Cloning and Characterization of the Promoter Region of Human Telomerase Reverse Transcriptase Gene

Izumi Horikawa,^1 P. LouAnn Cable, Cynthia Afshari, and J. Carl Barrett^2

Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

Abstract

Activation of telomerase is one of the rate-limiting steps in human cell immortalization and carcinogenesis. Human telomerase is composed of at least two protein subunits and an RNA component. Regulation of expression of the catalytic subunit, human telomerase reverse transcriptase (hTERT), is suggested as the major determinant of the enzymatic activity. We report here the cloning and characterization of the 5′-regulatory region of the hTERT gene. The highly GC-rich content of the 5′ end of the hTERT cDNA spans to the 5′-flanking region and intron 1, making a CpG island. A 1.7-kb DNA fragment encompassing the hTERT gene promoter was placed upstream of the luciferase reporter gene and transiently transfected into human cell lines of fibroblastic and epithelial origins that differed in their expression of the endogenous hTERT gene. Endogenous hTERT-expressing cells, but not nonexpressing cells, showed high levels of luciferase activity, suggesting that the regulation of hTERT gene expression occurs mainly at the transcriptional level. Additional luciferase assays using a series of constructs containing unidirectionally deleted fragments revealed that a 59-bp region (∼208 to −150) is required for the maximal promoter activity. The region contains a potential Myc oncoprotein binding site (E-box), and cotransfection of a c-myc expression plasmid markedly enhanced the promoter activity, suggesting a role of the Myc protein in telomerase activation. Identification of the regulatory regions of the hTERT promoter sequence will be essential in understanding the molecular mechanisms of positive and negative regulation of telomerase.

Introduction

Telomeres are specialized structures at chromosome ends, which consist of tandemly repeated DNA sequences and associated proteins 1, 2. In normal human somatic cells, which show no or little telomerase activity to synthesize new telomeres, the telomeric DNAs progressively shorten with each cell division. Critically short telomeres are suggested to cause irreversible cell growth arrest and cellular senescence 3, 4. In contrast, most cancer cells have mechanisms that compensate for telomere shortening, most commonly through the activation of telomerase 5, allowing them to stably maintain their telomeres and grow indefinitely 3, 4, 6. Thus, activation of telomerase is a rate-limiting step in human carcinogenesis, and telomerase repression in normal human somatic cells can act as a tumor-suppressive mechanism. Three components of the human telomerase enzyme are thus far identified: the RNA component (hTERC), which acts as an intrinsic template for telomeric repeat synthesis 7; a telomerase-associated protein (TEP1) with similarity to the Tetrahymena telomerase protein p80 8, 9; and a telomerase catalytic subunit, hTERT,^3 which shares several motifs with other known reverse transcriptases 10, 11. Recent reconstitution experiments both in vitro and in vivo strongly suggest that hTERT is the major determinant of human telomerase activity 12, 13. Concomitant up-regulation or down-regulation of the hTERT mRNA expression and telomerase activity during cell immortalization or differentiation is observed 10, suggesting that control of the hTERT expression at the mRNA level mainly contributes to the regulation of telomerase enzymatic activity 14. We found that a putative senescence-inducing gene with a telomerase repressor activity on human chromosome 3p also acts through down-regulation of the hTERT mRNA 15. How the hTERT mRNA expression is controlled by factors that could play a role in cellular senescence, immortalization, and carcinogenesis (e.g., oncogene products, tumor suppressor gene products, and the telomerase repressor on chromosome 3p) is of great interest. Identification of cis-elements and trans-acting factors that regulate the hTERT gene transcription will help to answer this question. As a first step toward the better understanding of molecular mechanisms of the hTERT gene and telomerase expression, we describe here the cloning and characterization of the promoter region of the hTERT gene.

Materials and Methods

Isolation and Sequence Analysis of the hTERT Genomic Clones. Two independent methods were applied to obtain genomic DNA clones containing the 5′-flanking region of the hTERT gene: PCR-based genomic walking and BAC library screening. For the PCR-based method, we used the Human Genome Walker kit (Clontech Labs, Palo Alto, CA) according to the supplier’s protocol. The gene-specific primers within the 5′-region of hTERT cDNA were 5′-AGC ACT CGG GCC ACC AGC TCC T-3′ (primer GW1) for the initial PCR and 5′-AAC GTG GCC ACC GCG ACC ACC T-3′ (primer GW2, Fig. 1) for the nested PCR. The use of Advantage-GC Genomic PCR kit (Clontech Labs, Inc.) designed for PCR of highly GC-rich regions was critical to our successful amplification. The final PCR product (∼1.8 kb in length) was cloned into pCR2.1 vector (Invitrogen Corp., San Diego, CA) via the TA cloning method and sequenced in both strands by the dRhodamine terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA). The RPCI-11 human BAC library^4 was screened using a 430-bp hTERT cDNA fragment (nucleotides 18–447 in GenBank AF015950) as a hybridization probe at Research Genetics, Inc. (Huntsville, AL). Two resultant positive BAC clones were sequenced by the BigDye terminator cycle sequencing kit (PE Applied Biosystems) using the primers that were designed based on the sequences from the PCR-based clones described above. A DNA homology search was performed at the National Center for Biotechnology Information using the basic local alignment search tool (BLAST) network service. Potential transcription factor binding sites were predicted by TESS (Transcription Element Search Software)^5 search program. A search for the CpG island was carried out using

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2 To whom requests for reprints should be addressed, at Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, NC 27709. Phone: (919) 541-2992; Fax: (919) 541-7784; E-mail: barrett@nichs.nih.gov.

3 The abbreviations used are: hTERT, human telomerase reverse transcriptase; BAC, bacterial artificial chromosome; HBV, hepatitis B virus; bHLHZ, basic helix-loop-helix zipper.

4 http://bacpac.med.buffalo.edu.

5 http://www.cbil.upenn.edu/teiss.

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Fig. 1. Nucleotide sequence of the 5′-flanking region, exon 1, and intron 1 of the hTERT gene. The 5′-flanking region and exon 1 are shown by upper-case letters, and intron 1 is shown by lower-case letters. The translation initiation codon (ATG) is doubly underlined. A 1/2 EcoRV site (ATC) at the 5′ end, two SalI sites (AGGCGC), two Pfal sites (CAAGTC), and an EcoRI site (AGGCGT) are shown in Fig. 1. The region identical to the HBV integration site in the huI-4 cell line (GenBank X51995) is shown in bold and italic. The HBV genome is inserted adjacent to the position −307 (20), though the downstream end of the insertion is not precisely determined.

The 5′-end of this 59-bp region to the trans-

The 32 P-labeled RNA probe (460 bases in length), which corresponds to the 390-base antisense hTERT sequence shown in Fig. 1 (the end of GW primer) to −559 (PvuII site) and the pCR2.1 vector-derived 70-base sequence, was synthesized by in vitro transcription using the MAXIscript T7 kit (Ambion, Inc., Austin, TX). Hybridization between the probe (5 × 10^5 cpm/reaction) and total cellular RNA (50 μg/reaction) and digestion with RNaseA/RNaseT1 were performed using the RPA III kit (Ambion, Inc.). The protected fragments were detected on a 5% denaturing polyacrylamide gel.

**Construction of Luciferase Reporter Gene Constructs.** An ∼1.7-kb SacI/Eco47III fragment (∼1665 to 5 in Fig. 1; SacI site was at the multiple cloning site of the pCR2.1) was ligated to the SacI/Smal-digested pGL3-Basic vector (Promega Corp., Madison, WI) to allow transcription of firefly luciferase gene under the control of this fragment. The resultant plasmid (pGL3/TRTP) was digested with SacI and Stul and then divided into two reactions: one was end-polished by T4 DNA polymerase and self-circularized (pBT-SE); the other was subject to the unidirectional deletions by the Exonuclease III/Mung bean nuclease system (Stratagene Cloning Systems, La Jolla, CA) to make a series of constructs shown in Fig. 2 (pBTdel-X: X means the nucleotide number in Fig. 1 where the fragment starts). A 251-bp fragment (∼210 to 41) was PCR amplified and cloned into the SacI/Smal-digested pGL3-Basic to produce the plasmid p2XEB. All plasmid DNAs were purified with the QIAfilter plasmid kit (Qiagen, Inc., Chatsworth, CA) and confirmed to have correct sequences by nucleotide sequencing, and their quantity and quality were routinely checked by agarose gel electrophoresis.

**Cell Lines and Luciferase Assay.** Three human cell lines positive for both the telomerase activity and the hTERT mRNA expression were used: normal human primary fibroblasts derived from foreskin; an immortalized fibroblast cell line, CMV-Mj-HEL-1; and an RCC23-derived clone that was confirmed to have the expected structure by nucleotide sequencing. We also isolated two BAC clones carrying the hTERT gene, and their sequences corresponding to the 5′-flanking region, exon 1 and intron 1, were determined (Fig. 1). The nucleotide sequences from the PCR-based clones and the two BAC clones were identical except for a few possible polymorphisms. The transcription initiation sites were examined by the RNase protection assay with an RNA probe containing the 390-base sequence (135 to −255 in Fig. 1). Multiple protected fragments ranging from ∼120 to ∼190 bases were reproducibly observed in the hTERT mRNA-expressing CMV-Mj-HEL-1, RCC23, huH-4 (hepatocellular carcinoma, Ref. 19; see below) and SiHa cells, but not in the hTERT mRNA-negative SUSM-1, normal human fibroblasts, and RCC23 cells (Fig. 2 and data not shown), suggesting the presence of multiple transcription initiation sites. The most abundant fragment was ∼135 bases in length. This suggests that a G nucleotide, which lies 55 bp upstream of the transcriptional initiation shown in Table 1), the pGL3-Control plasmid (1 μg/well; Promega), which has the firefly luciferase gene under the transcriptional control of SV40 enhancer/promoter, was also transfected into each cell line and used for normalization of the activities shown by the hTERT promoter-luciferase construct. For experiments to identify DNA elements responsible for the full promoter activity, a human c-myc cDNA expression plasmid, pSVmcyCYP (a kind gift from Dr. Chi V. Dang, Johns Hopkins University, Baltimore, MD), or an empty vector (0.5 μg/well) was cotransfected with the hTERT promoter-luciferase constructs (0.5 μg/well) and the pRL-SV40 (1 ng/well; Promega) containing the Renilla reniformis luciferase gene under the transcriptional control of SV40 enhancer/promoter, was cotransfected with the hTERT promoter-luciferase constructs (1 μg/well, as described above). The level of firefly luciferase activity was normalized to that of Renilla reniformis luciferase activity for each transfection.
codon, is a major transcription initiation site, which matches the S'-end of the hTERT cDNA sequence reported by Nakamura et al. 11. We thus refer to this G as +1 in this manuscript. A 390-base fragment was protected only in the hepatocellular carcinoma cell line, huH-4, which suggests an alternate transcription initiation upstream of −255.

The most striking feature of the region is a highly GC-rich content. Search by GRAIL identified a CpG island of 1138 bp in length (−808 to 330 in Fig. 1), with a GC content of 71.3% and a ratio of observed versus expected CpGs of 0.79. A DNA homology search by BLAST of known sequences in GenBank showed that the 65-bp sequence (−371 to −307) is identical to the cellular sequence of the integration site of the HBV in the huH-4 cell line (GenBank X51995, Ref. 20), implying a genomic rearrangement of the hTERT gene promoter and possibly, taken together with the RNase protection assay described above, a transcription initiation from a viral gene promoter in this cell line. We indeed confirmed the genomic rearrangement by Southern blot analysis (data not shown). Although the hTERT gene promoter lacks a typical TATA box or a typical CCAAT box, as seen with many GC-rich promoters, the TESS search program predicts a number of potential transcription factor binding sites near or upstream of the major transcription initiation site, including potential binding sites for Sp1, MAZ (Myc-associated zinc finger protein), a bHLH class of transcription factors (E boxes), c-Ets-2, and AP-2 (activator protein-2; Fig. 1).

To examine the transcriptional activity of the hTERT gene promoter, we first put the 1670-bp fragment (−1665 to 5) upstream of the firefly luciferase gene in the pGL3-Basic vector. The resultant plasmid pGL3-TRTP was transiently transfected in the endogenous hTERT mRNA-expressing and nonexpressing cells, and the firefly luciferase activity obtained was compared with those from the pGL3-Basic vector alone and the pGL3-Control plasmid, which was used to monitor transfection efficiency (Table 1). In the hTERT mRNA-positive cells of both fibroblastic (CMV-Mj-HEL-1) and epithelial (SiHa and RCC23) origins, the pGL3-TRTP showed significant activities, i.e., 6.0–30.9% of the pGL3-Control, or 50–70-fold of the baseline activity of the pGL3-Basic vector. The highest, normalized luciferase activity observed in the CMV-Mj-HEL-1 cells is consistent with the highest expression of endogenous hTERT mRNA in this cell line, as shown in the RNase protection assay (Fig. 2), making our results convincing. In marked contrast, the pGL3-TRTP construct resulted in no or little luciferase activity (0.2–1.0% of the pGL3-Control; 1.3–3-fold of the pGL3-Basic) in the hTERT mRNA-negative cells including normal human fibroblasts, SUSM-1, and RCC23+3. These data suggest that the regulation of hTERT gene expression occurs mainly at the transcriptional level, rather than at the posttranscriptional level such as control of mRNA stability. This is also consistent with our finding that treatment of hTERT mRNA-negative cells with cycloheximide to diminish short-lived RNAs did not induce the hTERT mRNA expression (data not shown).

We next made a series of luciferase constructs containing unidirectional promoter activities of pGL3-Basic and pGL3B-TRTP were normalized with that of pGL3-Control for each cell line and are shown as a percentage in the parentheses.

We next made a series of luciferase constructs containing unidirectional promoter activities of pGL3-Basic and pGL3B-TRTP and used them for luciferase assay to determine the elements responsible for the hTERT gene promoter activity (Fig. 3). The plasmid pbTdel-279 (containing −279 to 5) showed the highest promoter activity in the CMV-Mj-HEL-1 and SiHa cells, and pbTdel-408 (containing −408 to 5) was highest in the RCC23 cells. Reduced activities up to ~50% of the full promoter activity shown by the constructs containing longer fragments might either reflect the presence of negative regulatory element(s) or be due to the lower transfection efficiency of the larger construct. It is notable that the deletion of the 59-bp region from −208 to −150 resulted in the remarkably decreased promoter activity in all of the cell lines tested, suggesting the presence of a cis-element(s) responsible for the maximal promoter activity within this 59-bp region. Interestingly, the region contains a typical E-box (CACGTG, −187 to −182 in Fig. 1), which is known as a potential binding site of the bHLH class of transcription factors such as the c-Myc oncoprotein and the upstream stimulatory factor 21. There is another typical E-box downstream of the major transcription initiation site (22

**Fig. 1. RNase protection assay to examine the transcription initiation sites of the hTERT gene.** A 460-bp RNA probe complementary to the 390-bp sequence of the hTERT gene (from −255 to 135 in Fig. 1) was hybridized to the following RNA samples: Lane 1, CMV-Mj-HEL-1; Lane 2, SUSM-1; Lane 3, RCC23; Lane 4, RCC23+3; Lane 5, huH-4. Lane 6, yeast RNA. *Left, undisgested probe. Size markers were transcribed in vitro from the Century marker template (Ambion). A 135-base major protected fragment and a 390-base fragment specific to huH-4 are indicated by an arrow and an asterisk, respectively. Signals near the bottom of the figure were common to both the hTERT mRNA-positive and -negative cell lines and thus appear to be nonspecific.
Fig. 3. Luciferase assay to identify the regions required for the hTERT gene promoter activity. The fragments cloned upstream of the firefly luciferase gene are shown by nucleotide positions that correspond to those in Fig. 1. For each transfection, the firefly luciferase activity was normalized with the Renilla reniformis luciferase activity by the cotransfected pRL-SV40. The relative activity of each construct is expressed as a ratio to the activity of the pBTdel-279 (CMV-Mj-HEL-1 and SiHa) or the pBTdel-408 (RCC23). The means from at least three independent experiments are shown for each construct; bars, SD.

Fig. 4. Induction of the hTERT gene promoter activity by c-Myc. A human c-myc cDNA expression plasmid or a vector alone was cotransfected with the firefly luciferase construct (pGL3-Basic, pBTdel-408, pBTdel-208, or pBTdel-149) and the pRL-SV40 into the SUSM-1 cells. As in Fig. 3, the firefly luciferase activity was normalized with the Renilla reniformis luciferase activity. Promoter activity of each combination of plasmids was expressed as fold induction relative to that of combination of the vector alone and the pGL3-Basic. Means from three independent experiments are shown; bars, SD.

to 27). The plasmid p2XEB (~211 to 40) containing both of the E-boxes showed similar activity to that of the pBTdel-208 (~208 to 5) or pBTdel-279 (~279 to 5) containing only an upstream E-box, suggesting that the downstream E-box does not have an additive or synergistic effect at least in our transient transfection experiments. As shown in the constructs pBTdel-149 and pBTdel-130, a low but significant transcriptional activity (20–35% of the full promoter activity) was observed, even without either E-box.

To examine a role of the Myc protein in the hTERT gene transcription, a human c-myc cDNA expression plasmid was cotransfected with the hTERT promoter-luciferase constructs in the endogenous hTERT mRNA-negative SUSM-1 cells (Fig. 4). We also observed a 3–7-fold increase of the promoter activity by the c-Myc expression in the endogenous hTERT-negative SUSM-1 cells (Fig. 4). This is consistent with the finding by Wang et al. 22 that retroviral expression of c-Myc increases the amount of hTERT activity in human immortal cells of both fibroblastic and epithelial origins. Among the potential transcription factor binding sites within this 59-bp region, a typical E-box (CACGTG, −187 to −182), to which the bHLHZ family of transcription factors bind 21, is likely to play an important role for the hTERT gene transcription. We showed that the expression of a member of this family, c-Myc oncoprotein, markedly induces the transcriptional activity of the E-box-containing hTERT gene promoter in the endogenous hTERT-negative SUSM-1 cells (Fig. 4). The expression of telomerase catalytic subunit gene, mainly regulates the expression of human telomerase enzymatic activity 10–15. Thus, investigation of the molecular mechanisms that regulate the hTERT gene transcription, probably through the E-box within the 59-bp region identified above. A slight induction observed in the pBTdel-149 might reflect an indirect effect of c-Myc protein by modulating other regulatory factors.

Discussion

The expression of telomerase activity is strongly associated with human cell immortalization and carcinogenesis 3–6. Recent studies have suggested that the expression of the telomerase catalytic subunit gene, hTERT, mainly regulates the expression of human telomerase enzymatic activity 10–15. Thus, investigation of the molecular mechanisms that regulate the hTERT gene expression could lead to a better understanding of telomerase regulation, cellular senescence and immortalization, and human carcinogenesis. The cloning and characterization of the hTERT gene promoter in this study is an essential step toward this goal. Our luciferase assays with hTERT mRNA-positive versus negative human cell lines of fibroblastic origin (CMV-Mj-HEL-1 versus SUSM-1 and normal human fibroblasts) or epithelial origin (SiHa and RCC23 versus RCC23 +3) indicate that the hTERT gene expression is controlled mainly at the transcriptional level. Thus, identification of the cis-elements and trans-acting factors that regulate the hTERT gene transcription is important to understand the regulation of this gene.

By using a series of unidirectionally deleted fragments derived from the hTERT gene promoter, we identified a 59-bp region (~208 to −150 in Fig. 1) that is responsible for the maximal promoter activity in human immortal cells of both fibroblastic and epithelial origins.
mRNA and activates telomerase in human mammary epithelial cells and fibroblasts, although their work did not include transcriptional regulation of the hTERT gene. Considering that activation of c-Myc is a common target of several oncogenic signals, e.g., mutant p53 proteins 23, viral oncoproteins 24, and defects in the adenomatous polyposis coli/β-catenin pathway 25, an attractive hypothesis is that these oncogenic signals converge on the transactivation of the hTERT gene promoter through c-Myc activation as a part of their oncogenic functions. Involvement of other factors is also possible, including other BHLH transcription factors such as upstream stimulatory factor 21, members of the Ets family of oncoproteins, which are known to cooperate with the BHLH proteins 26, 27, and the AP-2 family of transcription factors. We are presently investigating direct bindings of these factors, as well as c-Myc protein, to potential binding sites and their functional significance. Although the second typical E-box downstream of the major transcription initiation site appeared to contribute little to the hTERT gene promoter activity in the presence of the upstream E-box (see p2XEB in Fig. 3), there is a possibility that the downstream E-box also acts as a positive regulatory element but is functionally redundant with the upstream one. Additional experiments will be needed to address this issue. It should also be noted that the constructs without either E-box (pBTDel1-149 and pBTDel1-130) still showed low, but significant, promoter activity. Multiple potential binding sites for the Sp1 and MAZ proteins, which function cooperatively at some promoters 28, 29, are likely to be responsible for this basal activity of the hTERT gene promoter. Indeed, the Sp1 binding was confirmed in the hTERT mRNA-positive cell lines by a supershift on gel mobility shift assay using a 20-bp probe (~117 to ~98, data not shown).

Although in this study we focused our attention on the elements that positively regulate the hTERT gene transcription, the mechanisms involved in the transcriptional repression of the hTERT gene in normal human somatic cells are also of great interest. Although positive regulatory factors, e.g., c-Myc protein, may explain enhanced transcriptional activity, negative regulatory factors that function in telomerase-negative cells may play a role in the transcriptional repression. The hTERT gene promoter sequence and a series of constructs generated in this study may help to identify DNA elements and protein factors that negatively regulate the hTERT gene transcription. It has also been suggested that chromatin structure is a major determinant of gene activity 30. In this respect, the highly GC-rich content, forming a CpG island, of the hTERT gene promoter is notable. Increased DNA methylation and associated histone deacetylation could turn off the hTERT gene expression by establishing and maintaining an inactive state of chromatin structure.

Finally, we found a genomic rearrangement at the hTERT promoter region in a hepatocellular carcinoma cell line huH-4. The rearrangement probably resulted from the integration of the viral (HBV) genome into the promoter region, which is reminiscent of the activation of some oncogenes by insertion of viral genomes 31, 32. Determination of the precise structure of the HBV integration and examination of the transcriptional activity of the rearranged promoter will be needed to clarify functional significance of this rearrangement.

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