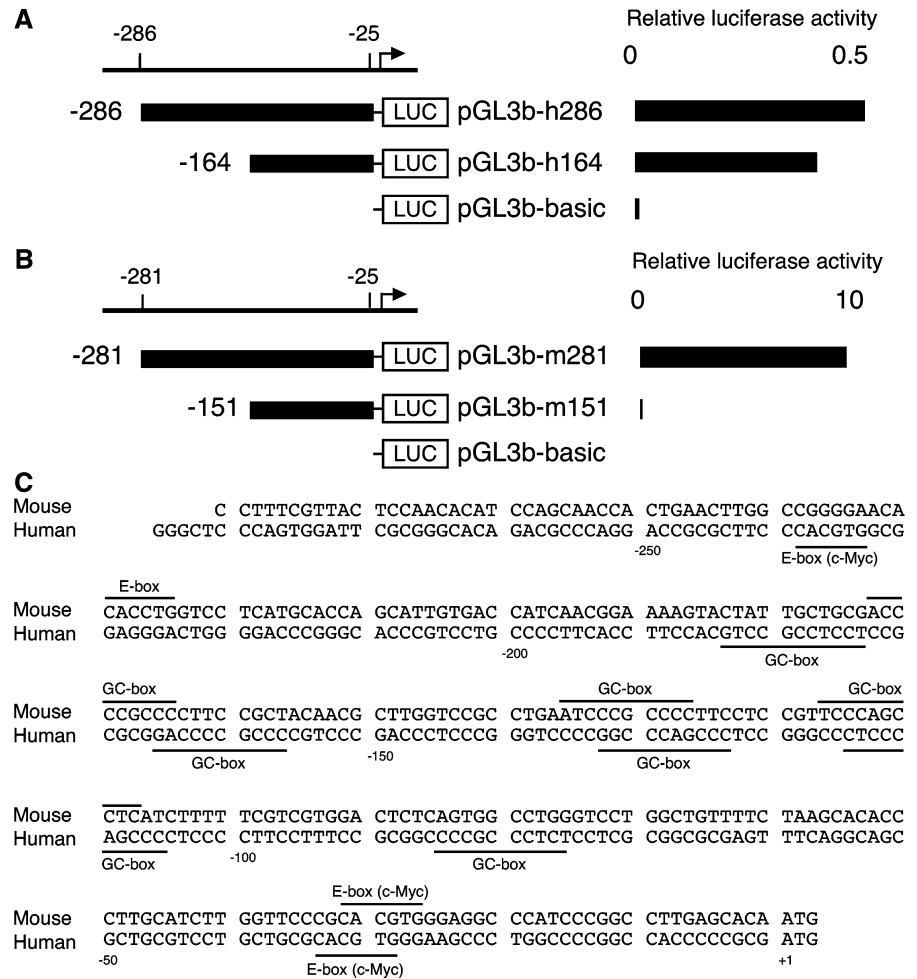


Fig. 1 Expression of the *hTERT* and *mTERT* genes in immortal and mortal cells. A549 cells and NIH3T3 cells (immortal cells) and TIG-1 cells and MEF (mortal cells) were investigated, and the expression of *hTERT* and *mTERT* was assessed by quantitative real-time PCR. The samples were analysed in triplicate, and the *hTERT* and *mTERT* levels were normalized to the corresponding beta-actin levels

-25 of the *hTERT* gene was reported to be the core promoter region (Horikawa et al. 1999; Takakura et al. 1999). TERT core promoter activity is known to be related to TERT mRNA expression in human cells (Takakura et al. 2005) and in mouse cells (Nozawa et al. 2001). As expected, we detected a strong promoter activity in the fragment region from -286 to -25 of the *hTERT* promoter. Next, we tested for the promoter activity of the fragment derived from the *mTERT* promoter. As shown in Fig. 2b, a fragment of the *mTERT* promoter (-281 to -25) with almost the same length as that of the *hTERT* core promoter demonstrated a similar strong promoter activity. These results indicate that an almost similar length of fragments located upstream of the ATG codon constitutes the core promoter in both the species. Sequence alignment of the core promoter regions of the *hTERT* and *mTERT* promoters indicates several conserved *cis* elements, including an E-box and GC box (Fig. 2c). E-boxes of the c-Myc/Max-binding type (CACGTG) in particular, are highly conserved between the *hTERT* and *mTERT* promoters and are located at -34 in the *hTERT* promoter and at -32 in the *mTERT* promoter. The second E-box identified at -242 of the *hTERT* promoter is also of the c-Myc/Max-binding type, while that identified at -230 of the *mTERT* promoter is of the canonical and not the c-Myc/Max-binding type. These single nucleotide differences in the second E-box might induce differential dependency of *hTERT* and *mTERT* promoters on c-Myc. Further, GC boxes are also conserved in both the core promoter regions; five GC boxes in the *hTERT* promoter and three GC boxes in the *mTERT* promoter. Sp1 was reported to play an important role in the activation of *hTERT* transcription through its binding to the GC box (Kyo et al. 2000), thereby suggesting that although Sp1 is also involved in the transcriptional regulation of the *mTERT* promoter, the difference in the number of GC boxes might elicit a distinct regulatory mechanism between *hTERT* and *mTERT*. Thus, sequence alignment demonstrated that the core promoter regions for the *hTERT* and *mTERT* promoters have approximately the same length and similar number and kinds of *cis* elements at almost the same position. This suggests common regulatory mechanisms between the *hTERT* and *mTERT* promoters. However, some differences in the *cis* elements and in the surrounding regions of both the promoters might elicit differential transcriptional regulation of these promoters.

Fig. 2 Core promoter regions for the *hTERT* and *mTERT* genes. **a** and **b**, A549 cells and NIH3T3 cells were transfected with luciferase reporter constructs containing the 5'-flanking region of the *hTERT* and *mTERT* genes. Experiments were performed in triplicate, and the representatives are indicated. **c** Sequence alignment of the *hTERT* and *mTERT* core promoters and the putative cis elements. The translation start site is indicated as +1. Consensus motifs for the E-box and GC box are *underlined*



E-box dependency of regulation of the *hTERT* and *mTERT* core promoters

c-Myc is reported to be a dominant transcriptional activator for *hTERT* expression (Greenberg et al. 1999; Horikawa et al. 1999; Kyo et al. 1999, 2000; Wang et al. 1998; Wick et al. 1999). Further, previous reports have demonstrated that Mad acts in an antagonistic manner in response to c-Myc-induced *hTERT* transactivation via binding to the E-box elements located in the core promoter (Xu et al. 2001). On the other hand, the functionality of c-Myc is complicated in mouse cells, and this is evident in the report by Ramakrishnan et al. (1998), which states that c-Myc activates *mTERT* expression in primary mouse cells, but not in some immortal mouse cell lines. The ability of c-Myc to regulate *mTERT* expression was suggested to be superseded by other

events associated with immortalization, adaptation to ex vivo culture, and/or cell context.

In the present study, we attempted to clarify the differences in c-Myc dependency between the *hTERT* and *mTERT* core promoters and then performed luciferase assays. As expected, the activity of the *hTERT* core promoter was activated by c-Myc and attenuated by Mad (Fig. 3a). Further, a mutant *hTERT* core promoter with mutated E-box elements did not respond to both c-Myc and Mad. These results indicate that the regulation of the *hTERT* core promoter is dependent on the E-box and the E-box binding factors. On the other hand, the *mTERT* core promoter activity did not respond to both c-Myc and Mad (Fig. 3b). Unexpectedly, the introduction of mutations in E-box elements augmented the *mTERT* core promoter activity. These results indicate that the *mTERT* core promoter is regulated independent of the

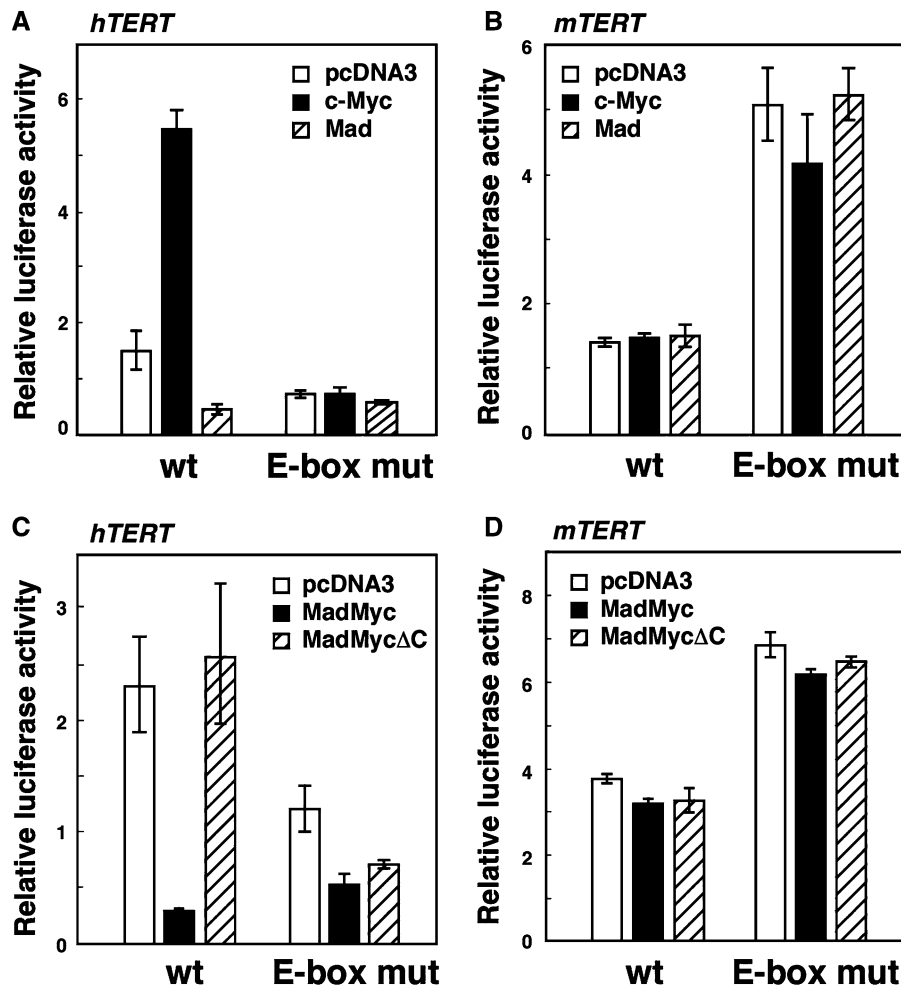


Fig. 3 Regulation of *hTERT*, but not *mTERT*, core promoter is dependent on the E-box and c-Myc **a** the *hTERT* core promoter is regulated in a c-Myc-dependent manner. A549 cells were transfected with a vector expressing c-Myc or Mad along with pGL3b-h286 or its E-box mutant, and these transfected cells were then used for the luciferase assay. The relative luciferase activity is shown. **b** the *mTERT* core promoter is not regulated in a c-Myc-dependent manner. Transfection into NIH3T3 cells and luciferase assay using pGL3b-m281 containing the *mTERT* core promoter were performed as described in **a**. **c** MadMyc

represses *hTERT* core promoter activity. A549 cells were transfected with a plasmid expressing MadMyc or MadMyc Δ C along with pGL3b-h286 or its E-box mutant, and the transfected cells were then used for the luciferase assay. **d** MadMyc does not affect the *mTERT* core promoter activity. Transfection into the NIH3T3 cells and the luciferase assay using pGL3b-m281 were performed as described in **b**. All results were expressed as the bars indicating mean \pm SD of three independent experiments

E-box and c-Myc. Because we could not find any transcription factors that bind to the mutated E-box site, unidentified factors other than c-Myc would bind to the E-box and repress its promoter activity.

To further clarify the E-box-dependency of *hTERT* and *mTERT* core promoters, a luciferase assay was performed using a dominant negative c-Myc, named MadMyc, which represses the c-Myc target genes. MadMyc Δ C with a mutation in the DNA binding and heterodimerization domain was used as the control. As

shown in Fig. 3c, MadMyc, but not MadMyc Δ C, suppressed the *hTERT* core promoter activity. Further, both MadMyc and MadMyc Δ C slightly repressed the mutant *hTERT* promoter. These results also indicate that the regulation of the *hTERT* core promoter is dependent on the E-box and c-Myc; they also suggest that MadMyc might attenuate the *hTERT* core promoter activity in an E-box independent manner, possibly by capturing transcriptional activators. On the other hand, both MadMyc and MadMyc Δ C did not affect the *mTERT*

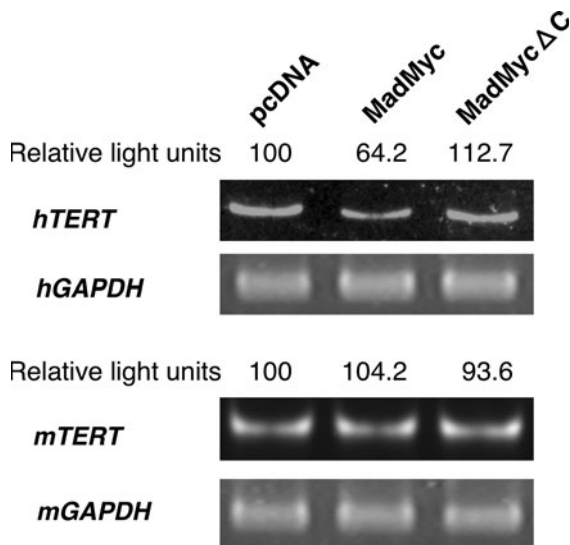


Fig. 4 Effect of MadMyc on the transcription of the *hTERT* and *mTERT* genes. A549 cells and NIH3T3 cells were transfected with a plasmid expressing MadMyc or MadMycΔC. After culturing for 72 h, the transfected cells were applied to RT-PCR analysis in order to detect the transcription of the *hTERT* and *mTERT* genes

core promoter activity (Fig. 3d) and also demonstrated that the *mTERT* core promoter activity is regulated independent of the E-box and c-Myc.

Next, we investigated the c-Myc dependency of *hTERT* and *mTERT* gene transcription. After transfecting A549 cells and NIH3T3 cells with a vector expressing MadMyc or MadMycΔC, *TERT* expression was assessed. As shown in Fig. 4, MadMyc, but not MadMycΔC, repressed the transcription of the *hTERT* gene. On the other hand, neither MadMyc nor MadMycΔC affected the transcription of the *mTERT* gene. These results also indicate that the regulation of the *hTERT*, but not the *mTERT*, core promoter is dependent on E-box and c-Myc. All these results demonstrate that the dominant transcriptional activator of c-Myc for the *hTERT* core promoter is not involved in the regulation of *mTERT* gene transcription. These results also suggest that immortalization and tumorigenesis are regulated by distinct regulatory mechanisms and molecules in human and mice.

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