

Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase *hTERT* gene

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ABSTRACT

Expression of *hTERT* is the major limiting factor for telomerase activity. We previously showed that methylation of the *hTERT* promoter is necessary for its transcription and that CTCF can repress *hTERT* transcription by binding to the first exon. In this study, we used electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) to show that CTCF does not bind the methylated first exon of *hTERT*. Treatment of telomerase-positive cells with 5-azadC led to a strong demethylation of *hTERT* 5'-regulatory region, reactivation of CTCF binding and down-regulation of *hTERT*. Although complete *hTERT* promoter methylation was associated with full transcriptional repression, detailed mapping showed that, in telomerase-positive cells, not all the CpG sites were methylated, especially in the promoter region. Using a methylation cassette assay, selective demethylation of 110 bp within the core promoter significantly increased *hTERT* transcriptional activity. This study underlines the dual role of DNA methylation in *hTERT* transcriptional regulation. In our model, *hTERT* methylation prevents binding of the CTCF repressor, but partial hypomethylation of the core promoter is necessary for *hTERT* expression.

INTRODUCTION

Telomeres are nucleoprotein complexes that 'cap' and stabilize the termini of linear chromosomes. They are also involved in chromosome replication, maintenance of

nuclear architecture, chromosome stability, gene expression, aging and cell division (1,2). In normal cells, each division is associated with the loss of 50–100 base pairs of telomere length. This shortening of the telomeres acts as a mitotic counter and limits life span. Telomerase, a complex consisting of a reverse transcriptase bound to its own RNA template, allows the maintenance of the telomere length (3). In most somatic cells, telomerase activity is not detectable (4). In contrast, telomerase is expressed in highly proliferative cells, such as germ cells and stem cells, and in the cells of about 85% of cancers (5). *In vitro*, two components are absolutely essential for telomerase activity: the catalytic subunit, *hTERT*, containing the reverse transcriptase activity, and the RNA component, *hTERC*, containing a complementary template for the telomeric DNA sequence (TTAGGG) (6,7).

Following the characterization of the genomic sequence of *hTERT* and the elucidation of the organization of the gene (8–11), many studies have shown that expression of *hTERT* represents the limiting factor for telomerase activity, and that the regulation of *hTERT* expression occurs primarily at the transcriptional level. Transient transfection experiments have identified a minimal promoter encompassing the 283 bp region upstream of the ATG initiation codon (9–11). The 5'-*hTERT* regulatory region contains numerous binding sites for transcription factors. Activators of *hTERT* transcription include c-Myc, Sp1, hALP, Hif-1, Mbi-1, USF1/2 and estrogen response element. Repressors for *hTERT* have also been identified and include the tumor suppressor protein p53, Mad1, myeloid-specific zinc finger protein 2 (MZF-2), TGF- β , Wilms' Tumor 1 (WT1) and CTCF. (12–20). Recently, Horikawa *et al.* described an E-box binding sequence located upstream of the transcriptional start site, as inhibitor of *hTERT* but

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not in *mTERT*, suggesting a differential regulation of TERT in human and mouse (21). We previously showed that 5' exonic sequences might be involved in the regulation of the *hTERT* gene through an inhibitory effect on transcriptional activity of the minimal *hTERT* promoter (22). Moreover, this region inserted immediately downstream of *CMV* and *CDKN2A* promoters repressed their activity in normal, immortalized and cancer cells. We observed that the 11-zinc finger factor CTCF binds this region in telomerase-negative cells, but not in telomerase-positive cells (18). Since CTCF represses transcriptional activity when it binds DNA downstream of transcriptional start sites (23,24), these findings suggest that CTCF might function as a transcriptional repressor for *hTERT* in normal cells. However, the mechanisms that might prevent CTCF from binding to the *hTERT* gene in telomerase-positive cells have not been determined. Therefore, it is essential to note that the identification of these activators and repressors of *hTERT* do not take in account the methylation profile of the *hTERT* promoter described in numerous studies (25–27).

The fact that the *hTERT* promoter is located within a CpG island suggests that transcription of the gene might be regulated by DNA methylation. Previous studies showed that the *hTERT* promoter is hypermethylated in most telomerase-positive tumors and hypomethylated in telomerase-negative normal tissues (25–27). These observations contrast with the general association between promoter methylation and gene silencing (28), which prompted us to study how DNA methylation of the *hTERT* promoter can lead to its expression.

To this end, we initiated a detailed evaluation of the methylation pattern of each CpG site in the areas that appear to contribute to the transcriptional regulation of the *hTERT* gene. Based on our previous analyses (22), two regions of the *hTERT* CpG island seemed essential: one is the core promoter that is necessary to its expression; the other is the 5' exonic region where the CTCF repressor binds. The *hTERT* region at –441 to –218 from the ATG translational start site examined in our previous studies was located immediately upstream of the regions we considered now as essential (29).

Although the first two exons play a role in the repression of the *hTERT* transcription by CTCF, which binds to two sites, one in the first exon and the other at the beginning of the second (18); in the present study, only the effect of CTCF on the first exon was investigated. The presence of an intron between these two CTCF sites complicate the analyses since, in transient transfections, splicing of this intron is only partial.

In the present report, we tested the hypothesis that *hTERT* methylation prevents binding of CTCF inhibitor and that a partial methylation of the *hTERT* promoter region can result in some level of transcriptional activity. The results have allowed us to identify an unexpected dual role for DNA methylation in the transcriptional regulation of the *hTERT* promoter.

MATERIALS AND METHODS

Cell culture

The human tumor cell lines HeLa (cervical adenocarcinoma), SW480 (colorectal adenocarcinoma) and normal BJ fibroblasts were obtained from the ATCC. All these cells were grown in the medium recommended by the ATCC. The HLF/*hTERT* cells were kindly provided by Dr. Joachim Lingner (ISREC, Epalinges, Switzerland), and were cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (Invitrogen, Basel, Switzerland). HeLa and SW480 cell lines are telomerase positive, whereas BJ is telomerase negative. HLF/*hTERT* cells, obtained through stable transfection of HLF cells with an MSCV-*hTERT* retrovirus, constitutively express *hTERT* and have high telomerase activity.

Tissue samples

Tumor tissues (breast, bladder and cervix) were obtained from the Tissue Bank of the Institute of Pathology of Lausanne. Microdissection and DNA extraction were performed as described previously (30). Briefly, frozen tissue sections (7- μ m thickness) were stained with toluidin blue. Then, normal cells were removed by scratching. Verification that the remaining cells were indeed tumor was performed before the cells were harvested.

Plasmid construction

For stable transfection, *hTERT* sequences and firefly luciferase gene were extracted from the pTERT-297/ex2 vector, containing the *hTERT* minimal promoter and the 1071 bp downstream of the ATG (22), by digestion with *Asp718I* and *BamHI*. Then, this fragment was cloned into the pcDNA5/FRT vector (Invitrogen), previously deleted of the CMV promoter, in order to create the pTERT-297/ex2/FRT that is used in stable transfection experiments with or without *in vitro* methylation of all the CpG sites with the *SssI* methylase (Promega, Madison, WI, USA). Full methylation was confirmed by digestion with *MspI* (Promega, Madison, WI) and *HpaII* (Amersham Biosciences Buckinghamshire, England) restriction enzymes.

pCpG-LacZ, a CpG-free vector (InvivoGen, San Diego, CA, USA), was used to study the effect of *hTERT* methylation on transcriptional activity. *hTERT* minimal promoter and exon 1 were generated by PCR, using primers containing either the *SdaI* or the *XbaI* sites. These fragments were cloned into pCpG-LacZ to produce pCpG-Tm (*hTERT* promoter) and pCpG-Tmex1 (*hTERT* promoter and the first 80 bp of the exon 1).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described (18). Then, purified DNA was analyzed by PCR with specific primers for the co-amplification of the first exon of the *hTERT* gene to generate a 171-bp fragment (TERT-FW 5'-CTGCTGCGCACGTGGGAA GCC-3' and TERT-REV 5'-GTCCCCGCGCTGCA CCAGCC-3') or the H19 gene as a 149-bp control (H19-FW 5'-CACCGCCTGGATGGCACGGAATTG-3' and

H19-REV 5'-TGCGACGCGTGGCTTGGGTGAC-3'). Amplification was performed with the following PCR conditions: 94°C for 30 s, 62°C for 45 s and 72°C for 60 s. The cycle number and the amount of template were varied to ensure that results were within the linear range of the PCR. PCR products were analyzed on 2% agarose gel. ChIP experiments were carried out in triplicate to ensure reproducibility.

DNA methylation analysis

DNA was extracted from culture cells and modified with sodium bisulfite, as previously described (31). After bisulfite modification, PCR were performed with primers specifically designed to amplify bisulfite-modified DNA sequence of the promoter and first exon of *hTERT* 5'-CTACCCCTTCACCTTCCAA-3' and 5'-GTTAGTTT TGGGGTTTTAGG-3'. The amplified region corresponded to nucleotide positions 3791-4105 of the unmodified *hTERT* gene sequence (GenBank AF097365). Amplification was performed using the master mix (Promega), with the following PCR conditions: 40 cycles of 94°C for 30 s, 57°C for 45 s and 72°C for 50 s. PCR products were cloned into the pGEM-T vector using the pGEM-T vector system II (Promega). DNA extracted from bacterial clones (QIAprep Spin Miniprep Kit, Qiagen) was analyzed by sequencing with the M13 forward primer (5'-GTAAACGACGGCCAG-3'), using a Big Dye Terminator Cycle Sequencing Kit, and an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA).

To verify the methylation status of the *hTERT* stable transfectant, DNA from cells were extracted with the DNeasy extraction kit (Qiagen) and analyzed by MS-SSCA (Methylation-Sensitive Single Strand Conformation Analysis) (31). The forward Primer used was specific to the plasmid sequence (5'-TGTATGA AGAATTTGTTTAGGGTTA-3'), and the reverse primer was located within the second exon of *hTERT* (5'-AACCACCAACTCCTTCAA-3').

Electrophoretic mobility shift assay (EMSA)

The F1 fragment, which contains part of the first exon of *hTERT*, was synthesized by PCR using the plasmid pTERT-297/ex2 as template, previously sequenced and published (22) and with the following primers previously used (18): 5'-CCTGCTGCGCACGTGGGAAGCC-3' and 5'-GGCAGCACCTCGCGGTAGTGG-3'. Purified fragments were verified by sequencing. Five µg were methylated with 2 units/µg SssI methyltransferase in the presence of 180 µM S-adenosyl-L-methionine for 16 h at 37°C. Following termination of methylation reaction by heating at 65°C for 15 min, the methylation status of plasmid constructs was analyzed by digesting overnight with an excess amount of BstUI. EMSA was then performed as previously described (18).

Transfection and β-galactosidase assays

Stable transfection experiments were performed with the Flp-In system, allowing integration of the gene of interest, always at the same site, in the genome of a mammalian

cell, following the manufacturers' recommendations (Invitrogen). A HeLa Flp-In host cell line containing a single FRT site was created with this system. Positive clones were selected using 500 µg/ml of Zeocin antibiotic. The vector containing the gene of interest was integrated into the genome via Flp-recombinase-mediated DNA recombination at the FRT site. Integration confers hygromycin resistance and Zeocin sensitivity. Cells were selected with 600 µg/ml of Hygromycin antibiotic.

Transient transfection assays were performed with cells seeded at a concentration of either 200 000/3.8 cm² for HeLa and SW480, or 50 000 cells/3.8 cm² for BJ. Cells were then cultured overnight. Transient transfection of reporter plasmids (0.75 µg/well) was carried out in triplicate using JetPEI Cationic Polymer Transfection reagent (4 µl/well) (Polyplus-transfection, Illkirch, France). All experiments were performed at least twice and in triplicate. Analysis of LacZ reporter plasmids was performed with a β-galactosidase assay. Briefly, protein was extracted from cells using 500 µl of the 5X passive lysis buffer (Promega). In a 96-wellplate, 50 µl of cell lysate was analyzed with 100 µl of the β-galactosidase substrate buffer (NaH₂PO₄ 0.2 M, pH 8, β-mercaptoethanol 0.1 M, MgCl₂ 2 mM, ONPG 1.33 mg/ml). The kinetics of the β-galactosidase activity was followed with a colorimeter at 414 nm during 15 min. The kinetics defined a linear curve, and the mean was calculated with values taken at fixed time points. β-galactosidase activity of the different constructs was compared to the level of the pCpG-LacZ vector containing the synthetic LacZΔCpG gene under the control of a mammalian promoter (combination of the CMV enhancer, the human elongation factor 1 alpha core promoter and 5'UTR containing a synthetic intron) and to the level of the pCpG-basic vector, corresponding to that of the pCpG-LacZ vector without its promoter region.

Methylation cassette assay

A methylation cassette assay was used to determine the effect of hypomethylation of a specific regulatory region on *hTERT* transcription. Two restriction sites were introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, CA, USA) in pCpG constructs. The mut1 primers set (FW-mut1 5'-ACCTTCCAGCTCCGGATCC TCCGCGCGGAC-3' and REV-mut1 5'-GTCCGCGC GGAGGATCCGGAGCTGGAAGGT-3') created the upstream site *Bam*HI (indicated in bold) and the mut2 primers set (FW-mut2 5'-CCGCCCTCTCCTCGACGG CGCGAGTTTTCAGG-3' and REV-mut2 5'-CCTGAAA CTCGCGCCGTCGAGGAGAGGGCGG-3') created the downstream site *Taq*I (indicated in bold). The generated plasmids, pCpG-Tm Δ (containing the *hTERT* minimal promoter) and pCpG-Tmex1 Δ (containing the *hTERT* minimal promoter and the first 80 bp of exon 1), were methylated *in vitro* with the *Sss*I methylase following the manufacturers instructions (Promega). The methylated and unmethylated cassette *Bam*HI-*Taq*I 110 bp fragment (−183 to −74 bp of the *hTERT* sequence) were extracted through enzymatic digestion and ligated back into the methylated or unmethylated vector, using the Ligafast

Rapid Ligation System (Promega). The efficiency of the ligation reaction was assessed by analysis of an aliquot of the ligation reaction mixture on a 1% agarose gel. The methylated, unmethylated and partially methylated constructs were confirmed by digestion with *HpaII* restriction enzyme. The remainder of the ligation reaction mixtures was then transfected into HeLa cells. β -galactosidase activities were measured as described.

5-azadC treatment

Treatment with the demethylating agent, 5'-aza-2'-deoxycytidine (5-azadC), was performed on HeLa and SW480 cell lines. Cells were treated 24 h after seeding with 3 μ M of 5-azadC (Flucka, Buchs, Switzerland) in 48-h intervals and for four passages. Cells were then collected and treated for ChIP or used for DNA and RNA extractions (as described below).

RNA extraction, reverse transcription and real-time PCR

RNA was extracted from cells before and after 5-azadC treatment, using the TRIzol LS Reagent (Invitrogen). RNA was then converted to cDNA using random primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR analysis was performed using the TaqMan[®] Universal PCR Master Mix (Applied Biosystems, Foster city, CA) and using the Applied Biosystems 7900HT Real-Time PCR system. CTCF expression was determined using the following primers and probe: 5'-TGACACAGTCATAGCCCGAAAA-3' (FW), 5'-TGCCTTGCTCAATATAGGAATGC-3' (REV) and 6FAM-TGATTTGGGTGTGCACTTGCG AAAGC-MGB (probe). Human Glycerhaldehyde-3-phosphate dehydrogenase (hGAPDH: 99999905) and human Telomerase Reverse transcriptase (*hTERT*: 00162669) primers/probe mixtures were purchased as Predevelopped Assay (Applied Biosystems, Foster city, CA).

RESULTS

Interaction of CTCF with the first exon of *hTERT* is methylation dependent

To evaluate binding of CTCF to sequences within the first exon of *hTERT*, ChIP assays were performed on telomerase-positive tumor cell lines (HeLa, SW480), normal telomerase-negative cells (BJ) and telomerase-positive cells immortalized by *hTERT* transfection (HLF/*hTERT*). CTCF binding to the ICR of *H19* gene was used as a positive control for the efficiency of the experimental protocol. PCR reactions were performed under conditions in which the negative control samples (no antibody) always showed negligible levels of background amplification (data not shown). ChIP experiments were performed in a way that the positive control, H19, was coamplified with the *hTERT* exon 1 in a single reaction and PCR fragments were resolved on the same gel. Results show that CTCF bound to the first exon of the endogenous *hTERT* gene of *hTERT*-negative BJ and HLF/*hTERT* cells but not of the tumor cell lines

tested (Figure 1A). These results support our previous data (18) and are more convincing because positive controls and experimental fragments were amplified in the same reaction.

As methylation of CpG dinucleotides within the CTCF recognition sequence can influence its binding (32), we examined the methylation status of CpG sites within the *hTERT* minimal promoter and the first exon sequences from -200 to +100 from the ATG using bisulfite modification, PCR amplification, and subsequent cloning and sequencing of the PCR products. Clones from two tumor cell lines, HeLa and SW480, two normal cell lines, BJ and HLF/*hTERT*, as well as tumor tissues from breast, bladder and cervical cancer were sequenced and are presented in Figure 2. In tumor tissues and tumor cell lines, almost all CpGs between -100 and +100, including the CTCF binding site (Region B) were methylated (75 to 100% methylated). By comparison, most of the CpGs from approximately -165 to -100 (Region A) were unmethylated (0 to 55% methylated). In BJ and HLF/*hTERT*, very few CpGs in the entire region were methylated (3 to 23%). These findings suggested that methylation of CpGs in Region B might inhibit CTCF binding while leaving unexplained the selective hypomethylation of Region A.

To test whether methylation might interfere with CTCF binding, we used *SssI* methylase to modify a PCR fragment encompassing the first exon of the *hTERT* gene, previously described to be bound by CTCF (18), and compared the native (F1) and modified sequences (F1-met) for their ability to bind CTCF in EMSAs. These studies revealed a marked preference of CTCF for the unmethylated site (Figures 1B and C).

We also tested the effect of CpG methylation on the affinity of CTCF binding *in vivo*. HeLa cells were stably transfected with an *hTERT* construct, pTERT -297/ex2/FRT (Figure 1D) that was methylated *in vitro* before transfection. Genomic DNA of stable transfectants was extracted after 30 population doublings (PD) and the methylation status was analyzed by Methylation-Sensitive Single-Strand Conformation Analysis (MS-SSCA). To differentiate endogenous *hTERT* gene sequences from the transfected sequence, we used a specific forward primer located in the reporter vector (Figure 1D). The transfected *hTERT* sequence remained methylated even after 30 PDs, whereas the unmethylated control transfectant stayed unmethylated (Figure 1E). ChIP assays were performed on methylated and unmethylated stable transfectants. CTCF bound to the *hTERT* exon1 region of the vector sequences only when this region was unmethylated (Figure 1F). Taken together, these results indicated that methylation of the first exon of *hTERT* prevented binding of CTCF both *in vitro* and *in vivo*.

CTCF binding is restored after treatment with 5-azadC

As was shown in previous studies (33–35), treatment of telomerase-positive cells with the demethylating agent 5-azadC inhibits *hTERT* transcription and reduces telomerase activity. We hypothesize that demethylation of the first exon of *hTERT* restores CTCF binding. To test this

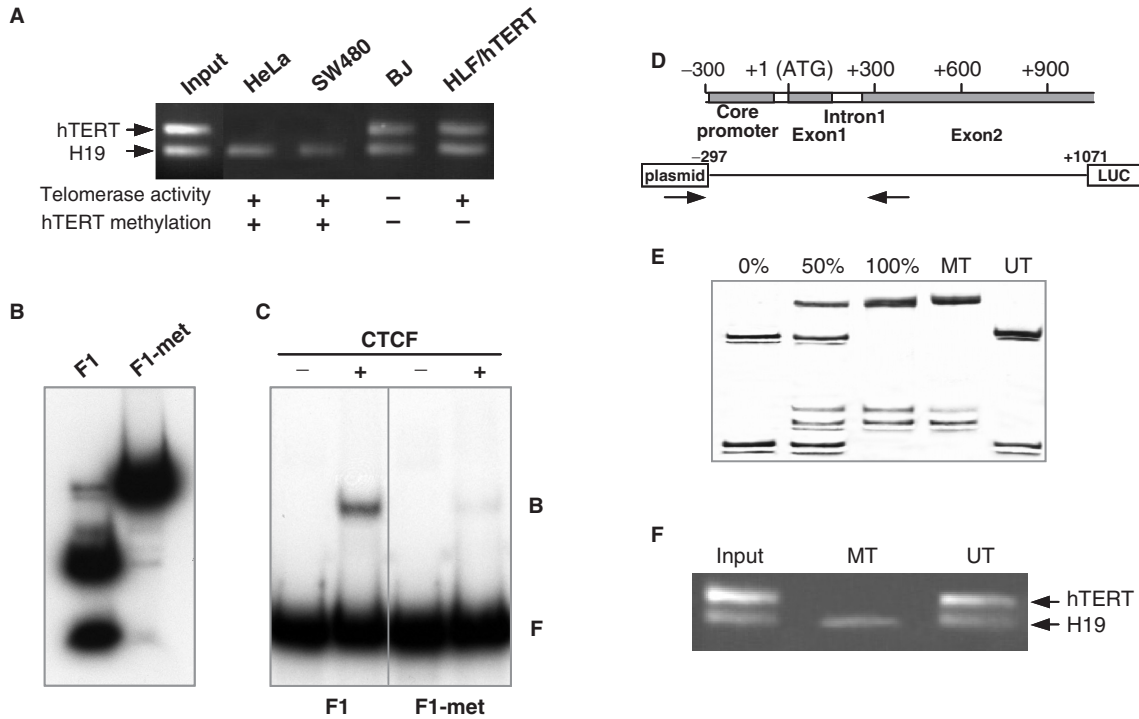


Figure 1. Methylation-sensitive binding of CTCF to the first exon of *hTERT*. **(A)** *In vivo* binding of CTCF to the first exon of *hTERT* in telomerase-positive and negative cell lines was analyzed by ChIP assay using anti-CTCF antibody. PCR coamplification of the test fragments (*hTERT* and H19) using as template DNA input fraction and DNA recovered from immunoprecipitated fractions bound by the anti-CTCF antibody. **(B)** 5'-end-labeled control (F1) and SssI methylase-treated (F1-met) fragments were digested with methylation-sensitive BstUI and analyzed on polyacrylamide gels to verify efficiency of *in vitro* methylation. **(C)** Control unmethylated (F1) or SssI-methylated (F1-met) fragments were analyzed by gel-shift assay (EMSA). F, free probe; B, CTCF-bound probe. **(D)** Representation of the *hTERT* sequence cloned into the pTERT-297/ex2/FRT. Arrows represent the localization of the primers used for *hTERT* methylation analysis of stable transfectant. **(E)** The methylation status of the stable transfectants was verified by MS-SSCA. Unmethylated and fully methylated controls were obtained from plasmids used for stable transfection. UT and MT represent, respectively, the unmethylated and methylated plasmids stably transfected into HeLa cells, and after 30 population doublings. **(F)** Binding of CTCF to the first exon of *hTERT* in stably transfected cell line was analyzed by ChIP assay using anti-CTCF antibody.

hypothesis, we treated HeLa and SW480 cells with 5-azadC for 4 weeks. Methylation of *hTERT* as determined by bisulfite sequencing showed that CpG methylation in Region B was reduced from that in untreated cells (54 and 64% methylated after 5azadC treatment versus 93 and 90% methylated in untreated HeLa and SW480 cells, respectively) (Figure 2 and 3A). ChIP analyses revealed significant CTCF binding to *hTERT* first exon sequences from treated cells (Figure 3B). In addition, real-time PCR analyses of *hCTCF* mRNA levels showed no significant differences before and after 5azadC treatment. In contrast, the *hTERT* mRNA level, which is high in HeLa and SW480 before 5azadC treatment, cannot be detected after 5azadC treatment (Figure 3C).

Total methylation of *hTERT* inhibits transcriptional activity: a region of the *hTERT* minimal promoter must be hypomethylated to allow its transcriptional activity

To evaluate the importance of methylation for *hTERT* activation, HeLa cells were transiently transfected with CpG-free plasmids containing a *hTERT* promoter that was previously methylated *in vitro*. The *hTERT* minimal

promoter did not show any activity if all the CpG sites were methylated (Figure 4A). Likewise, no transcriptional activity was observed in a construct with methylated promoter and the first exon of *hTERT*. These results showed that transcription of *hTERT* was completely repressed by full methylation of the promoter. Sequencing of bisulfite-modified DNA from telomerase-positive tumor cell lines (HeLa and SW480) and tumor tissues (breast, bladder and cervix) showed partial demethylation of the promoter, between -160 to -80 bp (Figure 2, Region A). A recent study has also described partial methylation of the promoter region in telomerase-positive cell lines (35). This hypomethylation, just upstream of the transcriptional start sites, might permit the low transcriptional activity of the *hTERT* promoter observed in tumor cells (36,37). To test the role of methylation of this region in the regulation of *hTERT* transcriptional activity, we performed a methylation cassette assay (38). A β -galactosidase reporter plasmid was generated, containing a cassette with sequences from -183 to -73 bp, encompassing 12 CpG sites and representing the region A described in Figure 2. The methylated or unmethylated cassette was excised and ligated back into the vector, which was then transfected

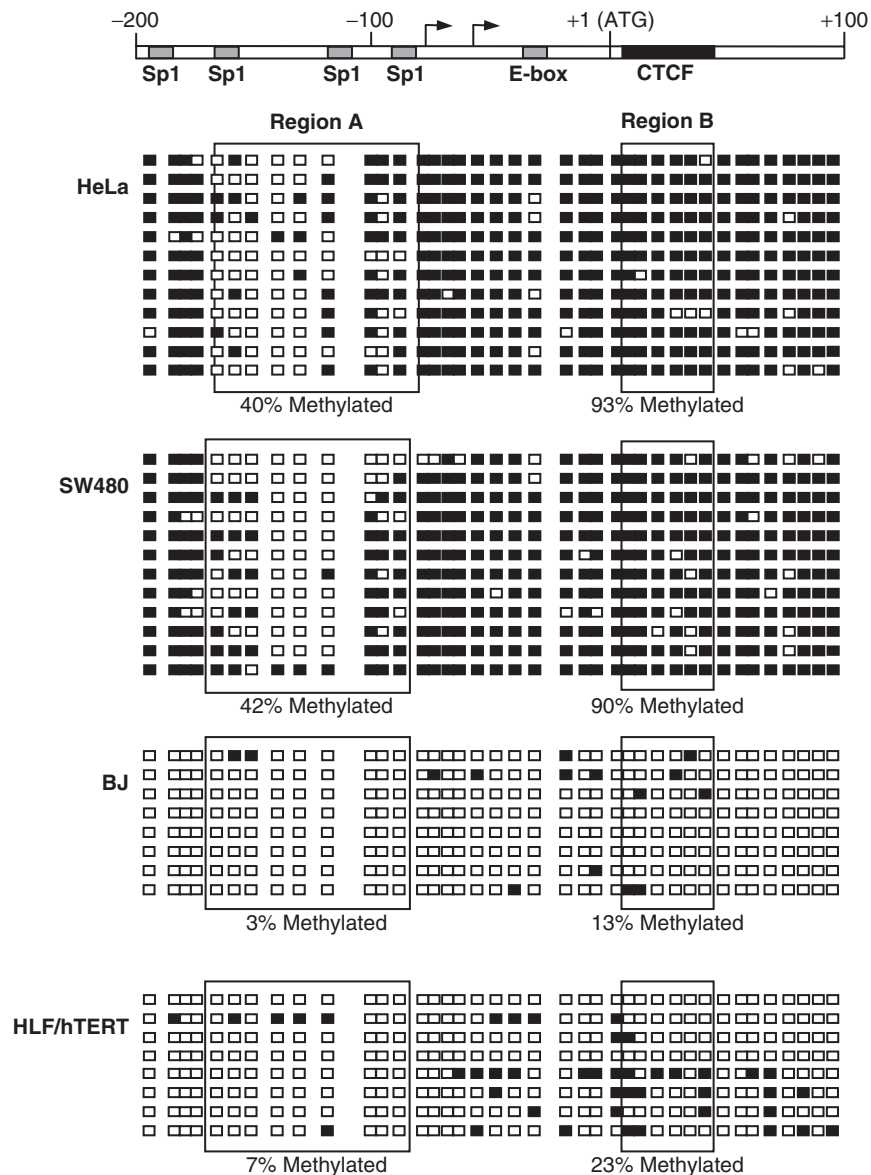


Figure 2. Genomic bisulfite sequencing of *hTERT* promoter and 5' exon region (–200 to +100 nucleotide bases around the ATG translational start site). After PCR amplification of bisulfite-modified DNA and cloning into pGEM-T vector, 12 clones of telomerase-positive cell lines (HeLa and SW480), 8 clones of endogenously telomerase-negative cells (BJ and HLF/*hTERT*) and 8 clones from telomerase-positive tumor tissues (breast, bladder and cervix) (Figure 2) were analyzed by sequencing. Clones of each are shown on the figure. Each square represents one CpG site. Filled squares: methylated; open squares: unmethylated. Region A: in this region (–80 to –165 bp), multiple CpG sites were found to be unmethylated in tumors tissues and cell lines. Region B: localization of the CTCF binding site within the first exon of the *hTERT* gene, most of the CpG sites were found to be methylated in telomerase-positive tumor tissues and cell lines. Percentages of methylated CG are indicated for regions A and B.

into HeLa cells. Methylation of the cassette reduced the activity of the *hTERT* promoter to the background levels, a result similar to that obtained with the fully methylated promoter (Figure 4B). In contrast, the unmethylated cassette, ligated to the methylated vectors, reduced transcriptional activity of the minimal promoter nine-fold compared to the activity obtained with the same unmethylated reporter. When exon 1 is present in the construct, the activity is reduced only two-fold. Moreover, if we compare fold difference between the minimal promoter activity and the minimal promoter+exon1 in cassette methylation experiments, the difference is very

significant when the plasmids are unmethylated; but when the exon1 is methylated, there is almost no difference (Figure 4C). These results suggested that the hypomethylation of the region A (Figure 2), which contains three of the four Sp1 binding sites, was permissive for low levels of *hTERT* expression, comparable to those seen *in vivo* (36,37), and that the methylation of the exon1 allowed the transcriptional level to be likely the same than with the minimal promoter only. These results also suggested that the regions surrounding the region A in the minimal promoter might be sites for a strong activator that might have binding sites sensitive to methylation.

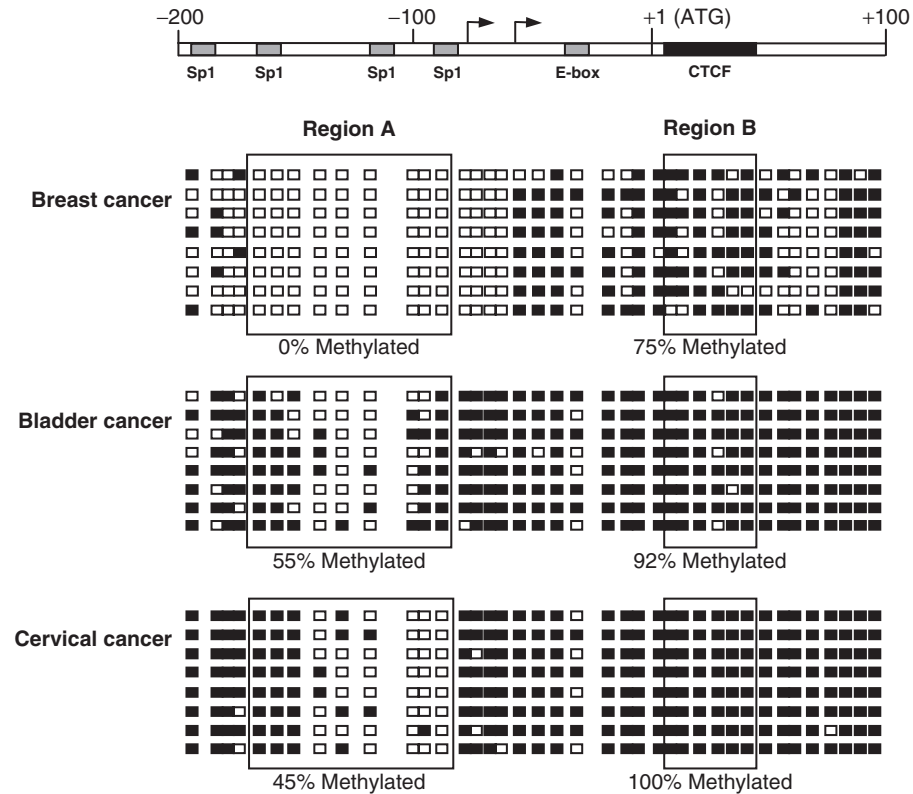


Figure 2. Continued.

DISCUSSION

The aim of the present study was to define more precisely the role of CTCF and DNA methylation in transcriptional regulation of the *hTERT* gene. CTCF binds preferentially to GC-rich DNA regions, exerts an inhibitory effect when bound downstream of a transcriptional start site (39) and may be released when its binding site is methylated (32). In a previous study, we showed that CTCF binds to GC-rich regions within exons 1 and 2 of the *hTERT* gene, irrespective of the cell type or the promoter used. In the *hTERT* CpG island, binding of CTCF might be influenced by the methylation status of its sites. In the present study, ChIP assays revealed that CTCF binds to the first exon of *hTERT* when the *hTERT* CpG island is not methylated. In contrast, CTCF no longer binds its recognition sequence when this site is methylated. Hypermethylation of its binding site can therefore abolish CTCF repressor activity. This was confirmed by inducing demethylation with 5-azadC, which allowed CTCF to bind to the first exon region and repress *hTERT* expression. Therefore, the main role of *hTERT* methylation in tumor cells is probably to prevent binding of the CTCF repressor and, as a consequence, to allow transcription of the *hTERT* gene.

The observation that 5-azadC treatment results in *hTERT* gene repression was confirmed by other groups (33–35). Nevertheless, these results are apparently in marked contrast to two other reports showing activation of *hTERT* following treatment of telomerase-negative

ALT cells upon 5-azadC (25,26). These seemingly contrasting effects might be explained as a result of the balance between demethylation of the *hTERT* first exon region allowing binding of CTCF and, possibly, demethylation of genes necessary for *hTERT* transcription. Indeed, in luciferase reporter gene assays, it was shown that the *hTERT* promoter is much less active in ALT cells (e.g. U2-os) than in telomerase-positive tumor cell lines (21). Further studies of ALT cells should be undertaken to develop a better understanding of this phenomenon.

Promoter methylation is an epigenetic process most commonly associated with transcriptional repression. Methylation of DNA helps to stabilize chromatin in an inactive configuration, thereby inhibiting gene expression (28). As expected, our results clearly show that methylation of all the CpG sites within the *hTERT* promoter CpG island resulted in complete transcriptional repression, in spite of the inability of CTCF repressor to bind to the *hTERT* gene. In this condition, *hTERT* does not provide a real exception to the general model of gene silencing by promoter methylation. However, even if *hTERT* is generally hypermethylated in telomerase-positive tumor cells (29), we showed in the present study that a region between –165 to –80 bp upstream of the translational start site was hypomethylated in tumor tissues and cell lines. This region contains three of the four Sp1 sites present in the *hTERT* core promoter. We used cassette methylation assay to demonstrate the importance of this

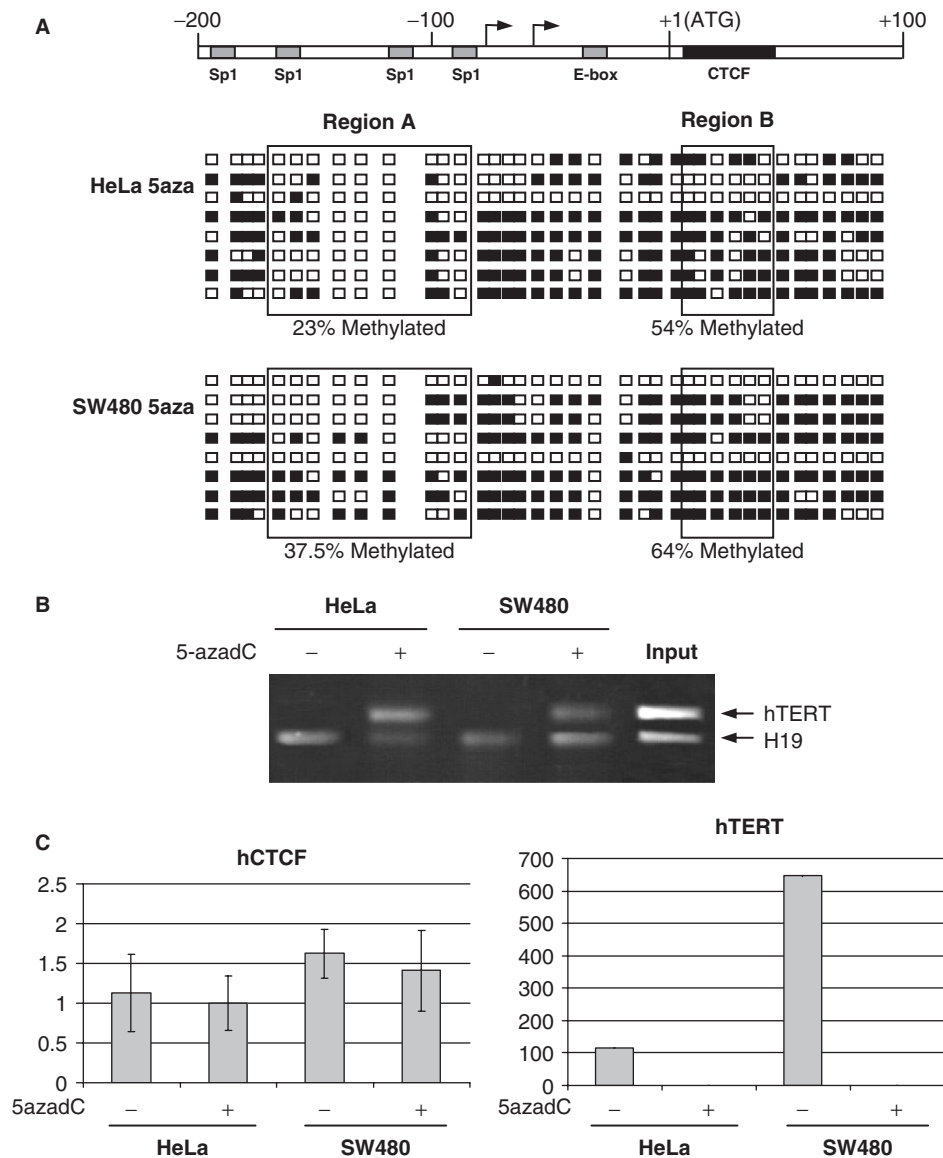


Figure 3. CTCF binding to the exogenous *hTERT* sequence after 5-azadC treatment. **(A)** Genomic bisulfite sequencing of *hTERT* promoter and first exon region (-200 to +100 nucleotide bases around the ATG translational start site). After PCR amplification of bisulfite-modified DNA and cloning into pGEM-T vector, 8 representative clones of HeLa and SW480 are shown. Each square represents one CpG site. Filled squares: methylated; open squares: unmethylated. Region A and region B were represented for an easy comparison with Figure 2, and percentages of methylated CG are indicated for regions A and B. **(B)** Binding of CTCF to the first exon of *hTERT* in 5aza-dC cell lines was analyzed by ChIP assay using anti-CTCF antibody. PCR coamplification of the test fragments (*hTERT* exon1 and H19) using as template DNA input fraction and DNA recovered from immunoprecipitated fractions bound by the anti-CTCF antibody. **(C)** Quantitative reverse transcription-PCR analyses of *hCTCF* and *hTERT* expression before and after 5azadC treatment. GAPDH expression is used to normalize samples.

region for the transcriptional activity of the *hTERT* promoter. A recent study also showed that *hTERT* transcription might require partial methylation of its promoter (35). However, our data clearly indicate that selective partial demethylation of 12 CpGs limited to the small region upstream the transcription start site, defined as region A, significantly activates *hTERT* promoter in a reporter plasmid. Nevertheless, the activity of the promoter in these conditions is significantly lower than the activity obtained with the unmethylated minimal promoter. These results suggested that the regions

surrounding the minimal promoter region A might be the target of transcriptional factors other than CTCF, whose binding are sensitive to methylation. The low expression level of the *hTERT* showed with this partial demethylation of the core promoter corresponded rather well to the level of *hTERT* expression observed *in vivo*. Indeed, the *hTERT* mRNA levels detected in telomerase-positive cell lines are very low, 0.2 to 6 copies per cell (36,37). This is in stark contrast with the high level of transcriptional activity obtained in transient transfection of the *hTERT* core promoter in

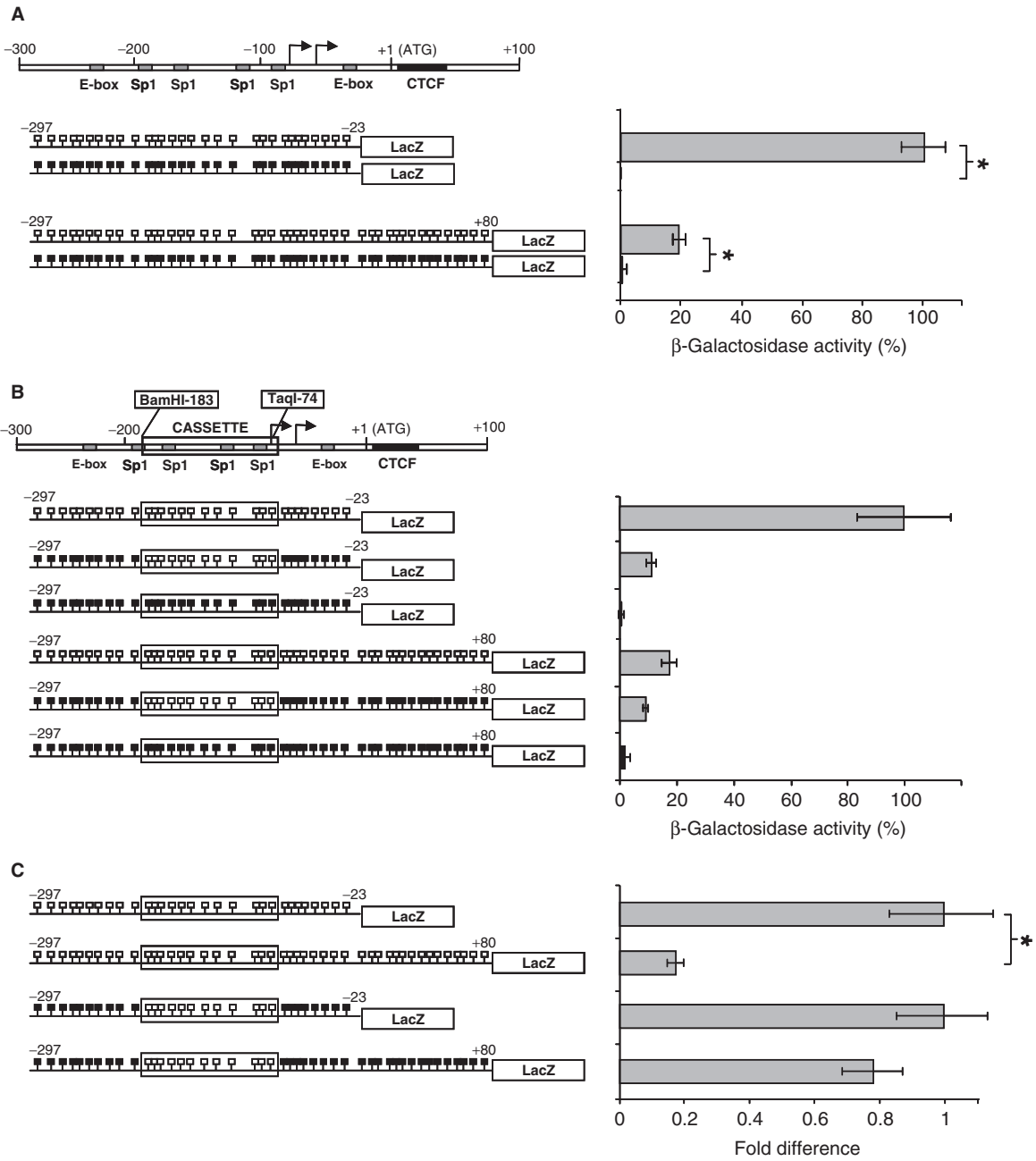


Figure 4. Effect of the *hTERT* promoter methylation on transcriptional activity. **(A)** Transcriptional activity of the *hTERT* minimal promoter was assayed, with or without the presence of the *hTERT* exon 1 in an unmethylated CpG free plasmid. Methylated and unmethylated constructs were transiently transfected in HeLa cells. The 100% activity is represented by the activity of unmethylated promoters. **(B)** Transcriptional activity of the partially methylated *hTERT* minimal promoter was assayed in an unmethylated CpG free plasmid. Different constructs were transiently transfected in HeLa cells. The 100% activity is represented by the activity of unmethylated plasmid. **(C)** Fold difference activities between unmethylated and partially methylated plasmids. The *hTERT* minimal promoter is taken as 1 in each case. Empty squares represent unmethylated CpG sites, and solid squares represent methylated CpG sites. * $P < 0.005$ by Student's t-test.

telomerase-positive cell lines, a level comparable to that induced by strong SV40 early promoter (8).

Methylation cassette experiments shows that the expression levels of the construct with partially methylated *hTERT* minimal promoter and methylated exon 1 in transient transfection is very close to the levels of the endogenous *hTERT* in human cancers showing similar methylation pattern. The methylation of the exon1 of

hTERT seems to be sufficient to allow the transcription from the minimal promoter, and does no more exert its inhibitory effect as seen in unmethylated constructs. Moreover, a CTCF site located at the beginning of exon 2 also plays an important role in the downregulation of the constructs with unmethylated *hTERT* (18). However, as previously mentioned, the presence of an intron between the two CTCF sites complicates the

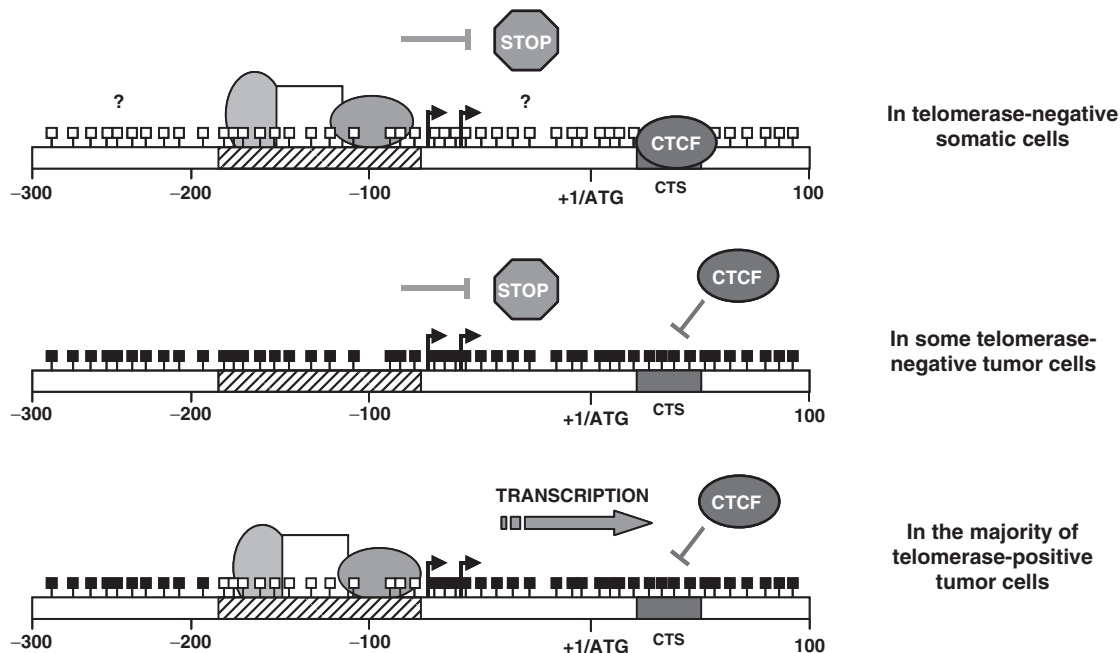


Figure 5. Proposed model of *hTERT* transcriptional regulation. In telomerase-negative somatic cells, CTCF binds to unmethylated CTCF binding site and inhibits *hTERT* transcription. Full methylation of the *hTERT* minimal promoter and exon1 in some telomerase-negative tumor cells cannot lead to *hTERT* transcription. A majority of the tumor CTCF sites within the *hTERT* gene are methylated, thus preventing CTCF binding. Partial demethylation of the *hTERT* promoter region with the formation of an active transcriptional complex can lead to *hTERT* transcription and telomerase activity. CTS stands for CTCF target sites. The hatched box represents the region A showed as unmethylated in cancer cell lines and tumor tissues. Question marks represent the two regions surrounding the region A, and that might have a strong influence on the *hTERT* promoter activity, most probably methylation-sensitive binding sites to transcription factors. Empty squares represent unmethylated CpG sites, and solid squares represent methylated CpG sites.

interpretation of the transfection assays (22). Nevertheless, in our proposed model for the transcriptional regulation of the *hTERT* gene (Figure 5), we took the effect of the two CTCF sites into account.

Although promoter methylation might be one of the main mechanisms involved in *hTERT* regulation in tumor tissues and cell lines (27,40,41), methylation-independent mechanisms have also been identified (25,26,41–43). In normal tissues, we observed that the *hTERT* gene was not methylated in testis, a site where telomerase is highly expressed (unpublished data). It is interesting to note that expression of an *hTERT* transgene in mice remained high, especially in testis, regardless of whether the transgene comprised only an 8-kbp region of the *hTERT* promoter or the entire *hTERT* gene promoter (21,44). According to Horikawa *et al.*, the use of the entire *hTERT* promoter in mouse leads to a human-like pattern of *hTERT* expression, suggesting that specific regulatory sequences, rather than mouse background, determines differences in *hTERT* and *mTERT* expression. Together, these data suggest that tissue-specific factors present in testis and in some tumor cells could prevent binding of the CTCF repressor to the first two exons of *hTERT* and thus lead to *hTERT* expression. According to previous data, the testis-specific factor BORIS, a paralog of CTCF (45), might be a good candidate, and further studies will be necessary to investigate its potential role in the *hTERT* regulation.

In summary, *hTERT* expression is induced when the *hTERT* CpG island is sufficiently hypermethylated to

avoid binding of the CTCF repressor and when a small part of the core promoter region is hypomethylated to allow the transcription complex to be formed. Under these conditions, additional regulators can come into play to effectively induce *hTERT* expression. Figure 5 illustrates the dual role of DNA methylation in the transcriptional regulation of the telomerase *hTERT* gene. The complexity of *hTERT* regulation might hamper the development of anticancer therapies targeting telomerase and calls for further studies.

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