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Identification of a Novel Transcription Factor Binding Element Involved in the Regulation by Differentiation of the Human Telomerase (hTERT) Promoter

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Three different cell differentiation experimental model systems (human embryonic stem cells, mouse F9 cells, and human HL-60 promyelocytic cells) were used to determine the relationship between the reduction in telomerase activity after differentiation and the regulation of the promoter for the hTERT gene. Promoter constructs of three different lengths were subcloned into the PGL3-basic luciferase reporter vector. In all three experimental systems, all three promoter constructs drove high levels of reporter activity in the nondifferentiated state, with a marked and time-dependent reduction after the induction of differentiation. In all cases, the smallest core promoter construct (283 nt upstream of the ATG) gave the highest activity. Electrophoretic mobility shift assays revealed transcription factor binding to two E-box domains within the core promoter. There was also a marked time-dependent reduction in this binding with differentiation. In addition, a distinct and novel element was identified within the core promoter, which also underwent time-dependent reduction in transcription factor binding with differentiation. Site-directed mutagenesis of this novel element revealed a correlation between transcription factor binding and promoter activity. Taken together, the results indicate that regulation of overall telomerase activity with differentiation is mediated at least in part at the level of the TERT promoter and provides new information regarding details of the regulatory interactions that are involved in this process.

INTRODUCTION

Eukaryotic telomeric DNA is composed of repetitive stretches of guanine-rich sequences (e.g., TTAGGG in mammalian cells), which, together with an associated set of proteins, form a DNA–protein complex that is thought to maintain the integrity of chromosome ends (Greider, 1991; Levy *et al.*, 1992; Blackburn *et al.*, 1997). The shortening of telomere ends, with progressive rounds of cell division, has been proposed to serve as a mitotic clock by which cell divisions are counted, eventuating in a state of replicative arrest that is known as cellular senescence (Harley *et al.*, 1990; Harley, 1991; Allsopp *et al.*,1992). Therefore, mechanisms for the maintenance and restoration of functional telomeres are essential in those situations, in which the requirements for DNA replication exceed the ability to maintain a telomere length that is consistent with chromosome stability. Thus, for example, all eukaryotic species must possess at least a germ-line mechanism to overcome progressive telomere shortening. The best characterized of such mechanisms involves telomerase and associated telomeric proteins. Telomerase is a ribonucleoprotein enzyme with reverse transcriptase activity that is capable of extending chromosome ends with specific telomeric DNA sequences by using a portion of its RNA component as a template (Kim *et al.*, 1994; Autexier and Greider, 1996; Lundblad and Wright, 1996a). Telomerase activity is readily detected in male and female germ-line cells, ensuring the maintenance of integrity through successive generations. The activation of telomerase also has been implicated as the major mechanism for attainment of cellular immortalization in the molecular pathogenesis of most, but not all, malignant tumors. In contrast, most normal adult human somatic cells lack significant telomerase activity. Even in rapidly proliferating normal (nontransformed) tissues, only low levels of telomerase activity are detected postnatally, representing the contribution of the small subset of stem cells that replenish the rapidly proliferating population (Vaziri *et al.*, 1994). However, as a notable exception,

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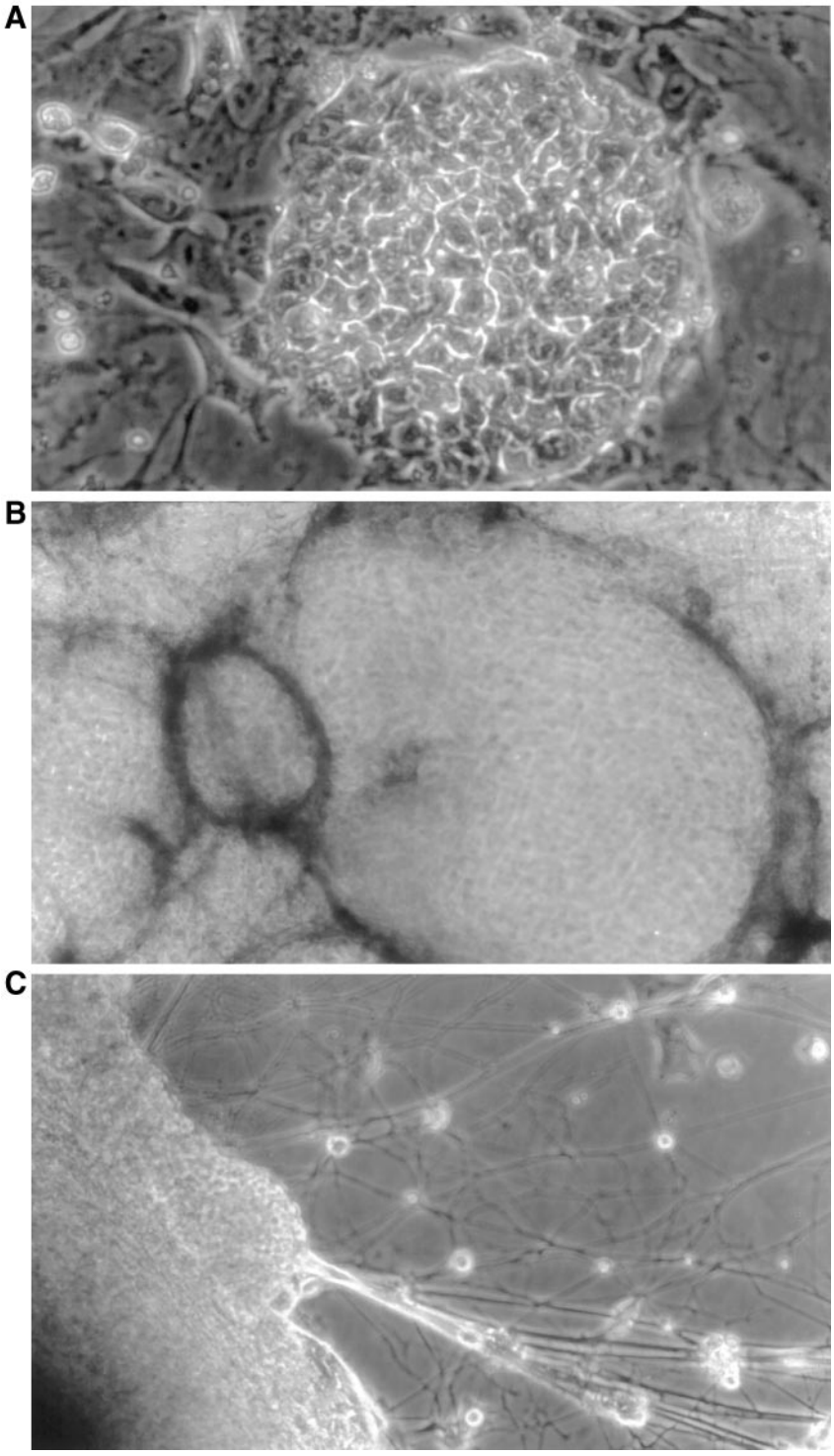

Figure 1. Differentiation of hES, F9, and HL-60 cells. (A) Pluripotent undifferentiated hES cells grown on an MEF feeder layer. (B) Differentiated hES cells cultured for 12 d in the absence of MEFs and neuron-like cells 25 d after differentiation (C) (Bar = 36 μm). F9 cells (D) (Bar = 100 μm) were grown in the presence of 1 μM RA for 7 d for induction of differentiation (E) (Bar = 25 μm), and F9 30-d EBs (F) (Bar = 250 μm). Differentiation of HL-60 cells after exposure to 10% DMSO assessed by monitoring of the cell surface CD11B integrin expression profile by fluorescence-activated cell sorter analysis (G). (H) Differentiation of hES cells is associated with the down-regulation of telomerase activity. TRAP assay was performed using extracts from hES cells cultured on MEF cells (day 1) and from hES cells that were allowed to differentiate for 7,9, 11, and 14 days. As controls, TRAP activity was measured in extracts of MEF, buffer control (bc), and of telomerase-positive cells supplied in the kit (pc). In preliminary experiments, telomerase activity was measured using increasing amount of extracts (1, 5, 10, and 50 μg) to ensure that the assay was performed in the linear range. The results shown were obtained using 5 μg of each extract without and with 15 min at 85°C heat inactivation. A 36-bp internal control for amplification efficiency and quantitative analysis was run for each reaction, as indicated by the arrowhead. The reaction products were separated on 10% nondenaturing polyacrylamide gel.

telomerase is active in human lymphocytes during development, differentiation, activation, and aging (Weng *et al.*, 1997; Liu *et al.*, 1999).

Although the roles of telomere shortening and telomerase have been extensively investigated with respect to the process of aging, cellular senescence, and neoplastic transformation, there has been a relative paucity of information with respect to its role during embryonic and fetal development, as well as during cellular differentiation. The expression of telomerase activity has been detected at the earliest gesta-

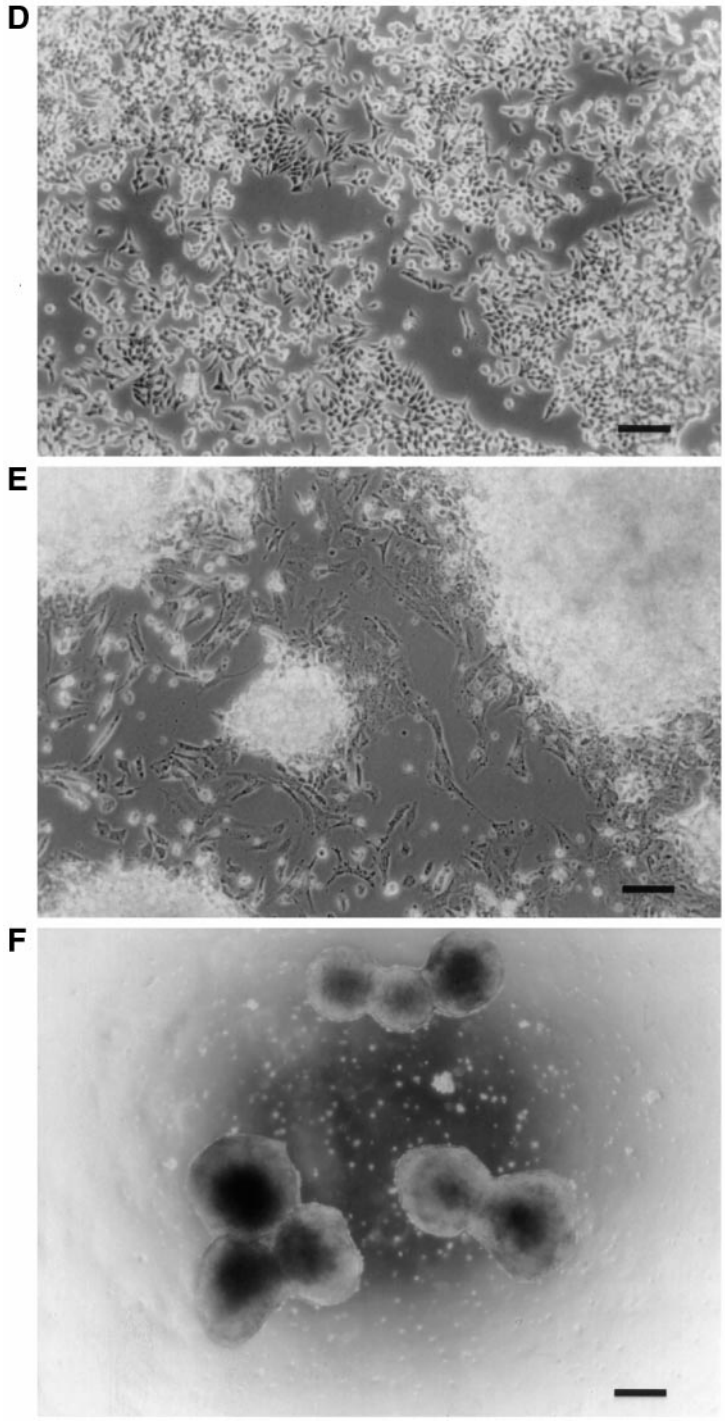

Figure 1 (caption on facing page).

tional stages during human embryonic development, with variable down-regulation of telomerase activity during subsequent fetal development (Wright *et al.*, 1996; Ulaner and Giudice, 1997; Youngren *et al.*, 1998). A similar pattern of suppression of telomerase activity also has been reported after the induction of differentiation in cultured cell lines (Sharma *et al.*, 1995; Holt *et al.*, 1996, 1997; Horikawa *et al.*, 1998; Tanaka *et al.*, 1998).

The protein (TERT) and mRNA (TER) components of the telomerase ribonucleoprotein are each encoded at a separate genetic locus and are under independent regulatory control. Studies comparing TER and TERT message levels with te-

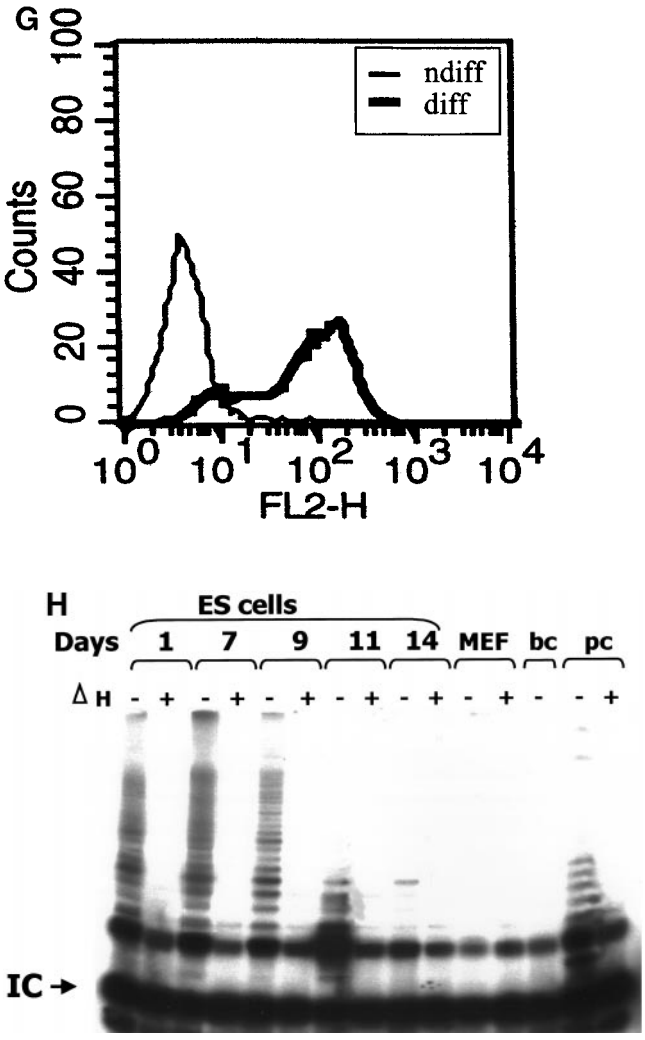

Figure 1. (cont)

lomerase activity, as well as ectopic TERT expression, strongly suggest that the overall regulation of telomerase activity is achieved, at least in part, through rate-limiting mechanisms governing the expression of the human TERT (hTERT) gene (Nakayama *et al.*, 1998; Greider, 1999). The 5′-flanking region of the hTERT gene has been characterized in part using sequence analysis, transient transfection of promoter–reporter constructs, as well as electrophoretic mobility shift and DNAse footprint analysis (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). These studies have identified a core promoter extending from −283 nt upstream of the transcription initiation site to 78 nt of the first exon, containing several putative transcription factor-binding elements. The finding of multiple putative c-Myc binding elements was considered to be of particular interest, in view of the role of c-Myc in cell proliferation and transformation (Greenberg *et al.*, 1999; Kyo *et al.*, 2000; Oh *et al.*, 1999; Wu *et al.*, 1999). Transient transfection of a variety of hTERT promoter–reporter constructs in different cell types has shown a correlation of promoter activity with endogenous hTERT gene expression and telomerase activity. These findings lend further support to the contention that transcriptional regulation of hTERT gene expression may serve as a rate-limiting step for overall telomerase activity.

In the current study, we use three different cell differentiation experimental model systems (human embryonic stem [hES] cells, mouse F9 cells in culture, and human HL-60 promyelocytic cells in suspension culture) to determine whether the reduction in telomerase activity reported to occur with the induction of differentiation (Sharma *et al.*, 1995; Holt *et al.*, 1996; Holt *et al.*, 1997; Horikawa *et al.*, 1998; Tanaka *et al.*, 1998) can be attributed to a decrease in TERT promoter activity. In conducting these studies, we identified a novel motif within the core-promoter sequences that specifically binds protein from nuclear extracts of undifferentiated telomerase positive, but not from the corresponding telomerase negative cells, after the induction of differentiation.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The H9 cell line of hES cells (Thomson *et al.*, 1998) were maintained in the undifferentiated state by propagation in culture on a feeder layer of irradiated primary mouse embryonic fibroblasts (MEFs). MEFs were prepared according to the method of Robertson (1987) and were plated on gelatin-coated, six-well plates. hES cells were grown in knockout DMEM supplemented with 20% serum replacement, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol (GIBCO-BRL, Grand Island, NY), 1 mM glutamine (Biological Industries, Ashrat, Israel), and 4 ng/ml human recombinant bFGF (PeproTech Inc., Rocky Hill, NJ). Cultures were grown in 5% CO_2 at 95% humidity and were subcultured every 4–5 days after treatment with 0.1% collagenase type IV (GIBCO-BRL). F9 murine teratocarcinoma and HEK293 human embryonic kidney cell lines were grown in DMEM containing 2 mM glutamine, 50U/ml penicillin, 50 μg of streptomycin, and 10% fetal calf serum (Biological Industries). HL-60 human promyelocytic cells in suspension culture were grown in RPMI supplemented with 10% fetal calf serum (Biological Industries).

Induction of Differentiation

Undifferentiated hES cells spontaneously differentiate when passaged in the absence of MEFs and formed multicellular aggregates, from which emerged multiple identifiable differentiated cell types. Alternatively, undifferentiated hES cells were disaggregated and were transferred in suspension into bacterial grade Petri dishes (Greiner, Frickenhausen, Germany) for the induction of synchronous differentiation that results in the acquisition of a configuration of simple embryoid bodies (EBs). F9 cells were induced to differentiate by adding 1 μM retinoic acid (RA) to the medium for 7 d. After 7 d, cells were collected and transferred in suspension into bacterial grade Petri dishes to form EBs. Differentiation was assessed by the microscopic observation of changes in cellular morphology. HL-60 cells were induced to differentiate by exposure to 1.3% DMSO (Sigma Chemical, St. Louis, MO) for 7 d. Differentiation was assessed by monitoring the CD11b (M01) integrin expression profile by fluorescence-activated cell sorter analysis using CD11b (Leu™-15) monoclonal antibody (Becton Dickinson, Franklin Lakes, NJ).

Telomerase Repeat Amplification Protocol Assay

Telomerase activity was measured by a modified telomerase repeat amplification protocol (TRAP) using the TRAPeze telomerase detection kit (Oncor, Gaithersburg, MD) (Kim *et al.*, 1994). Total cel-

lular extracts were prepared from undifferentiated and differentiated cells according to the manufacturer's instructions.

Recombinant Plasmids

hTERT Promoter–Luciferase Reporter Constructs. A human genomic library (Clontech, Palo Alto, CA) in EMBL3 (7.5×10^6 phages) was screened using a labeled 450-bp fragment corresponding to the 5′ hTERT cDNA as a probe. The sequence of the 5.8-kb promoter fragment was subjected to computer analysis (Wisconsin Package, version 8.0, Genetics Computer Group, Madison, WI). Three 5′ flanking fragments, 5.8-kb, 3.3-kb, and 283-bp fragments, upstream of the ATG were generated using restriction enzymes, were filled in, and were ligated into the *Sma*I site of pGL3-basic reporter plasmid (Promega, Madison, WI).

Mutated Core Promoter–Luciferase Reporter Constructs

Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, La Jolla, CA) to introduce mutations into the E-box and the MT-box motifs of the hTERT core promoter–luciferase construct (a 283-bp promoter fragment). The mutated oligonucleotide primers that were used for disruption of the proximal E-box and the MT-box motifs were as follows: 5′GTCCTGCTGCG*AT*CGTGGGAA GCCCTGGC3′ for M1 (mutant1), 5′GTCCTGCTGCGCACG*AT*GGAAGCC CTGGC3′ for M3, 5′GTCCTGCTGCGCACGTGGCAAGCCCTGGC3′ for M4.

Transient Transfections and Reporter Assays

HEK293 cells (6×10^4), F9 cells (2.5×10^4), and HNF cells (5×10^4) were plated 24 h before transfection in 24-well plates in the appropriate medium. Cotransfections were performed using FuGENE™6 transfection reagent (Boehringer Mannheim, Mannheim, Germany) with a 0.07-μg promoter–luciferase reporter construct and a 0.14-μg pCMV–β-galactosidase expression vector for calibration of transfection efficiencies. Transfection mixtures were adjusted with pBluescript DNA to a total of 0.3 μg per well. At 24 h after transfection, the medium was changed and the cells were incubated for an additional 48 h. Cell extracts were prepared, and luciferase or β-galactosidase activities were measured.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from the various cell lines as previously described (Schreiber *et al.*, 1989). Ten micrograms of protein were incubated with 300 ng of poly(dI-dC) for 10 min at 4°C in a 20-μl reaction volume containing 25 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM phenylmethyl sulfonyl fluoride, 1 mM dithiothreitol, and 10% glycerol with or without a 200-fold molar excess of unlabeled competitor DNA. After incubation, 1×10^5 cpm of the ^{32}P-labeled probe was added, and the reaction was incubated for an additional 20 min at 4°C. The DNA–protein complexes were separated by electrophoresis on a 5.3% polyacrylamide gel and were visualized by autoradiography. For supershift assays, samples were preincubated with 5 μg of antiserum against c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. The sequences of the oligonucleotides used as probes were: 5′GGACCGCGCTCCC*ACGTG*GCGGAGGGACTGGG3′ (−177 to −145) for the distal E-box designated as oligo-1, and 5′GTCCTGCTGCG*CA CGTG*GGAAGCCCTGGC3′ (32–61 nt of exon 1) for the proximal E-box designated as oligo-2.

RESULTS

Differentiation in Three Cell Culture Model Systems Is Associated with Suppression of Telomerase Activity

To examine the mechanisms for suppression of telomerase activity with tissue differentiation, three cell culture model systems for differentiation were utilized: hES cells, mouse embryonal carcinoma F9 cells, and HL-60 human promyelocytic leukemia cells. hES cells are defined as being derived from pluripotent cells of the early mammalian embryo, with the capacity for unlimited proliferation in vitro in the undifferentiated state when grown on a feeder layer of MEFs (Figure 1A). These cells maintain the ability to differentiate after propagation in the absence of the feeder layer and, as a result, display the outgrowth of cells deriving from each of the major developmental lineages (Figure 1, B and C). hES cells also can be induced to form EBs when grown on bacterial-grade Petri dishes. F9 teratocarcinoma cells can be induced to differentiate when incubated in the presence of 1 μM retinoic acid for 7–12 d, and the differentiation can be visualized by microscopic changes in cellular morphology with the subsequent formation of EBs in bacterial-grade Petri dishes (Figure 1, D–F). HL-60 cells can be induced to differentiate to mature granulocyte-like cells that cease proliferating and express the CD11b antigen after exposure to 1.3% DMSO for 7 d (Figure 1G). We measured telomerase activity before and at varying time points after the induction of differentiation in these three model systems. As shown in Figure 1H, pluripotent hES cells are strongly telomerase positive. The induction of differentiation by growth in the absence of MEFs is associated with a striking and time-dependent decline in telomerase activity, with the disappearance of activity by day 14 of differentiation. As shown, the MEF feeder layer cells are telomerase negative. As has been previously reported (Sharma *et al.*, 1995), we also obtained a similar pattern of results before and after differentiation in both F9 and HL-60 cells (our unpublished results). Therefore, we sought to determine in each of these model systems whether differentiation is also accompanied by inhibition of the hTERT promoter.

Isolation and Characterization of the hTERT Promoter

A human genomic library (7.5×10^6 phages) was screened using a 450-bp hTERT cDNA fragment as a probe. Three positive clones were selected and characterized using restriction enzymes and Southern blot analyses that yielded a 5.8-kb 5′-flanking genomic fragment upstream of the hTERT translation initiation site. Restriction enzyme and DNA sequence analysis yielded results identical to those that have been recently reported for the hTERT promoter (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999). Three subfragments of varying sizes were subcloned in the pGL3-basic vector upstream of the luciferase reporter gene: 5790 nt (full-length fragment), 3345 nt (intermediate fragment), and 283 nt (core promoter) upstream of the ATG translation initiation site (Figure 2A). The ability of these hTERT 5′-flanking genomic fragments to drive transcription was examined using transient transfection in several telomerase-

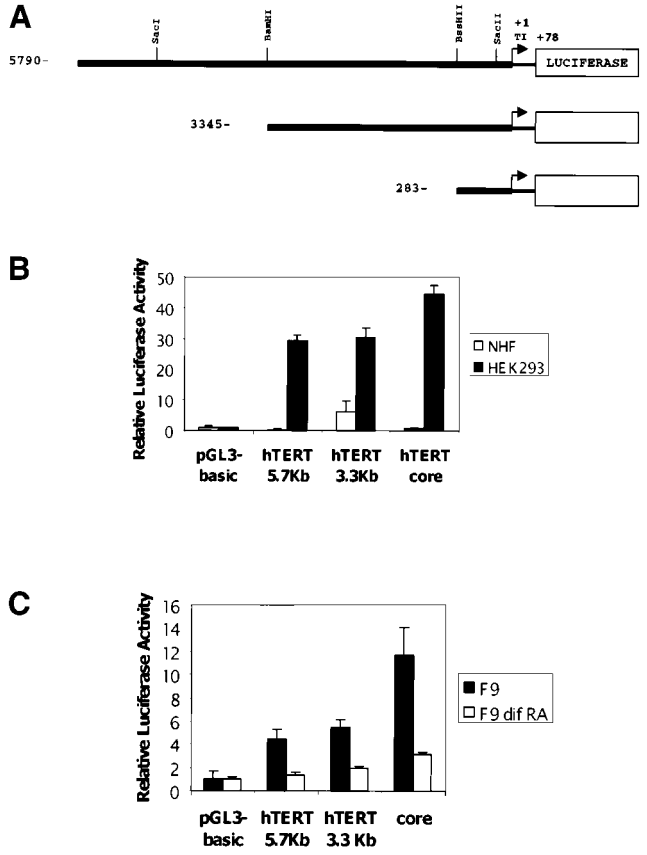

Figure 2. Transcription activation of hTERT promoter. Promoter fragments of decreasing size from the 5′ end (5790 bp, 3345 bp, and core promoter of 283 bp) to the ATG (78), were inserted into a pGL3-basic vector in front of luciferase reporter gene (A). hTERT promoter constructs were transiently cotransfected together with the β-galactosidase expression vector (CMV-β-gal) in HEK293 and NHF cells (B) and in F9 cells before and after differentiation (C). Cell extracts were assayed for luciferase activity, which was normalized for transfection efficiency by the corresponding β-galactosidase value. Data represent normalized relative luciferase fold activity compared with the promoterless pGL3 basic plasmid. Results are means ± SE of three independent experiments, each performed in quadruplicate.

negative and telomerase-positive cell lines. The results for telomerase-negative normal human fibroblasts and telomerase-positive HEK 293 cells are shown in Figure 2B. Using the PGL3-basic vector as a control, all three promoter constructs drove high levels of luciferase reporter activity in telomerase-positive HEK 293 cells compared with telomerase-negative fibroblasts. Of note, the highest level of promoter activity was consistently observed using the core promoter fragment (46-fold) compared with the control level. Relatively lower levels of activity for the two larger fragments suggest the possibility of inhibitory elements in the further 5′-flanking sequence contained in these larger elements compared with the core promoter. The core promoter also yielded the highest activity in several telomerase-positive malignant cell lines, as previously had been reported (Horikawa *et al.*, 1999; Wick *et al.*, 1999), with lower levels of activity evident using the larger full-length and intermediate-length fragments.

We next examined the effect of differentiation on activity of the three promoter fragments using transient transfection of F9 cells. Differentiation was induced by the addition of 1 μM retinoic acid for 7–12 d, as outlined in the previous section. As shown in Figure 2C, in undifferentiated F9 cells, the highest levels of promoter activity were obtained using the core promoter construct, and differentiation was associated with a marked inhibition of promoter activity, which is evident using all three promoter constructs and suggests that inhibition of telomerase activity during differentiation is mediated at least in part at the transcriptional level.

The Effect of Differentiation on Transcription Factor Binding

The sequence of the core promoter fragment was analyzed for the identification of putative transcription factor-binding elements. Two E-boxes with palindromic core sequences CACGTG, were identified at −177 nt (distal E-box) and at 32 nt (proximal E-box) (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). Oligonucleotides corresponding to these E-boxes were synthesized, labeled, and used in electrophoretic mobility shift assays (EMSAs), using nuclear extracts from cell lines in the undifferentiated telomerase-positive state and the corresponding cell lines, after the induction of differentiation, in the telomerase-negative state.

The oligonucleotide used for the distal E-box, designated as oligo-1, corresponded to the promoter sequence from −177 to −145, as follows: 5′GGACCGCGCTCCCC*ACGTG*GCGGAGGGACTGGG3′. The oligonucleotide for the proximal E-box, designated as oligo-2, corresponded to the promoter sequence from 32 nt to 61 nt, with the following sequence: 5′GTCCTGCTGCG*CACGTG*GGAAGCCCTGGC3′.

Figure 3A, shows the effect of differentiation on transcription factor binding to the distal E-box using oligo-1. A clear band shift was observed using nuclear extracts from telomerase-positive, undifferentiated F9, hES, and HL-60 cell lines. This band shift was markedly reduced using nuclear extracts from the corresponding telomerase-negative differentiated cell lines. Specificity of binding was confirmed using competition with excess cold oligonucleotide. No supershift was observed using antibodies to c-Myc.

We next examined the proximal E-box, using the corresponding labeled oligo-2 in the EMSA. In this case, two distinct shifted bands were noted. The upper band appears similar to the band shift noted using oligo-1, containing the distal E-box. However, in addition, there appeared another distinct, faster migrating band in all three cell lines in the undifferentiated state (Figure 3B). In all three cell lines, the induction of differentiation was associated with a marked reduction in the intensity of this band as well. Furthermore, in F9 cells, accompanying the reduction in intensity there appeared an additional faster migrating band that could potentially represent a degradative or covalently modified product derived from the binding protein(s).

Specificity of Binding and Mapping of Novel Transcription Factor Binding Recognition Sequence

Two approaches were used to determine whether the faster migrating gel-shift band represented binding to a novel

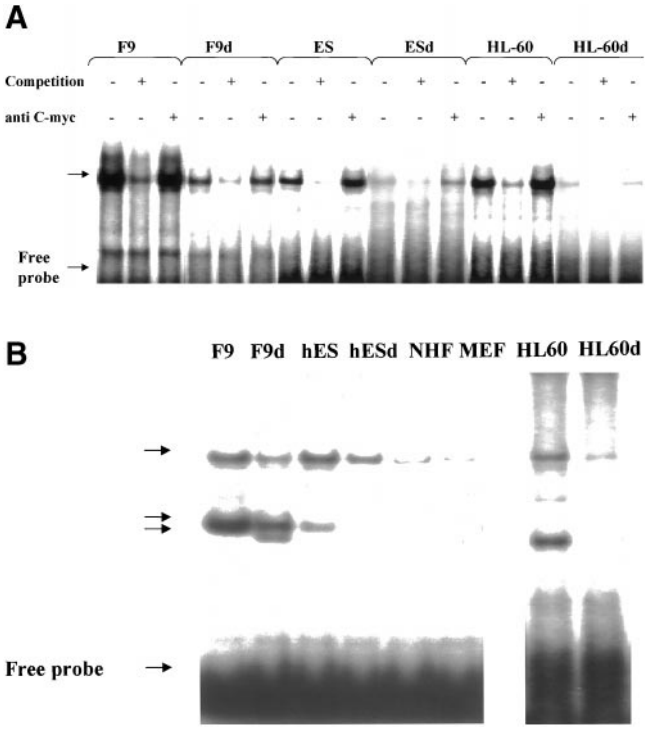

Figure 3. Identification of transcription factor binding to the E-box motifs of the proximal hTERT promoter. Nuclear extracts from F9 cells, hES cells, and HL-60 cells, before and after differentiation, were incubated with ^{32}P-labeled oligonucleotide corresponding to position −177 to −146 (oligo-1) (A) or with ^{32}P-labeled oligonucleotide corresponding to position 32–58 (oligo-2) (B) of the hTERT promoter that includes the E-box motif. Incubation was performed without or with competitor (150-fold excess of cold oligonucleotide) or with antibodies, as indicated above each lane. As a control, nuclear extracts from NHF and MEF cells were used. Arrowheads indicate specific retarded bands.

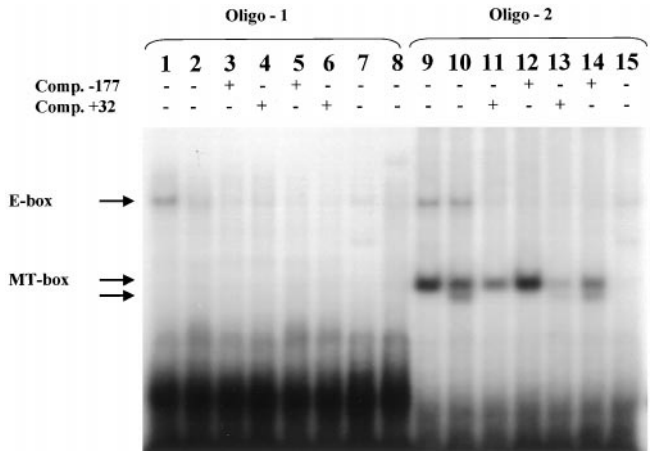

Figure 4. The effect of differentiation on transcription factor binding. Nuclear extracts from undifferentiated F9 cells (lanes 1, 3, 4, 9, 11, and 12) and from F9 cells after the induction of differentiation (lanes 2, 5, 6, 10, 13, and 14) were incubated with ^{32}P-labeled oligonucleotide corresponding to position −177 to −146 (oligo-1) or with ^{32}P-labeled oligonucleotide corresponding to position 32–58 (oligo-2) of the hTERT promoter. Incubation was performed with or without a competitor (150-fold excess of cold oligonucleotide) as indicated. As a control, a nuclear extract from telomerase-negative NHF cells was used (lanes 7 and 15). In addition, c-Myc in vitro transcription–translation product was used to examine binding to the oligo-1 E-box motif (lane 8). Arrowheads indicate specific retarded bands.

element within the oligo-2 sequence containing the proximal E-box. First, we performed binding competition experiments using undifferentiated F9 nuclear extracts and labeled proximal oligo-2, in which either excess unlabeled oligo-1 or excess unlabeled oligo-2 (containing the distal or proximal E-box consensus motifs, respectively) were added to the reaction mix. As shown in Figure 4, either excess unlabeled oligo-1 or oligo-2 reduced the upper gel-shift band. In contrast, only oligo-2 competed with itself in binding to the faster migrating transcription factor(s).

These results suggest that the upper band represents binding of the same transcription factor(s) to an element in common between the proximal and distal sequences, most likely the canonical CACGTG E-box sequence itself. In contrast, the lower gel-shift band, appears to represent recognition of an element not shared between the proximal and distal oligonucleotide sequences. Therefore, we used site-directed mutagenesis to map the distinct binding element in oligo-2 responsible for the faster migrating EMSA band. As shown in Figure 5A, a series of oligonucleotides was generated in which individual nucleotide pairs were systematically replaced. Each of the mutated oligonucleotides was used as an unlabeled competitor in EMSA reactions using undifferentiated F9 nuclear extracts with labeled wild-type oligo-2. As shown in Figure 5B, the mutations in mutants M1, M2, and M3 involve the E-box motif itself (CACGTG). These unlabeled mutant oligonucleotides failed to compete with binding to labeled oligo-2. Unlabeled oligonucleotides containing mutations outside of the known E-box motif (mutant oligonucleotides M4–M8) did compete with the upper gel-shift band. These findings confirm that the upper gel-shift band arises from specific transcription factor recognition of the CACGTG sequence corresponding to the E-box. In contrast, unlabeled mutant oligonucleotide M1, but not M2 or M3, competed with binding responsible for the lower gel-shift band, indicating that the two nucleotides in the center of the E-box (CG) represent the 5′-limit of a distinct binding element. Mutant oligonucleotides M4 and M5 failed to compete with binding at the lower gel-shift band, indicating that the two nucleotides AG represent the 3′-limit of the novel binding element. Mutant oligonucleotides M6–M8, downstream of the novel binding element, competed with binding to both the E-box and the novel elements. This analysis, suggests that the hTERT promoter sequence corresponding to oligo-2 includes two overlapping binding sites, as indicated in Figure 5A; namely, an E-box with sequence CACGTG and an overlapping but distinct binding element with sequence CGTGGGAAG. These results also were confirmed using mutant oligonucleotides as the labeled probes (our unpublished results). Since this sequence has not been previously described as a transcription factor-binding element, it was given a tentative new designation (MT-box). To further determine the significance of this novel binding mo-

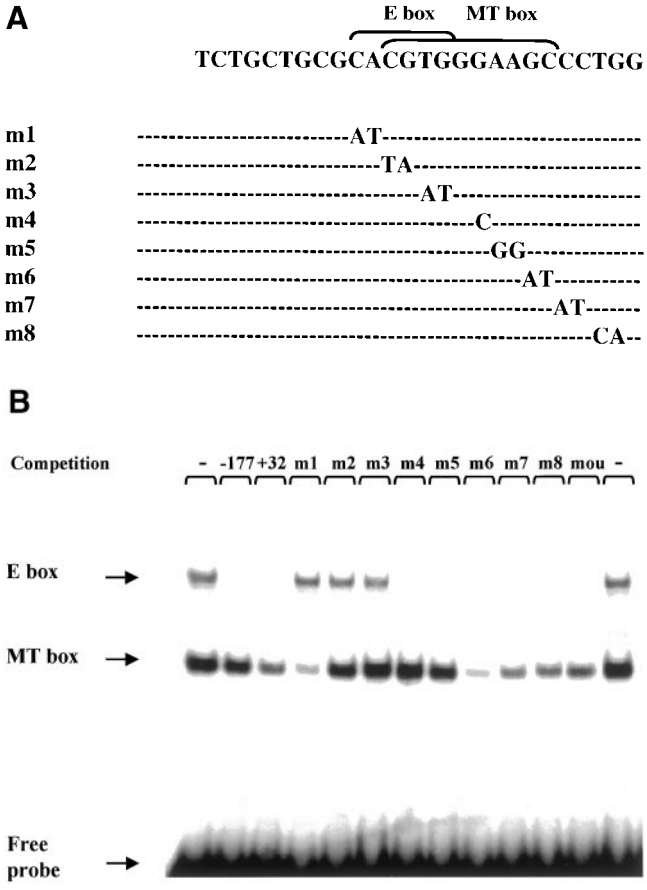

Figure 5. Mapping of novel transcription factor binding element. (A) Sequences of mutated oligonucleotide used for competition in EMSA analysis for mapping the transcription factor binding sites included in oligo-2. (B) F9 nuclear extracts were incubated with ^{32}P-labeled oligonucleotide without or with a 50-fold excess of unlabeled oligo-1 (−177), oligo-2 (32), or the mutated sequences M1–M8 indicated in the figure, or the homologous mouse oligo-2. Arrowheads indicate specific retarded bands.

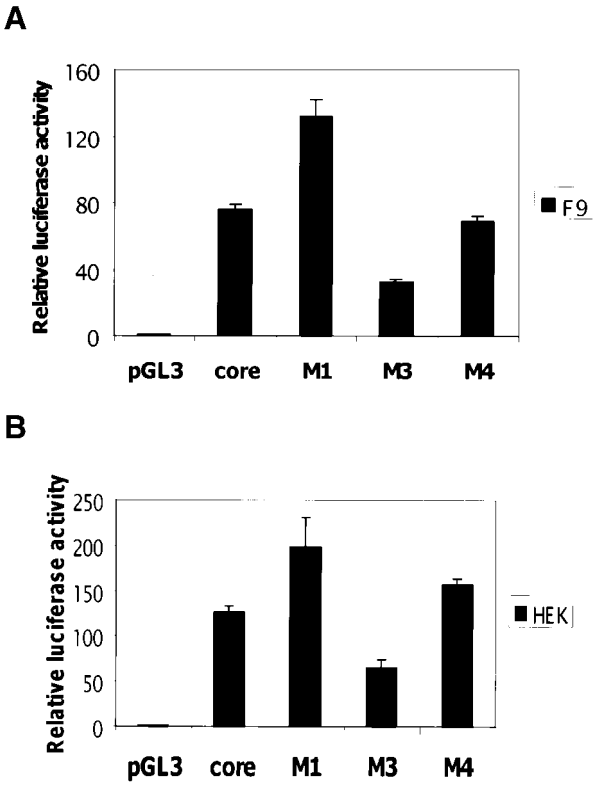

Figure 6. Transcription activation of wild-type and mutated hTERT core promoter. F9 and HEK cells were transiently cotransfected with pGL-3 construct containing as insert either the wild-type hTERT core promoter or the mutant core promoter sequences M1, M3, or M4, as indicated. Cells were cotransfected with the β-galactosidase expression vector (CMV-β-gal) for calibration of transfection efficiency. Cell extracts were assayed for luciferase activity, which was normalized by the corresponding β-galactosidase value. Data represent normalized relative luciferase fold activity compared with the promoterless pGL3 basic plasmid. Results are means ± SE of two independent experiments, each performed in quadruplicate.

tif, we compared the corresponding sequence upstream of the ATG translation initiation site in the mouse TERT gene. We found a homologous sequence containing the identical proximal E-box and a single nucleotide substitution within the homologous MT-box (CGTGGGAGG). To examine whether this A-to-G transition interfered with MT-box binding, we conducted an EMSA using a 32-nt oligonucleotide carrying this mutation as a cold competitor. As shown in Figure 5B, there was no interference with competition at either gel-shift band. In addition, a labeled probe corresponding to the mouse homologous MT-box sequence gave the identical EMSA results as shown for the labeled oligonucleotide corresponding to the human sequence (our unpublished results).

Role of MT-Box in Promoter Activity

The functional significance of the MT binding site was evaluated using transient transfection assays in F9 cells with corresponding mutations in the core promoter. As shown in Figure 6A, the M1 mutation, which interferes with transcription factor binding to the proximal E-box, results in enhanced promoter activity (173% compared with the core promoter activity), possibly reflecting augmented binding at the MT element on interference with binding to the overlapping E-box. Indeed, mutation M3, which abrogates binding to both elements, yields the lowest level of promoter activity (42% of core promoter activity). Residual promoter activity with the M3 mutation could represent activity emanating from the distal E-box at −177 bp. The M4 mutation, which eliminates binding only at the MT element, yields promoter activity similar to the wild-type core promoter (89%). Taken together, these results indicate an interaction between the binding of the two overlapping elements and suggest that the functional role of binding to the MT element is evident in the context of E-box binding and activity (M1 versus M3). A similar pattern was observed for HEK293 cells (Figure 6B).

DISCUSSION

A number of studies have been conducted, in which the hTERT 5′-flanking region has been analyzed for promoter activity (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). These studies have been conducted in telomerase-positive cancer cell lines and have identified a core 5′-flanking region, which yields greatest promoter activity, and which contains a number of binding elements that contribute to promoter activity. The greatest attention has been paid to E-box elements, and in particular to the E-box at −177 nt upstream of the translation initiation site (Greenberg *et al.*, 1999; Oh *et al.*, 1999; Wu *et al.*, 1999; Kyo *et al.*, 2000). Transcription factor binding to this element has been demonstrated previously in telomerase-positive immortal cell lines and cancer cell lines, and conflicting results have been obtained with respect to the role of c-Myc binding to this site.

In the current study, we have chosen to focus our attention on the behavior and role of the TERT promoter in differentiation, using three different cell culture model systems. This is of particular interest since, as first reported by Sharma *et al.* (1995), differentiation is associated with a marked reduction in telomerase activity in at least two different cell lines. Subsequent studies have confirmed these results using the TRAP assay to monitor telomerase activity, but they did not determine whether this reduction could be attributed to inhibition of the promoter (Holt *et al.*, 1996, 1997; Horikawa *et al.*, 1998; Tanaka *et al.*, 1998). Therefore, in the current study, we confirm this finding, extend it to hES cell differentiation, and show that this reduction is associated pari passu with a corresponding marked reduction in promoter activity. In the case of HL-60 cells, differentiation is associated with a loss of proliferative capacity. Since it can be argued that the differentiation of HL-60 cells simply represents the induction of a postmitotic state, we also examined the differentiation of F9 cells and hES cells. Differentiated F9 cells and hES cells continue to proliferate as multiple lineages after differentiation. In both of these cell lines, differentiation is nevertheless associated with a reduction in both telomerase enzyme and TERT promoter activities. These findings indicate that inhibition at the level of the TERT promoter contributes, at least in part, to the overall loss of telomerase activity with differentiation, although additional post-transcriptional mechanisms and mechanisms acting on other components of the telomerase complex could certainly also be involved.

In view of this close association between differentiation in three different model systems, and between the inhibition of the TERT promoter and the loss of telomerase activity, it will be of interest to determine the effect of exogenous hTERT overexpression on the differentiation of undifferentiated and pluripotent stem cells. Such experiments could elucidate whether inhibition of TERT is necessary for differentiation. The inhibition of the hTERT promoter and the loss of telomerase activity with the differentiation of hES cells could also have important practical implications with respect to the development from stem cells of differentiated cell lines for therapeutic tissue-engineering purposes. In particular, it will be important to know whether the introduction of exogenous hTERT in stem cells before differentiation, for purposes of immortalization, will interfere with the subsequent derivation of differentiated cell lines. In previous studies, it has been demonstrated that the immortalization of already differentiated cells using ectopic hTERT expression did not interfere with their differentiated functional phenotype (Thomas *et al.*, 2000).

These results are also of potential relevance to understanding the inhibition of telomerase activity that accompanies cell differentiation during fetal development and organogenesis. Telomerase activity has been detected in fetal, neonatal, and adult gonadal tissues, but not in mature spermatozoa or oocytes (Ulaner and Giudice, 1997). Telomerase activity was also detectable at high levels in human blastocysts, which were obtained from patients who had undergone in vitro fertilization, and in some, but not all, human somatic tissues during early, but not later, stages of prenatal development (Youngren *et al.*, 1998). Marked differences have been observed in the pattern of telomerase expression and timing of telomerase suppression among different human fetal tissues, with the greatest persistence of activity in the liver and intestine; lesser persistence of activity in lung, skin, muscle, adrenal, and renal cells; and little if any detectable activity in brain, bone, or placental extracts at 16 wk of gestation (Wright *et al.*, 1996a; Yashima *et al.*, 1998; Ulaner *et al.*, 1998). In all cases, telomerase activity was lower or absent in the corresponding neonatal or adult tissues, including placental tissue at birth. Indeed, it is now known that in the adult soma the persistence of low levels of telomerase activity is restricted to a limited number of somatic stem cells, which permit cell renewal during postnatal tissue loss and regeneration. However, even this level of telomerase activity is not sufficient to prevent the eventual exhaustion of telomeres in renewing tissues with a rapid cellular turnover. Of note, it has been shown that lysates of telomerase-negative tissues do not inhibit the activity of telomerase-positive cells taken during various stages of gestational development, suggesting that the suppression of telomerase activity is due to the loss of telomerase rather than to the presence of an inhibitor (Youngren *et al.*, 1998). This is in contrast to the results of studies using cell and microcell/hybrids, as well as cellular genes, in which suppression of endogenous telomerase activity was demonstrated (Wright *et al.* 1996b; Horikawa *et al.* 1998; Tanaka *et al.* 1998; Cuthbert *et al.* 1999). Taken together, these findings demonstrate tissue-specific and developmental regulation of telomerase in the human fetus, suggesting an important role for this ribonucleoprotein in human fetal tissue differentiation and development. Our findings, using hES cells, suggest that this may serve as a potential in vitro model for investigating molecular mechanisms of telomerase inhibition during fetal development. Of note, the inhibition of telomerase activity also has been shown during mouse fetal development (Prowse and Greider 1995). We also found in the mouse-derived F9 cell line that differentiation in vitro also was associated with inhibition of the hTERT promoter, which was accompanied by a similar pattern of reduced transcription factor binding. This is of particular interest, since it has been thought that there are less stringent requirements for telomere length maintenance in mouse strains with greater starting telomere lengths. Studies conducted using TERT promoter–reporter transgenic mice should provide further insight with respect to the transcriptional regulation of TERT during embryonic and fetal development.

Having observed the inhibition of hTERT promoter with differentiation, we sought to determine whether this inhibition was accompanied by a change in the pattern of transcription factor binding. As previously reported in telomerase-positive cancer cell lines, we found that a core region extending 283 nt upstream of the translation initiation site is sufficient to give maximum promoter activity. Larger promoter fragments, extending further 5′ upstream, result in inhibition compared with the core fragment. EMSA results revealed that the loss of promoter activity with differentiation was associated with the inhibition of transcription factor binding to both E-boxes contained within the core promoter. Although c-Myc can bind to E-box elements, supershift experiments did not identify the transcription factor as containing c-Myc. Moreover, in addition to E-box recognition, we also identified what appears to be a novel transcription factor-binding element. Transcription factor binding to this element also was found to diminish with cell-line differentiation and loss of promoter activity. The element was defined by a series of mutations, and extensive database searches failed to reveal prior identification of this sequence in the genome. Conservation of nearly identical sequences in the mouse and human TERT promoter further supports the functional importance of the element. The only difference between the murine and human sequences does not affect transcription factor binding.

The findings in the current study of a correlation between a decrease in transcription factor binding to these sites, on the one hand, and a loss of telomerase promoter activity, on the other hand, both occurring in association with differentiation in three different model systems, represent a necessary first step for further studies aimed at testing for a possible causal relationship. Site-directed mutagenesis, showing parallel decreases in transcription factor binding and promoter activity, further strengthen the basis for such a relationship and also reveals the mutual functional interaction between the E-box and the novel MT element. With respect to the proximal E-box, this is not surprising, given the overlap of the two binding motifs at this site and, hence, the possibility of competition between transcription factors for binding to each of the two motifs. However, definitive proof of an important causal relationship between binding to these elements and promoter regulation during differentiation or other cellular processes will require identification of the transcription factor(s) involved. Motivated by the current findings, further studies should determine whether these represent known or novel transcription factors and should clarify their mutual interaction.

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