Cloning of Human Telomerase Catalytic Subunit (hTERT) Gene Promoter and Identification of Proximal Core Promoter Sequences Essential for Transcriptional Activation in Immortalized and Cancer Cells

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Abstract

Telomerase activation is thought to be a critical step in cellular immortalization and carcinogenesis. Of the three major subunits comprising human telomerase, human telomerase catalytic subunit (hTERT) has been shown to be a rate-limiting determinant of the enzymatic activity of human telomerase. However, little is known concerning how expression of hTERT is regulated in human cells. To identify the regulatory elements controlling hTERT gene expression, we cloned the hTERT promoter region upstream of the transcription start site. Gel shift analysis revealed two major factors binding to core promoter, an E box (CACGTG) binding factor and Sp1. Overexpression of c-Myc resulted in a significant increase in transcriptional activity of the core promoter. These findings suggest that hTERT expression is strictly regulated at the transcriptional level, and that the proximal core promoter containing an E box and Sp1 sites is required for transactivation of hTERT.

Introduction

Telomeres are the distal ends of human chromosomes composed of tandem repeats of the sequence TTAGGG (1). Possible functions of telomeres include prevention of chromosome degradation, end-to-end fusions, rearrangements, and chromosome loss (2). Because DNA polymerase fails to fully synthesize DNA termini, human telomeres in somatic cells undergo progressive shortening with cell division (3). Reduction of telomere length may play significant roles in cellular replicative senescence. Synthesis and maintenance of telomeric repeats are mediated by a specialized ribonucleoprotein complex known as telomerase (4). Numerous studies have demonstrated that telomerase is activated in >90% of malignant tumors but is stringently repressed in normal somatic cells (5–7). Telomerase reactivation is thus thought to be essential for stabilization of telomere length in attaining cellular immortality and may therefore be a critical step in carcinogenesis (8, 9).

Recently, three major subunits comprising the human telomerase complex have been identified. The RNA component of human telomerase (hTR) provides the template for telomere repeat synthesis (10). Several studies have shown that disrupting the function of telomerase RNA leads to progressive shortening of telomeres, suggesting that this component plays an essential role in telomerase function (11). Most recently, targeting the telomerase RNA gene in mice has been shown to lead to progressive shortening of telomeres and to impair long-term viability of tissues with high rates of renewal such as testis and bone marrow (12, 13). Three proteins in different species associated with telomerase activity have also been identified. p80 and p95 were purified from the ciliate Tetrahymena (14), and the gene encoding a mammalian homologue of p80, TP1/TLP1, has also been cloned (15, 16). The functional significance of these telomerase-associated proteins remains unclear. Two related proteins, Est2p and p123, have been newly identified as catalytic subunits of telomerase in the yeast Saccharomyces cerevisiae and the ciliate Euplotes aediculatus, respectively. These proteins harbor several sequence motifs characteristic of catalytic regions of reverse transcriptase (17, 18). Disruption of these motifs has been shown to abolish enzymatic activity of telomerase. Most recently, the human homologue of Est2p and p123 has been cloned (hTERT; Refs. 19 and 20). Expression of hTERT is observed at high levels in malignant tumors and cancer cell lines but not in normal tissues or telomerase-negative cell lines, and a strong correlation was found between hTERT expression and telomerase activity in a variety of tumors such as cervical cancer, uterine cancer, and renal cell carcinomas (21–23). Introduction of hTERT cDNA into normal cells confers telomerase activity in these cells (24, 25). hTERT-expressing normal cell clones have an extended life span without any change in karyotypes (26). These findings strongly suggest that hTERT is a catalytic subunit homologue protein of human telomerase, and that up-regulation of hTERT might be a critical event in carcinogenesis.

Despite these findings, the molecular mechanisms by which hTERT is expressed remain to be determined. This is mainly due to the lack of information on transcriptional regulation of the hTERT gene. Identification of promoter sequences and transcription factors essential for transcriptional regulation of the hTERT gene may greatly contribute to understanding of molecular mechanisms of telomerase regulation. In the present study, we cloned the 5'-flanking sequence of hTERT and identified the core promoter region essential for transcriptional activation in immortalized and cancer cells.

Materials and Methods

Cell Culture. C33A, ME180, HeLa, and SiHa cells, all of which were derived from cervical cancers and obtained from the American Type Culture Collection, were grown in DMEM supplemented with 10% FCS in the presence of 5% CO2. Normal human primary keratinocytes and fibroblasts derived from the epidermis and normal human renal cortical epithelial cells were purchased from Clonetics (San Diego, CA) and were grown according to the manufacturer’s protocol.

Cloning of Sequences Encapsulating the Human Telomerase Reverse Transcriptase Genes. To identify hTERT genomic clone, a human genomic library in Lambda Fix II (Stratagene) was screened with a 32P-labeled
hTERT cDNA fragment that contained 600 bp of 5'-hTERT cDNA sequences. Screening of 500,000 plaques yielded one positive clone (TM-1) with a 5.6-kb insert. After three rounds of screening, TM-1 was plaque purified, subcloned into pGEM-3zf (+) (Stratagene), sequenced with a dRhodamine Terminator Cycle Sequencing FS Ready Reaction kit (ABI), and analyzed on an ABI Model 377 automated DNA sequencer. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequences with the accession number AB016767.

**Determination of Transcription Start Site.** To determine the transcription start site of hTERT mRNA, a CapSite Hunting method was used in accordance with the manufacturer’s protocol (NipponGene, Tokyo, Japan). Briefly, mRNAs from HeLa cells were isolated, and the 5'-terminal mGpppN cap structure was removed by the pyrophosphorolysis reaction of tobacco nucleases. The cap excised and recapped with the 3’ end of a 38-mer specific oligonucleotide (Oligo) by an RNA ligase reaction. A single-strand cDNA library was prepared from the recapped mRNA by the synthesis of first-strand cDNA with Moloney murine leukemia virus reverse transcriptase and random primer. To amplify hTERT-specific transcripts from the single-strand DNA library, nested PCR was performed using specific primers corresponding to the 38-mer Oligo and the 5’ cDNA sequence of hTERT between 63 and 84 (5’-CACGAACGTGCCCACGGCCAGCAGC-3’) for the first-round PCR and between 42 and 65 (5’-AGCACTCTCGGTTGAATGGTGCTGCCG-3’) for the second round PCR. Single bands of second round PCR products were isolated and subcloned into pGEM, and three independent clones were sequenced. The transcription start site was determined by identification of the boundary sequence between Oligo and hTERT mRNA sequences.

**Plasmid Construction.** The structures of hTERT promoter-luciferase constructs are shown in Figs. 3 and 7. Various lengths of DNA fragments upstream of the initiating ATG codon were PCR amplified and inserted into luciferase reporter vector PGL3-Basic, a promoter- and enhancerless vector (Promega) in sense orientation relative to the luciferase coding sequence at Mid and BglII sites. For the construction of reporter plasmids containing substitution mutations in factor binding sites, site-specific mutagenesis was performed by a PCR-based protocol (27). The sequence of each insert was checked by sequencing. The name of each reporter construct was assigned according to the PCR-based protocol (27). The sequence of each insert was checked by sequencing. The name of each reporter construct was assigned according to the PCR-based protocol (27).

**Luciferase Assay.** Transient transfection of luciferase reporter plasmids was performed using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD), according to the protocol recommended by the manufacturer. Briefly, 105 cells seeded in a 35-mm tissue culture dish were exposed to transfection with 2 µg of luciferase reporter plasmids and 0.5 µg of pSV-β-galactosidase control vector (Promega) for 5 h at 37°C. Then, 3 ml of growth media were added to the cells, followed by incubation for an additional 16 h. The cells were harvested 48 h after the transfection. Luciferase assays were performed according to the manufacturer’s protocols (Promega). β-Galactosidase assay was also performed with the same cell extracts to standardize for transcription efficiency. For c-Myc overexpression assay, 1 µg of c-Myc expression vectors was cotransfected with reporter plasmids, and luciferase assays were performed as described above. As an effector control, the same amounts of blank vectors without c-Myc cDNA insert were used. All experiments were performed at least three times in each plasmid and represent the relative luciferase activity as average.

**TRAP Assay.** TRAP assay was performed as described previously with some modifications (6). Cell pellets were washed in PBS and homogenized in ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (Sigma), and 10% glycerol]. After 30 min of incubation on ice, the lysate was centrifuged, and the supernatants were recovered. Assay tubes were prepared by sequestering 0.2 µg of CX primer (6) under a wax barrier (Ampliwax; Perkin-Elmer Cetus, Foster City, CA). Two µg of protein extracts were assayed in 50 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 1.5 mM MgCl2, 60 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 µM deoxynucleotide triphosphates, 0.2 µg of T3 primer (6), 1 µg of T4 ligase protein (Boehringer Mannheim), and 2.5 units of Taq DNA polymerase (Wako, Osaka, Japan). After 30 min incubation at 23°C, the reaction mixture was heated at 90°C for 3 min and then subjected to 31 cycles of PCR including denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 60 s. The PCR products were electrophoresed on a 12% polyacrylamide gel and visualized with SYBR Green I Nucleic Acid Gel Stain (FMC, BioProducts, Rockland, ME).

**Gel Shift Assay.** Nuclear extracts were prepared from C33A cells as described previously (28). Five µg of proteins were incubated with 1 µg of poly(dexoyinosinic-deoxycytidylic acid) in the presence or absence of a 100-fold molar excess of unlabeled competitor DNAs on ice for 20 min in a 25-µl reaction volume containing 10% glycerol, 25 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT. For supershift assay, specific antibodies against transcription factors were preincubated with nuclear extracts at 4°C for 60 min. After incubation, 20,000 c.p.m. of 3P end-labeled oligonucleotide probe was added, and the reaction was incubated at 4°C for an additional 30 min. The DNA-protein complexes were then separated from free probes by electrophoresis on a 4% polyacrylamide gel. The gel was dried and subjected to autoradiography. For competition assays, consensus oligonucleotides for AP1 (5’-CGCTTGTAGTACCGCAGGGA-3’; Promega), AP2 (5’-GATCGAATCTAGCGAGGGCCGCGG-3’; Promega), Sp1 (5’-ATTCGATCGGGGGCGGGGAGC-3’; Promega), Myc/Max (5’-GGAGACGACCGAGTCTGGCTTCCC-3’; Santa Cruz Biotechnology), and mutant oligonucleotides for Myc/Max (5’-GGAAGACGACCGAGGCAGTCTGGCTTCCC-3’; Santa Cruz Biotechnology) were used as competitors. The antibodies against c-Myc and Max were kindly provided by Dr. H. Ariga (Hokkaido University, Sapporo, Japan). The Sp1 antibody was purchased from Santa Cruz Biotechnology.

**Results**

**Cloning of 5′ Flanking Sequences of the hTERT Gene.** To obtain the hTERT 5′-flanking sequences, a human genomic DNA library was screened with a 5′-portion of hTERT cDNA as a probe, and an ~5.5 kb genomic clone (TM-1) encompassing 2135-bp genomic sequences of the hTERT gene including exon 1 (275 bp) and exon 2 (1355 bp) as well as the 3347-bp 5′-flanking sequence of hTERT gene was isolated (Fig. 1). The use of the CapSite Hunting method showed that the transcription start site is 77 bp upstream of the initiating ATG (Fig. 2). The first base in the mRNA is labeled +1, and the first base of the promoter is labeled −1 (Fig. 1). The 5′-flanking sequence with the first 200 bp is rich in GC content and lacks the typical TATA-box motif. A putative initiator element, which is thought to be strongly required for transcription initiator activity of TATA-less promoter, was found at position −3 (CCTCTCC), which conforms well with a consensus initiator sequence PyPyANA/TPyPy. Several potential transcription factor binding sites were found in this region, such as Sp1 consensus (GGGCGG) at positions −7 and −88, as well as their degenerated motifs at −36, −56, and −110. There were also several consensus motifs for AP2 and GC factor. Interestingly, two E boxes (CAGCTG) were found at positions −165 and −44.

**Transcriptional Activity of hTERT in Cancer and Normal Cells.** To examine the transcriptional activity of hTERT in normal and transformed cells, a transient expression assay was first performed using cancer cell lines and normal primary cells. Luciferase reporter plasmids containing a 1.4-kb region upstream of the initiating ATG codon were constructed (pGL3–1375; Fig. 3) and transfected into cervical cancer cell lines C33A, HeLa, ME180, and SiHa, all of which were confirmed to be telomerase positive (data not shown), as well as normal primary keratinoctyes and fibroblasts derived from skin, which lack telomerase activity, and the cell lysates were tested in luciferase assays. As shown in Fig. 4A, a 1.4 kb region (pGL3–1375) demonstrated significant transcriptional activity in these cancer cell lines. In particular, C33A and ME180 cells conferred the highest transcriptional activity, comparable with that in control reporter plasmids (pGL3-Control) driven by SV40 enhanced/promoter. HeLa and SiHa cells exhibited modest transcriptional activities equivalent to 10–20% of positive control activity, but these activities were still significant, because they were ~100-fold the activity in promoter-less reporter plasmid (pGL3-basic). In contrast, neither normal
primary keratinocytes nor normal primary fibroblasts conferred demonstrable transcriptional activity (Fig. 4B). These findings suggest that hTERT transcription is significantly activated in cancer cells but not in normal cells.

**Changes in Transcriptional Activity of hTERT at Various Stages of Transformation.** To examine the changes in hTERT transcriptional activity during the process of transformation, we developed cell lineages of normal skin fibroblasts derived from patients with hemizygous deletion of the RB gene locus (14.1–22) in chromosome 13q.2 Continued culture of primary cells (RB) resulted in replicative senescence at population doubling (PD) 60-70. The cells in early generations were transfected with the SV40 LT gene, and the clones with extended life span (PD 70-90) were selected (RBSV). With continued subcultures of RBSV cells, an immortal clone, RBI, which continues to divide over 300 PD, was obtained. We further obtained subclones of RBI with anchorage-independent growth (RBS). One of the RBS clones obtained tumorigenicity in mice (RBT) on continuous culture. The lineage of RB cells was confirmed to be derived from the same origin by checking the microsatellite polymorphism in chromosomes 18q12. Telomerase activity in this lineage was simultaneously examined, and the telomeric repeat amplification protocol assay revealed that RB and RBSV cells did not exhibit telomerase activity, whereas RBI, RBS, and RBT cells demonstrated significant telomerase activity.

**Identification of Core Promoter Region Essential for Transactivation of hTERT.** To identify the core promoter essential for transcriptional activation, 5' truncations of 1.4-kb fragments were prepared and tested by luciferase assay (Fig. 3). As shown in Fig. 4, transcriptional activity in C33A and ME180 cells decreased with 5' truncations, and the 776-bp fragment exhibited the minimal activity. However, transcriptional activity increased with more extended truncations, and the proximal 181-bp fragment conferred peak transcriptional activity, equivalent to 60–80% of control activity in the pGL3-Control. Truncations of this fragment (pGL-82 and pGL-31) resulted in a stepwise decrease in transcriptional activity, but the proximal 31-bp fragment still conferred the significant transcriptional activity. To exclude the possibility that sequences downstream of the transcription start site participate in transcriptional regulation, we tested a reporter plasmid containing untranslated downstream sequences (pGL3+19), but it did not exhibit transcriptional activity, suggesting that these sequences do not function as a part of the core promoter. Similar findings were observed for HeLa and SiHa cells, except that overall transcriptional activities are lower than for C33A and ME180 cells. In contrast, in normal primary keratinocytes and fibroblasts, no transcriptional activity was observed in either 1.4 kb or 5'-truncated fragments of hTERT promoter. These findings suggest that the proximal 181-bp region upstream of the transcription start site functions as the core promoter essential for transcriptional activation of hTERT.

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Identification of Nuclear Factors Binding Core Promoter Region.

To identify the transcription factors that bind the proximal core promoter, gel shift assays were performed using nuclear extracts from C33A cells. A total of seven probes were prepared to cover the entire region of core promoter as shown in Fig. 1. Using probe 1 containing E box (CACGTG) at position -165, a significant retarded band was observed, which was completely competed by addition of homologous competitors, but not those with substitution mutations in the E box. It was also competed by the addition of Myc/Max consensus oligonucleotides but not by Myc/Max mutant oligonucleotides or unrelated oligonucleotides (Fig. 6). However, neither competition nor supershift of the band was observed by addition of Myc or Max antibody (data not shown). As shown in Fig. 1, another E box is located at position 44 in 5’ untranslated region. Gel shift assay using this E box region as probe yielded the similar results.

On probe 2, no significant band was observed, whereas probes 3 to 7, each of which contained the Sp1 consensus motif, exhibited two retarded bands. The upper bands were more significant and competed by addition of Sp1 consensus oligonucleotides but not by addition of Sp1 mutant oligonucleotides or unrelated oligonucleotides. The bands were super-shifted by the addition of Sp1 antibody. The lower bands on each probe were faint and were not competed by any of the competitors used. At present, the significance of the lower bands remains unclear. No evidence of AP2 binding was observed because no bands were competed by AP2 consensus oligonucleotides. These findings suggest that the E box binding factor(s) which recognizes Myc/Max consensus motif and Sp1 are the major factors binding the proximal promoter.

Activation of hTERT Transcription by c-Myc. Recently, c-Myc has been reported to activate telomerase in normal epithelial and fibroblast cells through up-regulation of hTERT (28). To elucidate the mechanism of hTERT up-regulation by Myc, c-Myc was overexpressed in several cell lines as well as normal primary cells, and luciferase assays were performed. In SiHa cells, introduction of c-Myc expression vectors resulted in more than 3-fold activation of hTERT transcription in pGL3-1375 (Fig. 7). In HeLa and normal human renal cortical cells, more than 2-fold activation was observed. Similar results were obtained using the core promoter reporter plasmid (pGL3-181). These findings suggest that c-Myc interacts with core promoter to activate hTERT transcription.

Discussion

In the present study, we cloned the promoter of hTERT and examined transcriptional activity in a variety of cancer cells as well as normal cells. We found that the transcription of hTERT was activated in cancer cells and correlated with telomerase activity, although the levels of transcription varied among types of cancer cells. In contrast, the transcriptional activity of hTERT was repressed in normal cells. We also examined the timing of up-regulation of hTERT transcription and telomerase activity in the process of malignant progression using...
fibroblast lineage with the same genetic backgrounds. Transcription of hTERT was repressed in primary fibroblast strain (RB) and even in substrains (RBSV) that obtained increased proliferative capacity and obtained an extended life span by transfection of SV40 LT. Activation of hTERT transcription occurred with cells that had overcome replicative senescence and obtained characteristics of immortality. Telomerase activity status was consistent with that of transcriptional activity of hTERT at each stage of transformation. These findings suggest that expression of telomerase activity is strictly regulated through the transcriptional machinery of hTERT, and that activation of hTERT transcription followed by telomerase activation is a critical event in overcoming replicative senescence and progression of immortality.

Analysis of 5′-truncations of the promoter revealed that transcriptional activity decreased with deletion of sequences between −776 and −1375 and increased with the deletion of sequences between −378 and −776. These findings suggest that cis-acting and silencer elements, respectively, may exist in these regions. One important question is whether the potential silencer between −378 and −776 causes low transcriptional activity in normal cells. In normal cells, the proximal 181-bp region that lacks this silencer did not confer transcriptional activity, comparable with that in tumor cells. Therefore, the low transcriptional activity in normal cells is not simply explained by the action of this silencer. Transcriptional activity dramatically decreased with truncation of the 181-bp proximal region, and the untranslated downstream sequences did not contribute to transcriptional activity. These findings suggest that the 181-bp fragment upstream of the transcription start site is a core functional promoter essential for transcriptional activation of hTERT in cancer cells.

As shown in the present study, the promoter sequence of hTERT lacked a TATA box and TATA-like sequence. TATA-less promoters are frequently described as housekeeping genes, although hTERT is specifically up-regulated in tumors. TATA-less promoters often contain GC-rich regions in the proximal promoter (30), and an initiator element is usually found around the transcription start site. The promoter of hTERT conforms with this model. In the absence of a TATA box, it has been postulated that an initiator element helps position the RNA polymerase II and locate a transcription start site (31). In the hTERT promoter, an initiator-like sequence (CCTCTCC)

Fig. 4. Transcriptional activity of hTERT promoter in various cell lines and identification of core promoter region. Luciferase activity of reporter plasmids with 1.4-kb or 5′-truncated fragments of hTERT promoter was examined in cancer and primary cell lines. Luciferase activity in each plasmid was plotted as a percentage of the positive control plasmid (pGL3-Control) driven by SV40 enhancer/promoter (Promega). pGL3-Basic without enhancer/promoter was used for negative control.
was found around the transcription start site at position -3. It has also been demonstrated that TATA-less promoters still use TATA factors, and several factors have been proposed to help initiate transcription from TATA-less genes (31). In the present gel shift assay, we found that Sp1 binds at least five sites in the hTERT core promoter. It has been reported that Sp1 tethers the TATA factors and plays significant roles in transcriptional initiation (30). Sp1 might thus be one of the critical factors promoting initiation of hTERT transcription. Although Sp1 is known to be a ubiquitous factor, it has been shown to be up-regulated in fetal tissues, sperm, and hematopoietic cells in human early development, all of which are known to exhibit elevated levels of telomerase activity (32). It will thus be important to compare the levels of Sp1 expression between tumors and normal tissues.

Another factor we identified on gel shift assay is the E box binding factor. Several factors are known to bind the E box, such as Myc-related family members TFE3 and USF. In our competition assays, the retarded band on probe 1 was competed by Myc/Max consensus oligonucleotides. However, in the supershift assay, the band was not competed or supershifted by addition of Myc or Max antibody. These findings suggest that factor(s) other than Myc/Max heterodimer, which can recognize Myc/Max consensus motif, binds this E box. Because E box binding proteins are known to heterodimerize with a variety of factors with a bHLH-Zip leucine zipper domain (bHLH-Zip), careful biochemical analysis will be required to identify the factors binding this site. Recently, c-Myc has been reported to activate telomerase in normal epithelial and fibroblast cells.

\(^3\) The abbreviation used is: bHLH, basic helix-loop-helix.
References

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