Differential cis-regulation of human versus mouse *TERT* gene expression *in vivo*: Identification of a human-specific repressive element

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In vivo expression of human telomerase is significantly different from that of mouse telomerase. To assess the basis for this difference, a bacterial artificial chromosome clone containing the entire *hTERT* (human telomerase reverse transcriptase) gene was introduced in mice. In these transgenic mice, expression of the *hTERT* transgene was similar to that of endogenous *hTERT* in humans, rather than endogenous *mTERT* (mouse telomerase reverse transcriptase). In tissues and cells showing a striking difference in expression levels between *hTERT* in humans and *mTERT* in mice (i.e., liver, kidney, lung, uterus, and fibroblasts), expression of the *hTERT* transgene in transgenic mice was repressed, mimicking *hTERT* in humans. The transcriptional activity of the *hTERT* promoter was much lower than that of the *mTERT* promoter in mouse embryonic fibroblasts or human fibroblasts. Mutational analysis of the *hTERT* and *mTERT* promoters revealed that a nonconserved GC-box within the *hTERT* promoter was responsible for the human-specific repression. These results reveal that a difference in cis-regulation of transcription, rather than transacting transcription factors, is critical to species differences in tissue-specific *TERT* expression. Our data also suggest that the GC-box-mediated, human-specific mechanism for *TERT* repression is impaired in human cancers. This study represents a detailed characterization of the functional difference in a gene promoter of mice versus humans and provides not only important insight into species-specific regulation of telomerase and telomeres but also an experimental basis for generating mice humanized for telomerase enzyme and its pattern of expression.

Mouse models are widely used for understanding physiological and pathological processes in humans, including aging and cancer. However, there are some significant differences between mice and humans that are possible concerns in the use of mouse models in a variety of fields of current research. One of these differences is in the regulation of telomerase expression and telomere length (1). Telomeres, specialized structures consisting of repetitive DNA and associated proteins, function to protect chromosome ends from degradation and end-to-end fusion, thereby playing important roles in cell viability and genomic stability (2). The ribonucleoprotein enzyme telomerase synthesizes telomeric DNA repeat and can compensate telomere attrition associated with cell divisions. Most normal human somatic cells express undetectable or low levels of telomerase and undergo progressive loss of telomere length with cell divisions, eventually leading to telomere dysfunction (3). Telomere dysfunction induces permanent cell-growth arrest, termed cellular senescence, which may contribute to organismal aging in humans (4), and chromosome abnormalities, a hallmark of cancer cells (5). Activation of telomerase in human cells allows them to maintain telomere length and function, a critical step to immortalization and malignant transformation of human cells (6). In marked contrast, *Mus musculus*, mice used in research laboratories, have much longer telomeres and express robust levels of telomerase activity in a wider range of normal somatic tissues and cells than do humans (7–9). Telomere attrition is not a major cause of mouse-cell senescence (10), and mouse cells become immortalized and transformed more readily than human cells, at least in part, because of long telomeres and high levels of constitutive telomerase activity in mice (6, 11). These findings suggest that current mouse models for studying human cancer and aging do not always reflect the physiological and pathological status in humans (1). Indeed, knock-out mouse models of human genetic diseases associated with premature aging and cancer predisposition (e.g., Werner syndrome and Bloom syndrome) frequently do not recapitulate the human disease phenotypes; although, notably, in some instances, the disease phenotypes were manifest when mouse telomeres were shortened as a result of breeding with telomerase-null mice (12, 13). However, in this circumstance, telomerase is deficient in all tissues and cells, including those normally expressing telomerase activity in both mice and humans (e.g., germ-cell and stem-cell populations) and is, thus, discordant with the physiological status of telomerase and telomeres in humans. Thus, efforts to generate mouse models closer to humans in terms of telomere and telomerase regulation are of importance in the field of cancer and aging research.

Regulation of the telomerase reverse-transcriptase gene (*TERT*), which encodes the catalytic-protein subunit of telomerase enzyme, is a major determinant of telomerase activity (14). The expression level of *TERT* is highly correlated with telomerase activity in normal and cancer cells (15). In accordance with the differences in telomerase expression between mouse and human tissues, the expression profiles of mouse *TERT* (*mTERT*) in mouse tissues were reported to be different from those of human *TERT* (*hTERT*) in human tissues (16).

In this study, by generating transgenic mice carrying both an endogenous *mTERT* gene and an entire *hTERT* genomic transgene with considerable upstream and downstream regions and all exons and introns, we directly compare *in vivo* expressions of *mTERT* and *hTERT* to elucidate the molecular basis for the differential regulation. Moreover, through assays on transcriptional activities of a number of mutated *mTERT* and *hTERT* gene promoters, we identify a cis-acting DNA element that is responsible for species-specific regulation. This study provides essential data for understanding the significant difference in telomerase expression be-

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tween mice and humans and for making mouse models of cancer and aging closer to humans.

Materials and Methods

Generation of Transgenic Mice. A bacterial artificial chromosome (BAC) clone containing the ≥54-kb *hTERT* gene (including an at least 11-kb upstream region, all of the exons and introns, and 1.2-kb downstream region) was derived from a circular yeast artificial chromosome clone that was isolated by transformation-associated recombination cloning in yeast, as described in ref. 17. The linearized BAC DNA was microinjected into C57BL/6 mouse oocytes. The screening for founder lines containing the *hTERT* sequence was carried out by PCR genotyping (the PCR primer sequences are available at www.cephb.fr/poltel/Map_TERT.php) by using tail DNA. Mice from two independent *hTERT* transgenic founder lines were bred with $mTERT^{+/-}$ mice that were maintained on C57BL/6 background (18) to obtain $mTERT^{+/-}hTERT^+$ mice. $mTERT^{-/-}$-$hTERT^+$ mice were also generated by crossing $mTERT^{+/-}hTERT^+$ and $mTERT^{+/-}$ mice. As shown in Fig. 4, which is published as supporting information on the PNAS web site, spleen cells from wild-type, $mTERT^{-/-}$ (18), $mTERT^{+/-}hTERT^+$ and $mTERT^{-/-}$-$hTERT^+$ mice were used to examine hTERT expression by RT-PCR with the following primers: 5′-GCC TGA GCT GTA CTT TGT CAA-3′ and 5′-CGC AAA CAG CTT GTT CTC CAT GTC-3′, which amplified *hTERT* exons 5–9 (nucleotides 2,164–2,620 in GenBank NM_003219). Detection of mTERT and mouse actin mRNA was carried out as described in refs. 18 and 19. All animals were housed at Bioqual (Rockville, MD).

Real-Time Quantitative RT-PCR. Total RNA samples were extracted from mouse tissues and mouse embryonic fibroblasts (MEF) by using NucleoSpin kits (BD Biosciences Clontech, Palo Alto, CA). Human total RNA samples were obtained from BD Biosciences Clontech (sources of the samples are described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). One μg of total RNA was reverse-transcribed with random hexamers by using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). Each PCR reaction contained first-strand cDNA corresponding to 25 ng of RNA, TaqMan universal PCR master mix (Applied Biosystems), a set of primers and FAM/MGB probe for hTERT or mTERT, and a set of primers and VIC/MGB probe for human or mouse GAPDH (hGAPDH or mGAPDH, respectively) as the endogenous control (details of the primers and probes are available in *Supporting Materials and Methods*). Real-time detection of PCR products was performed by a PRISM 7700 sequence detector (Applied Biosystems). Reactions were in triplicate for each sample. Quantitative analysis of gene expression data was performed according to the standard-curve method in the supplier's protocol (User Bulletin #4303859B at www.appliedbiosystems.com/index.cfm) (standard curves are shown in *Supporting Materials and Methods*). The expression levels of mTERT or hTERT normalized with those of mGAPDH or hGAPDH (i.e., mTERT/mGAPDH, hTERT/mGAPDH, or hTERT/hGAPDH) are shown as average values ± SD in Fig. 1.

Promoter Constructs and Luciferase Assay. The 295-bp *hTERT* promoter cloned in pGL3-Basic vector (pBT-255, Promega) was described in ref. 20 (pBT-255). The 263-bp *mTERT* promoter (−248 to +15, shown in Fig. 2a) was PCR-amplified and cloned in the pGL3-Basic vector. Site-directed mutagenesis of *hTERT* and *mTERT* promoters was carried out by using the QuikChange site-directed mutagenesis kit (Stratagene). For the luciferase assay, cells were seeded on 24-well plates (8.0×10^4 to 1.5×10^5 per well, depending on cell-proliferation rate and cell size), cultured overnight, and transfected with the *TERT* promoter-luciferase plasmids (0.5 μg per well) or the pGL3-Control plasmid (in which the firefly luciferase reporter gene is driven by SV40 enhancer/promoter; 0.5
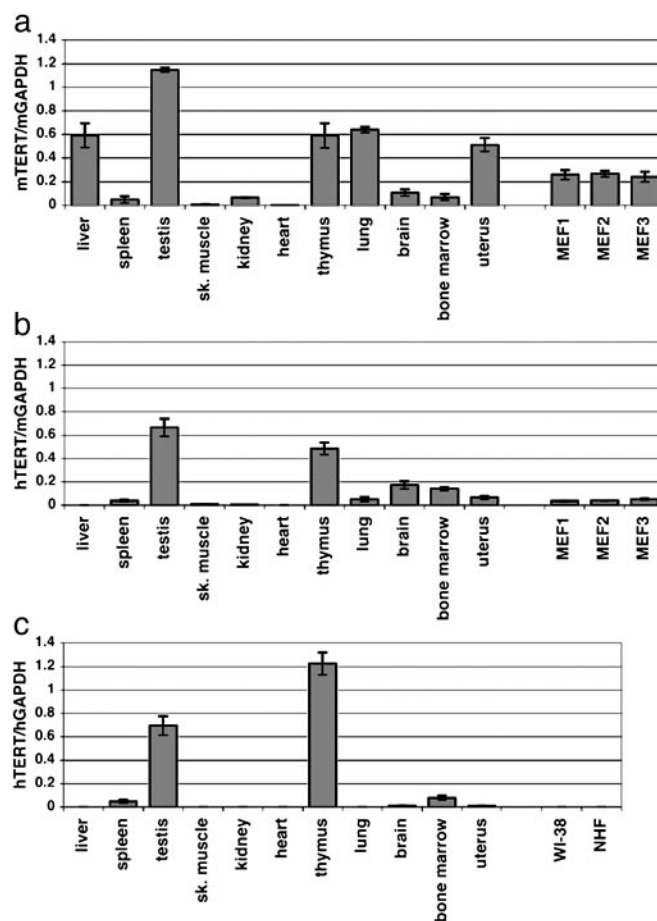

Fig. 1. Real-time quantitative RT-PCR analysis of mTERT and hTERT expression. RNA samples from various tissues in humans and transgenic mice and from fibroblasts (MEF1–3 and two human fibroblast strains WI-38 and NHF) were examined for mTERT and hTERT mRNA levels. (*a*) mTERT expression normalized with mGAPDH in $mTERT^{+/-}hTERT^+$ transgenic mice. (*b*) hTERT expression normalized with mGAPDH in $mTERT^{+/-}hTERT^+$ transgenic mice. In *a* and *b*, tissue RNA samples (except for testis and uterus) were pooled from one male and one female 8-week-old mouse (line BAC-C10). Testis or uterus sample was from one 8-week-old male or female mouse (line BAC-C10), respectively. Tissue RNA samples similarly pooled from the other line of $mTERT^{+/-}hTERT^+$ mice (BAC-C2) were also examined and confirmed to give similar levels of *mTERT* and *hTERT* expression to the data shown here. MEF1, MEF2, and MEF3 are three independent preparations of fibroblasts from BAC-C10 embryos. (*c*) hTERT expression normalized with hGAPDH in humans. See *Materials and Methods* for sources of human tissue RNA samples. For human liver, another RNA sample pooled from 23 individuals was also confirmed negative for hTERT expression (data not shown).

μg per well) by using FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis). The pRL-SV40 (3 ng per well; Promega) expressing *Renilla reniformis* luciferase driven by SV40 enhancer/promoter was included in each transfection as a control to normalize the transcriptional activity of *TERT* promoter fragments. Preparation of cell lysates and measurement of luciferase activity were performed by using the Dual Luciferase reporter assay system (Promega). In Fig. 2 *c–f*, the normalized activity of pGL3-Control was defined as 1.0 (not shown in the figures), and the activity of each *TERT* promoter fragment was expressed as a relative value. All of the data (mean ± SD) were from at least three independent experiments.

Results

Generation of *hTERT* BAC Transgenic Mice. After microinjecting the BAC clone containing the *hTERT* gene locus of at least 54 kb (17)
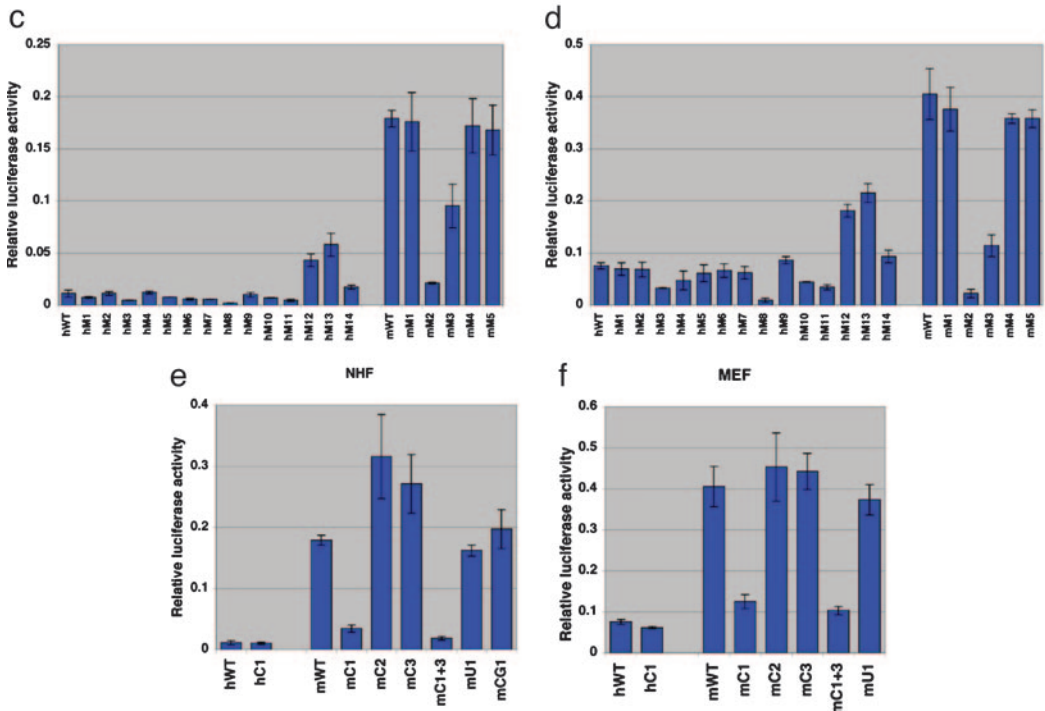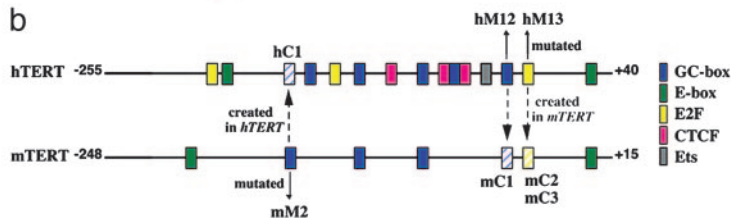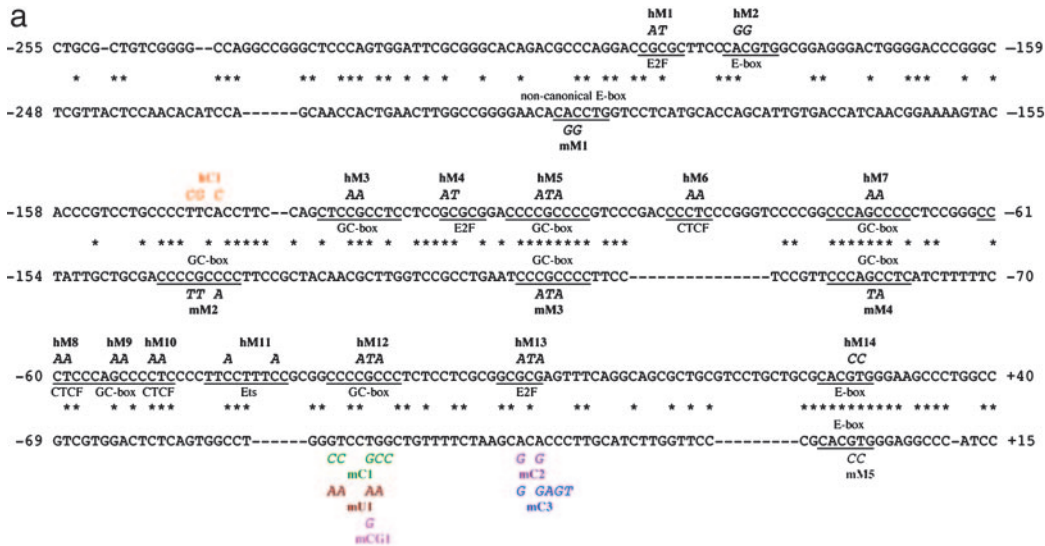
Fig. 2. Identification of a cis-element responsible for the differential transcriptional activity of *hTERT* versus *mTERT* promoter. (*a*) Sequence alignment of *hTERT* (above) and *mTERT* (below) promoters (24). Asterisks indicate identical nucleotides. Putative transcription-factor-binding sites are underlined and labeled along the sequences. Mutations destroying those sites are shown in italics (hM1–hM14 in *hTERT* and mM1–mM5 in *mTERT*). Mutations creating potential binding sites (hC1 in *hTERT* and mC1–mC3 in *mTERT*) and control mutations for mC1 (mU1 and mCG1) are shown in colored italics. A double mutant carrying both mC1 and mC3 mutations (mC1 + 3) was also made. (*b*) Schematic representation of transcription-factor-binding sites (colored rectangles) in *hTERT* and *mTERT* promoters. Also indicated are the mutations destroying (mM2, hM12, and hM13) or creating (hC1, mC1, mC2, and mC3) three candidate sites for a species-specific cis-regulatory element. The sites created in the promoter of another species are shown as hatched rectangles. (*c–f*) Luciferase reporter gene assays to examine the transcriptional activities of wild-type *hTERT* promoter (hWT) and wild-type *mTERT* promoter (mWT) and their derivatives with the mutations indicated in *a*. NHF were transfected in *c* and *e*, and MEF in *d* and *f*. In all experiments, the activity of the pGL3-Control plasmid (where SV40 enhancer/promoter drives the luciferase reporter gene) was defined as 1.0 (not shown in the figures). The activity of each promoter fragment is expressed as a relative luciferase activity (mean ± SD).

into ≈100 C57BL/6 mouse oocytes, PCR genotyping of the result-ing mice identified five founder lines containing the *hTERT* sequence. From two of them, the *hTERT* transgene was transmitted to the offspring. These offspring were found to express hTERT mRNA by RT-PCR analysis (data not shown; same primers as in Fig. 4). The hTERT-expressing offspring were bred with *mTERT*^{+/−} mice (18). Two independent lines of *mTERT*^{+/−}-*hTERT*[+] mice (BAC-C2 and BAC-C10, corresponding to the two different founder lines) were analyzed in this study. Fig. 4 shows hTERT and mTERT mRNA expression in spleen lymphocytes from wild-type, *mTERT*^{−/−}, *mTERT*^{+/−}*hTERT*[+], and *mTERT*^{−/−}-*hTERT*[+] mice. As expected, hTERT was expressed only in *hTERT*[+] transgenic mice, and mTERT was expressed only in a wild-type or *mTERT*^{+/−} background. In the *hTERT*[+] transgenic mice, PCR analyses using genomic DNA confirmed the presence of the 11-kb upstream region, all 16 exon sequences, and 1.2-kb downstream region contained in the original BAC clone, and additional RT-PCR analyses suggested the expression of full-length hTERT mRNA in spleen lymphocytes (data not shown).

Cis-Regulation Determines the Differential *in Vivo* Expression of *hTERT* and *mTERT*. To quantitatively compare the *in vivo* expression of *mTERT* and *hTERT* among various organs of *mTERT*^{+/−}-*hTERT*[+] transgenic mice, quantitative real-time RT-PCR assays of mTERT mRNA (Fig. 1*a*) and hTERT mRNA (Fig. 1*b*) were performed. For comparison, hTERT mRNA expression in human organs (Fig. 1*c*) was also examined. As expected, in the organs where both *mTERT* in mice and *hTERT* in humans are readily detected (i.e., spleen, testis, thymus, and bone marrow), the *hTERT* transgene was also expressed in the transgenic mice. In organs where little or no *mTERT* is expressed in mice and no *hTERT* in humans (i.e., skeletal muscle and heart), there was similarly little or no expression of *hTERT* in the transgenic mice. Most informative, however, was the analysis of *hTERT* transgene expression in the tissues in which endogenous *mTERT* is expressed in mice but in which *hTERT* is not expressed in humans. In liver, kidney, lung, and uterus of the *mTERT*^{+/−}*hTERT*[+] transgenic mice, endogenous *mTERT* was expressed, but the *hTERT* transgene was repressed completely or expressed much less abundantly, paralleling the pattern observed for endogenous *hTERT* in humans. Thus, the expression profiles of the *hTERT* transgene in most mouse organs followed those of *hTERT* in humans, suggesting that cis-regulation, rather than transacting factor environments, is responsible for much of the species-specific difference in *TERT* expression. There was an exception: The *hTERT* transgene in transgenic mouse brain was expressed similarly to the endogenous *mTERT*, in contrast to the repression of *hTERT* in human brain (discussed below). The difference between *hTERT* and *mTERT* expression was also observed in MEF derived from transgenic mouse embryos, in agreement with the repression of *hTERT* in normal human fibroblasts (WI-38 and NHF).

We also compared the relative abundance of mTERT and hTERT mRNA in each of the organs from *mTERT*^{+/−}*hTERT*[+] mice. For this purpose, we established an RT-PCR assay in which a single pair of primers amplified both mTERT and hTERT at similar efficiencies, and the resulting PCR products were digested with an *mTERT*- or *hTERT*-specific restriction endonuclease (see Fig. 5, which is published as supporting information on the PNAS web site). In spleen, testis, thymus, brain, and bone marrow, the expression level of *hTERT* was comparable to that of *mTERT*. In liver, kidney, lung, uterus, and MEF, no or little hTERT-derived products were detected in the presence of a large quantity of mTERT-derived products. These results are consistent with the expression profiles of *mTERT* and *hTERT* shown in Fig. 1 *a* and *b*. Thus, both the quantitative comparison of *mTERT* or *hTERT* among different organs and the relative comparison of *mTERT* versus *hTERT* in each organ suggest an organ-specific, differential cis-regulation of *mTERT* and *hTERT* genes.

A Nonconserved GC-Box Within the *hTERT* Promoter Is Critical to the Human-Specific Repression of *TERT* Transcription. To assess the basis for differential cis-regulated expression of *mTERT* and *hTERT*, we analyzed the function of mouse and human promoter sequences in a transcriptional reporter assay. When the 295-bp *hTERT* gene promoter and the corresponding 263-bp *mTERT* gene promoter (Fig. 2 *a* and *b*) were examined in a luciferase reporter gene assay, the activity of *mTERT* promoter (mWT) was 16-fold or 5.4-fold higher than that of *hTERT* promoter (hWT) in NHF or MEF cells, respectively (Fig. 2 *c–f*), suggesting that the difference between mTERT and hTERT mRNA expression is attributable largely to the differential transcriptional activity of *hTERT* and *mTERT* promoters. These findings further suggest the critical role of a cis-acting element in determining the *TERT* gene-expression profiles in mice and humans.

To identify the cis-element responsible for the differential transcriptional activity (either an *hTERT*-specific repressive element or an *mTERT*-specific activating element), a number of site-directed mutations were made within conserved or nonconserved potential regulatory elements on the 295-bp *hTERT* promoter (hM1–hM14) and 263-bp *mTERT* promoter (mM1–mM5), including GC-boxes (putative Sp1 sites) (21, 22), putative E2F-binding sites (23), canonical and noncanonical E-boxes (21, 24), putative Ets-binding sites (25), and putative binding sites for a zinc-finger repressor CTCF (26) (Fig. 2*a*). The luciferase assay using these mutant promoter constructs in NHF (Fig. 2*c*) and MEF (Fig. 2*d*) found three nonconserved sequences as candidates for the species-specific regulatory element (Fig. 2*b*): Two *hTERT* mutants, hM12 (a GC-box) and hM13 (a putative E2F site), significantly increased promoter activity and thus appeared to abrogate repression observed with the wild-type *hTERT* promoter. In addition, an *mTERT* mutant mM2 (a GC-box) showed much weaker promoter activity than the wild-type *mTERT* promoter. However, a GC-box created in the *hTERT* promoter at the corresponding position to mM2 (hC1 in Fig. 2 *a* and *b*) did not increase transcriptional activity (hWT and hC1 in Fig. 2 *e* and *f*), inconsistent with the idea that this GC-box is an *mTERT*-specific activating element. Whereas the creation of a putative E2F site in the *mTERT* promoter at the corresponding position to hM13 (mC2 and mC3 in Fig. 2 *a* and *b*) did not reduce the *mTERT* promoter activity (Fig. 2 *e* and *f*), a GC-box created at the corresponding position to hM12 by itself (mC1 in Fig. 2 *a* and *b*) or in combination with the mC3 mutation (mC1 + 3) markedly reduced the *mTERT* promoter activity, making it similar to the *hTERT* promoter activity (Fig. 2 *e* and *f*). An unrelated mutation at the same position (mU1) or the creation of a CpG site, but not a GC-box (mCG1), had no effect on the promoter activity (Fig. 2 *e* and *f*). These results identified the nonconserved GC-box located at −31 to −24 in the *hTERT* promoter as the human-specific repressive element, at least in fibroblasts in culture. Electrophoretic mobility-shift assays showed that the Sp1/Sp3 protein complex binds to this GC-box more strongly than either Sp1 or Sp3 alone (see Fig. 6, which is published as supporting information on the PNAS web site), similar to the preferential binding of the Sp1/Sp3 complex to another GC-box within the *hTERT* promoter (22). It is likely that the repressive effect of the GC-box is position-dependent, because the same GC-box sequences (CCCCGCCC), located upstream, did not act as a repressive element (see hM5 and mM2 in Fig. 2 *c* and *d*).

The GC-Box-Mediated Mechanism for *hTERT* Repression Is Impaired in Human Cancers. To examine whether the GC-box-mediated, *hTERT*-specific repressive mechanism is inactivated in human cancers, a variety of types of telomerase-expressing human cancer and immortalized cell lines, including those of fibroblastic origin (HT-1080 and CMV-Mj-HEL-1) that could be compared with a normal counterpart of the same cell type (NHF) were examined in the luciferase reporter gene assay using the wild-type *hTERT* promoter (hWT), the GC-box-mutated *hTERT* promoter (hM12),
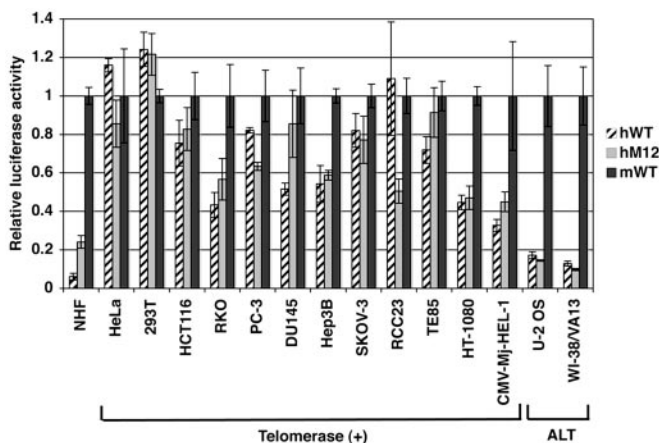
Fig. 3. The GC-box-mediated mechanism for *hTERT* repression is impaired in human cancer and immortalized cells. The transcriptional activities of the wild-type *hTERT* promoter (hWT), the GC-box-mutated *hTERT* promoter (hM12) and the wild-type *mTERT* promoter (mWT) were examined by the luciferase reporter gene assay in the following human cell lines: HeLa, uterine cervical cancer; 293T, immortalized embryonic kidney cells expressing SV40 T-antigen; HCT116, colon cancer; RKO, colon cancer; PC-3, prostate cancer; DU145, prostate cancer; Hep3B, hepatocellular carcinoma; SKOV-3, ovarian cancer; RCC23, renal cell carcinoma; TE85, osteosarcoma; HT-1080, fibrosarcoma; CMV-Mj-HEL-1, cytomegalovirus-immortalized fibroblasts; U-2 OS, osteosarcoma; and WI-38/VA13, SV40-immortalized fibroblasts. U-2 OS and WI-38/VA13 are telomerase-negative and maintain telomeres by the alternative lengthening of telomeres (ALT) mechanism. All of the other cell lines are telomerase-positive. The promoter activities in NHF are from data in Fig. 2 and are shown for comparison. For each cell line, the activities of hWT and hM12 are expressed as the relative values to the activity of mWT (defined as 1.0). Data shown are mean ± SD of triplicate assays.

and the wild-type *mTERT* promoter (mWT) (Fig. 3). As is evident in NHF, the GC-box-mediated, *hTERT*-specific repression is characterized by two features in the assay: The wild-type *hTERT* promoter is much less active than the wild-type *mTERT* promoter (hWT/mWT ratio = 0.06 in NHF), and the GC-box mutation results in a significant increase in the *hTERT* promoter activity (hM12/hWT ratio = 3.95 in NHF). In each of the 12 telomerase-expressing cell lines examined, these two features were lost or quantitatively diminished. The hWT/mWT ratio ranged from 0.33 (CMV-Mj-HEL-1) to 1.24 (293T), and the hM12/hWT ratio ranged from 0.46 (RCC23) to 1.66 (DU145). A decrease in the *hTERT* promoter activity was associated with the GC-box mutation in RCC23, PC-3, and HeLa, suggesting that the GC-box may act as a positive regulatory element rather than as a repressive element in some human cancers. Interestingly, two telomerase-negative alternative lengthening of telomeres (ALT) cell lines still showed a greater repression of hWT activity (hWT/mWT ratio = 0.17 in U-2 OS and 0.12 in WI-38/VA13) than the telomerase-expressing cancer cell lines, whereas the GC-box no longer appeared to function as a repressive element in these cells (Fig. 3). Collectively, these results suggest that human-specific repression of *TERT* transcription is frequently abrogated in telomerase-expressing human cancer cells, but not in ALT cells, and are consistent with the notion that the telomerase component genes (i.e., *hTERT* and *hTERC*) can be repressed in ALT cells by a different mechanism than that which is active in normal cells (14, 27).

Discussion

Distinct differences exist in the *in vivo* expression of telomerase activity in mice versus humans (1, 7, 8). These differences are, at least in part, because of differential regulation of the *TERT* gene (14, 16), but the basis for the regulation of this critical gene is unknown. In this study, we generated mice carrying both the mouse

and human *TERT* genes, each under the control of its own regulatory sequences, to study the regulation of these genes in the same cell context *in vivo*. Given that the regulation of the *hTERT* gene is cell-type-specific in humans (28) and that a transgene of large size (i.e., yeast artificial chromosome or BAC) tends to allow integration that is position-independent, copy-number-dependent, and tissue-specific in its expression (29, 30), we reasoned that introduction of the entire gene would be important for these studies. Therefore, we cloned and used a ≥54-kb genomic region containing all exons and introns and at least 11-kb upstream and 1.2-kb downstream sequences of the *hTERT* gene (17). Whereas the absence or instability of some human DNA sequences in conventional genomic libraries has hampered studies on functions and regulations of an entire gene locus and has left a number of gaps in the human genome sequence database, transformation-associated recombination (TAR) cloning enables the efficient, reproducible cloning and stable maintenance of large genomic regions of interest and, thus, is a powerful approach to solve these problems in genome research (31, 32). This study presents a successful application of an entire human gene isolated by TAR cloning in *in vivo* analysis of gene regulation. When we made transgenic mice carrying a BAC with the entire *hTERT* gene, we observed that the tissue-specific expression of the *hTERT* versus *mTERT* was maintained in the transgenic mice, with the possible exception of the brain (discussed below), demonstrating that the differential regulation of *TERT* was because of cis-acting elements of *mTERT* versus *hTERT* and enabled us to further dissect the basis for this difference.

Several cis-acting elements within the *hTERT* promoter have been suggested to function as repressive elements of *hTERT* transcription in normal human cells (14). In agreement with our previous identification of the proximal E-box (+22 to +27) as a repressive cis-element of the *hTERT* promoter (20), the mutation of this E-box sequence (hM14) increased the *hTERT* promoter activity in NHF (Fig. 2c). However, this E-box element is conserved in mice and humans (Fig. 2 a and b), and the effect of its mutation was much less than when a nonconserved GC-box was mutated (hM12). A nonconserved, potential E2F-binding site located at −13 to −9 in the *hTERT* promoter was a candidate for the human-specific repressive element, because its mutation hM13 abrogated the repression of *hTERT* promoter activity as efficiently as the GC-box mutation hM12 (Fig. 2 c and d), consistent with previous findings (23). However, creation of this E2F-binding site at the corresponding position in the *mTERT* promoter failed to transform its transcriptional activity into *hTERT*-like function (mC2 and mC3 in Fig. 2 e and f). Although one conserved (at −112 to −104) and two nonconserved (at −132 to −124 and at −31 to −24) GC-boxes in the *hTERT* promoter were suggested to act as repressive elements by another study (22), the nonconserved GC-box at −31 to −24 (see hM12 in Fig. 2 a–d), but not the other two (see hM3 and hM5 in Fig. 2 a–d), was found to contribute substantially to the tight repression of *hTERT* transcription in our *in vitro* reporter-gene assays. Most importantly, when this human-specific GC-box was created at the corresponding position in the *mTERT* promoter (Fig. 2 a and b), its transcriptional activity became repressed to levels similar to that of the *hTERT* promoter activity (mC1 and mC1 + 3 in Fig. 2 e and f). Based on these results, we conclude that the *hTERT*-specific GC-box is a cis-acting element that determines the differential expression of *TERT* genes in humans and mice. Further details of the GC-box- and Sp1/Sp3-mediated, *hTERT*-specific repression remain to be investigated. Such investigation would also be important for better understanding of *hTERT* activation during human carcinogenesis, because many of the human cancer cell lines studied here appeared to undergo functional inactivation of the factor or signaling pathway acting on the GC-box to repress *hTERT* (Fig. 3). Whereas the vast majority of studies of transcriptional regulation of a given gene in different mammalian species have emphasized conserved regulatory mechanisms, this study is a detailed characterization of the

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functional difference in a gene promoter of mice versus humans. It will be interesting to examine whether the chromatin status *in vivo* differs between *mTERT* and *hTERT* and, if so, whether and how the nonconserved GC-box at the *hTERT* promoter is involved in the chromatin regulation.

Ritz *et al.* (33) recently generated transgenic mice carrying a *lacZ* reporter gene driven by the 8-kb *hTERT* promoter (*hTERTp-lacZ* mice) and found that the activity of the *hTERT* promoter in the mouse tissues generally recapitulates the expression of *hTERT* in human tissues, consistent with our findings. The BAC transgenic mice generated in this study, which carried the longer promoter region, all of the exons and introns, the downstream region of the *hTERT* gene, and the quantitative analysis of mTERT and hTERT expression, rather than detection of a reporter gene, allowed us to compare *in vivo mTERT* and *hTERT* expression in more physiological settings. This study provides an important insight into species-specific features of telomere biology. It has been demonstrated that the expression level of *TERT* can affect telomere length, because a decrease in *mTERT* gene dosage (heterozygous knockout mice) led to reduction in telomere length *in vivo* (34, 35). Our findings from *hTERT* transgenic mice revealed the differential cis-regulation of the mouse and human *TERT* genes in a tissue-specific manner, suggesting a critical mechanism that determines the differential telomerase activity in many, but not all, tissues between mice and humans (8) and may also contribute to the difference in telomere-length homeostasis between the species (9). The data on the differential transcriptional activity of *mTERT* and *hTERT* promoters, attributed to an *hTERT*-specific repressive cis-element, provide an experimental basis for creating mouse models of cancer and aging that more closely simulate human physiology (1). Engineering of an endogenous *mTERT* promoter that recapitulates the properties of the *hTERT* promoter (i.e., creation of the *hTERT*-specific repressive element or replacement with the *hTERT* promoter) could achieve the generation of mice expressing mouse telomerase in a human-like manner. Alternatively, introduction of a human telomerase RNA component gene into *mTERT*$^{-/-}$ mice carrying the *hTERT* BAC (Fig. 4), which are telomerase-negative because of incompatibility between hTERT and the mouse RNA component (36) (Y.J.C., unpublished observation), could also reconstitute the human telomerase enzyme in mice.

In addition to germ line, immune system, and hematopoietic organs, some human adult organs likely contain a stem-cell-like population with a potential to express hTERT and telomerase (37). Our real-time quantitative RT-PCR assay using whole normal organs as RNA sources did not efficiently detect hTERT expression from such a stem-cell-like population in liver, skeletal muscle, kidney, heart, lung, brain, or uterus (Fig. 1*c*), thus allowing us to observe and analyze the differential *in vivo* expression of *mTERT* and *hTERT*. In combination with *in situ* detection of hTERT expression, the transgenic mice described in this study would also be an excellent model for dissecting *in vivo hTERT* regulation in adult stem-cell-like populations and the role of this regulation in human aging and carcinogenesis (38).

It is unknown why the *hTERT* transgene in mouse brain behaves like endogenous *mTERT* rather than the *hTERT* in human brain, in contrast to the findings in all other tissues analyzed. The *hTERT* promoter-driven *lacZ* reporter gene was also expressed in brain in an *hTERTp-lacZ* mouse (33). A brain-specific transacting factor(s) may exist to regulate *TERT* transcription. Alternatively, the brain-specific expression pattern may reflect different subpopulations of TERT-expressing cells. For example, a unique structure exists in the subventricular zone in the adult human brain (39), where telomerase-expressing neural stem cells are enriched (40). Finally, although this study identified the species-specific regulation of *TERT* expression as a candidate for the determinant of the difference in telomere homeostasis between mice and humans, the structural or functional divergence of mouse and human telomere-binding proteins (41) may also contribute to the species-specific telomere biology. Possible differences in the regulation and function of TERT proteins also deserve investigation. Despite these issues still to be addressed, this study provides important, mechanistic insight into the species-specific and tissue-specific regulation of telomerase and telomeres.

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