hTERT Is Expressed in Cancer Cell Lines Despite Promoter DNA Methylation by Preservation of Unmethylated DNA and Active Chromatin around the Transcription Start Site

Rebekah L. Zinn,[1,2] Kevin Pruitt,[1] Sayaka Eguchi,[1] Stephen B. Baylin,[1,2] and James G. Herman[1,2]

[1]Sidney Kimmel Comprehensive Cancer Center and [2]Graduate Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

hTERT, which encodes the catalytic subunit of telomerase and is expressed in most immortalized and cancer cells, has been reported to have increased DNA methylation in its promoter region in many cancers. This pattern is inconsistent with observations that DNA methylation of promoter CpG islands is typically associated with gene silencing. Here, we provide a comprehensive analysis of promoter DNA methylation, chromatin patterns, and expression of *hTERT* in cancer and immortalized cells. Methylation-specific PCR and bisulfite sequencing of the *hTERT* promoter in breast, lung, and colon cancer cells show that all cancer cell lines retain alleles with little or no methylation around the transcription start site despite being densely methylated in a region 600 bp upstream of the transcription start site. By real-time reverse transcription-PCR, all cancer cell lines express *hTERT*. Chromatin immunoprecipitation (ChIP) analysis reveals that both active (acetyl-H3K9 and dimethyl-H3K4) and inactive (trimethyl-H3K9 and trimethyl-H3K27) chromatin marks are present across the *hTERT* promoter. However, using a novel approach combining methylation analysis of ChIP DNA, we show that active chromatin marks are associated with unmethylated DNA, whereas inactive marks of chromatin are associated with methylated DNA in the region around the transcription start site. These results show that DNA methylation patterns of the *hTERT* promoter (−150 to +150 around the transcription start) are consistent with the usual dynamics of gene expression in that the absence of methylation in this region and the association with active chromatin marks allow for the continued expression of *hTERT*. [Cancer Res 2007;67(1):194–201]

Introduction

It has been well documented that cytosine residues in the context of CpG dinucleotides can become methylated by DNA methyltransferases (DNMT). Normally, DNA methylation occurs at CpG sites scattered throughout the genome, usually within noncoding regions, whereas CpGs that exist in clusters over small stretches of DNA, termed "CpG islands," tend to be unmethylated. However, during cancer progression, there is a genome-wide hypomethylation of CpG sites and hypermethylation of CpG islands in promoter regions of genes (1). Hypermethylation of CpG islands in gene promoters is associated with gene silencing (2). Silencing of such genes can be associated with the loss of tumor suppressor function, which has been shown for many tumor suppressor genes, including *p16*, a cell cycle control gene, and *hMLH1*, a mismatch repair gene (3, 4).

In addition to DNA methylation as a form of epigenetic control of gene expression, histones also play a role. Modification of amino acid residues on histone tails, such as acetylation, methylation, phosphorylation, or ubiquitination, can serve as a code for gene expression or repression (5). Hyperacetylated histones and histone H3 methylated at lysine 4 are common marks of active chromatin and have typically been associated with unmethylated DNA. In contrast, hypoacetylated histones and methylation of histone H3 at lysine 9 and 27 are generally associated with inactive genes and hypermethylated DNA (6–10).

The regulation of telomerase in cancer has seemingly challenged some of these well-established patterns of gene silencing. Telomeres, which are tandem repeat sequences of DNA that protect the ends of chromosomes, are maintained by telomerase, a RNA-dependent DNA polymerase (11). Telomerase consists of two essential components: *hTERT*, the catalytic subunit that has reverse transcriptase activity, and *hTR*, a RNA component that serves as a template for the repeat sequence (12). Expression of *hTERT* determines telomerase activity because *hTR* is ubiquitously expressed in most cells (13, 14). *hTERT* is expressed at high levels in embryonic stem cells and germ cells, down-regulated during differentiation and development, and silenced in fully differentiated somatic cells. However, *hTERT* is frequently reactivated through an unknown mechanism in 80% to 95% of immortalized and cancer cells, allowing these cells to survive with shortened telomeres (12).

Like many human genes, *hTERT* contains a CpG island in its promoter region. Surprisingly, some reports suggest increased DNA methylation in this area in *hTERT*-positive cancer cells and lack of methylation in normal *hTERT*-negative cells (15–19). However, other reports of *hTERT* promoter DNA methylation suggest that methylation of the *hTERT* promoter is associated with gene silencing (16, 17, 20–22). Collectively, these studies leave in question the involvement of DNA methylation in the regulation of *hTERT*. These unusual correlations between DNA methylation and expression in cancer cells result in part from the varied methods used to study differing regions of the *hTERT* promoter and from not using quantitative measures of *hTERT* expression. Furthermore, key aspects of chromatin modifications and their association with DNA methylation patterns and gene expression have not been thoroughly studied for *hTERT*. Therefore, we examined these relationships in more detail, including allele-specific patterns in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: James G. Herman, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Suite 541, 1650 Orleans Street, Baltimore, MD 21231. Phone: 410-955-8506; Fax: 410-614-9884; E-mail: hermanji@jhmi.edu.

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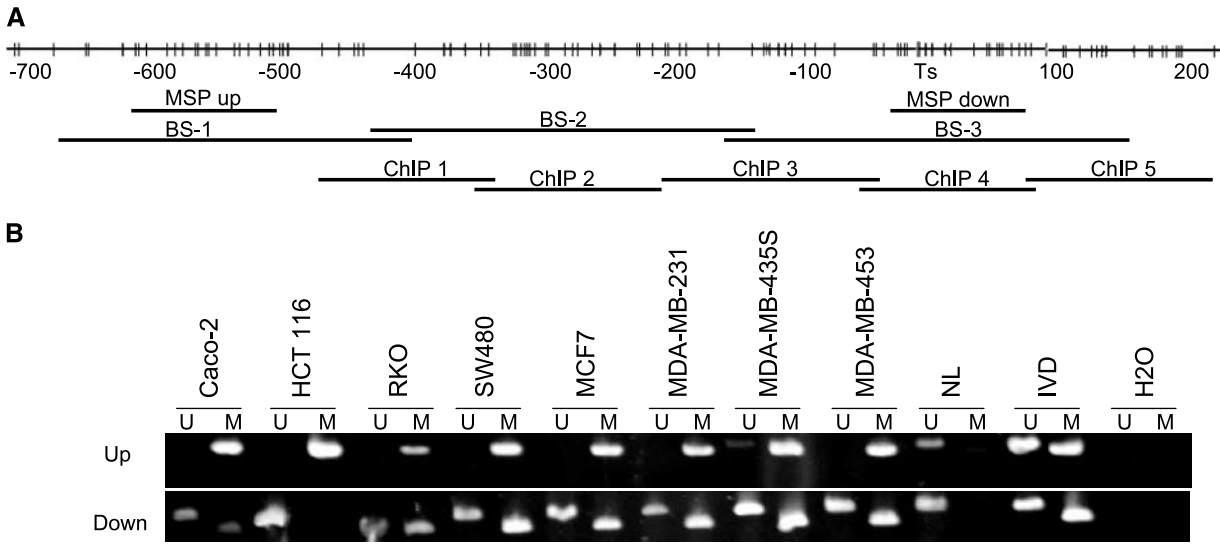

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Cell Line	MSP Up	MSP Down
Caco-2		
RKO		
SW480		
HCT 116		
DNMT1 -/-		
DNMT3b -/-		
DKO		
MCF7		
MDA-MB-231		
MDA-MB-435S		
MDA-MB-453		
H82		
H157		
H209		
H146		
H358		
H417		
H549		
H727		
H1299		
U1752		
DMS53		
HL-60		
KG-1a		
Jurkat		
Raji		
LCL		
VA13		
NL		
HMEC		

Figure 1. Cancer cell lines are densely methylated in a region upstream of the transcription start site, whereas the region containing the transcription start site is only partially methylated or completely unmethylated. *A,* map of *hTERT* promoter region showing location of CpG sites (*tick marks*), transcription start site (*Ts*), and primers used for MSP (*MSP up* and *MSP down*), bisulfite sequencing (*BS-1, BS-2*, and *BS-3*), and ChIP (*ChIP-1, ChIP-2, ChIP-3, ChIP-4*, and *ChIP-5*). The transcription start site is nucleotide 1,666 in National Center for Biotechnology Information accession no. AF098956. *B,* MSP results for colon cancer cell lines (Caco-2, HCT 116, RKO, and SW480), breast cancer cell lines (MCF7, MDA-MB-231, MDA-MB-435S, and MDA-MB-453), normal lymphocytes (NL), and, as a control, *in vitro* methylated DNA (IVD). *Top,* results using the MSP up primer set; *bottom,* results using the MSP down primer set. *C,* summary of methylation in cell lines determined using MSP. Cell lines included colon cancer cells listed above and HCT 116 DNMT knockout cells (DNMT1 −/−, DNMT3b −/−, and DKO), breast cancer cells listed above, lung cancer cells (H82, H157, H209, H146, H358, H417, H549, H747, H1299, U1752, and DMS53), leukemia cells (HL-60, KG-1a, Jurkat, and Raji), an EBV-immortalized cell line (LCL), a SV40-transformed lung fibroblast (VA13), normal lymphocytes (NL), and HMECs. *Black box,* complete methylation; *gray box,* partial methylation; *white box,* lack of methylation.

neoplastic cells. Our findings resolve what has seemed, in the past, a paradox for the relationship of *hTERT* expression and promoter DNA methylation in cancer cells.

Materials and Methods

DNA isolation and bisulfite treatment. Genomic DNA was isolated from cells using Wizard Genomic DNA Purification kit following the manufacturer's instructions (Promega, Madison, WI). Bisulfite modification was done as described previously (23). Briefly, ~1 μg of genomic DNA was denatured by NaOH (final concentration, 0.2 mol/L) for 10 min at 37°C. Hydroquinone (10 mmol/L, 30 μL) and 520 μL of 3 mol/L sodium hydroxide (pH 5) were added, and samples were incubated at 50°C for 16 h. Modified DNA was purified using Wizard DNA Clean-Up System following the manufacturer's instructions (Promega) and eluted into 50 μL water. DNA was treated with NaOH (final concentration, 0.3 mol/L) for 5 min at room

temperature, ethanol precipitated, and resuspended in 20 μL water. Modified DNA was used immediately or stored at −20°C.

Methylation-specific PCR. Primers specific for unmethylated and methylated alleles of *hTERT* were used to amplify bisulfite-modified DNA. Methylation-specific PCR (MSP) primers were designed for an upstream (~600 bp from transcription start site) and downstream region (encompassing the transcription and translation start sites) of the *hTERT* promoter. The primer sequences and conditions are shown in Supplementary Table S1 and location can be seen in Fig. 1*A*. PCR was done using 1 μL of bisulfite-treated DNA and JumpStart Red Taq DNA Polymerase (Sigma, St. Louis, MO). PCR products were analyzed by PAGE.

Bisulfite sequencing. Bisulfite-modified genomic DNA was analyzed using three primer sets spanning a region from −650 to +150 from the transcription start site of *hTERT*. The primer sequences and conditions are shown in Supplementary Table S1 and their locations are shown in Fig. 1*A*. All PCRs were done using JumpStart Red Taq DNA Polymerase. PCR products were run on 1.5% agarose gels and bands were excised using QIAquick Gel Extraction kit following the manufacturer's instructions (Qiagen, Valencia, CA). Purified bands were cloned using TOPO-TA cloning kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Colonies were selected and grown overnight in Luria-Bertani medium containing ampicillin (100 μg/mL) with shaking at 37°C. Plasmid DNA was isolated using QIAprep Spin Miniprep kit following the manufacturer's instructions (Qiagen). Plasmids were screened for inserts by *Eco*RI digestion and sequenced using the M13 universal reverse primer (Invitrogen).

Real-time reverse transcription-PCR. RNA was isolated from cells using RNeasy Mini kit following the manufacturer's instructions (Qiagen). RNA (~2.5 μg) was reverse transcribed to cDNA using SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) using the oligo(dT) primer according to the manufacturer's instructions (Invitrogen). Real-time RT-PCR was done using HotStar Taq DNA Polymerase (Qiagen) and SYBR Green (Molecular Probes, Carlsbad, CA) in an iCycler Optical Module (Bio-Rad, Hercules, CA). Reactions were done in triplicate using 1 μL cDNA per reaction and primers specific for *hTERT* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; see Supplementary Table S1). *hTERT* expression levels were normalized to *GAPDH* for each cell line and calculated relative to normal lymphocytes (control) using the following equation: relative expression = $2^{-(\text{Sample } \Delta Ct \ - \ \text{Control } \Delta Ct)}$, where ΔCt = average *C*t (*hTERT*) − average *C*t (*GAPDH*).

Single-cell cloning. SW480 (colon cancer) cells were maintained in McCoy's 5A modified medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Mediatech). MDA-MB-231 (breast cancer) cells were grown in DMEM (Mediatech) supplemented with 10% bovine calf serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin. All cells were grown at 37°C in 5% CO_2 atmosphere. Conditioned medium was collected

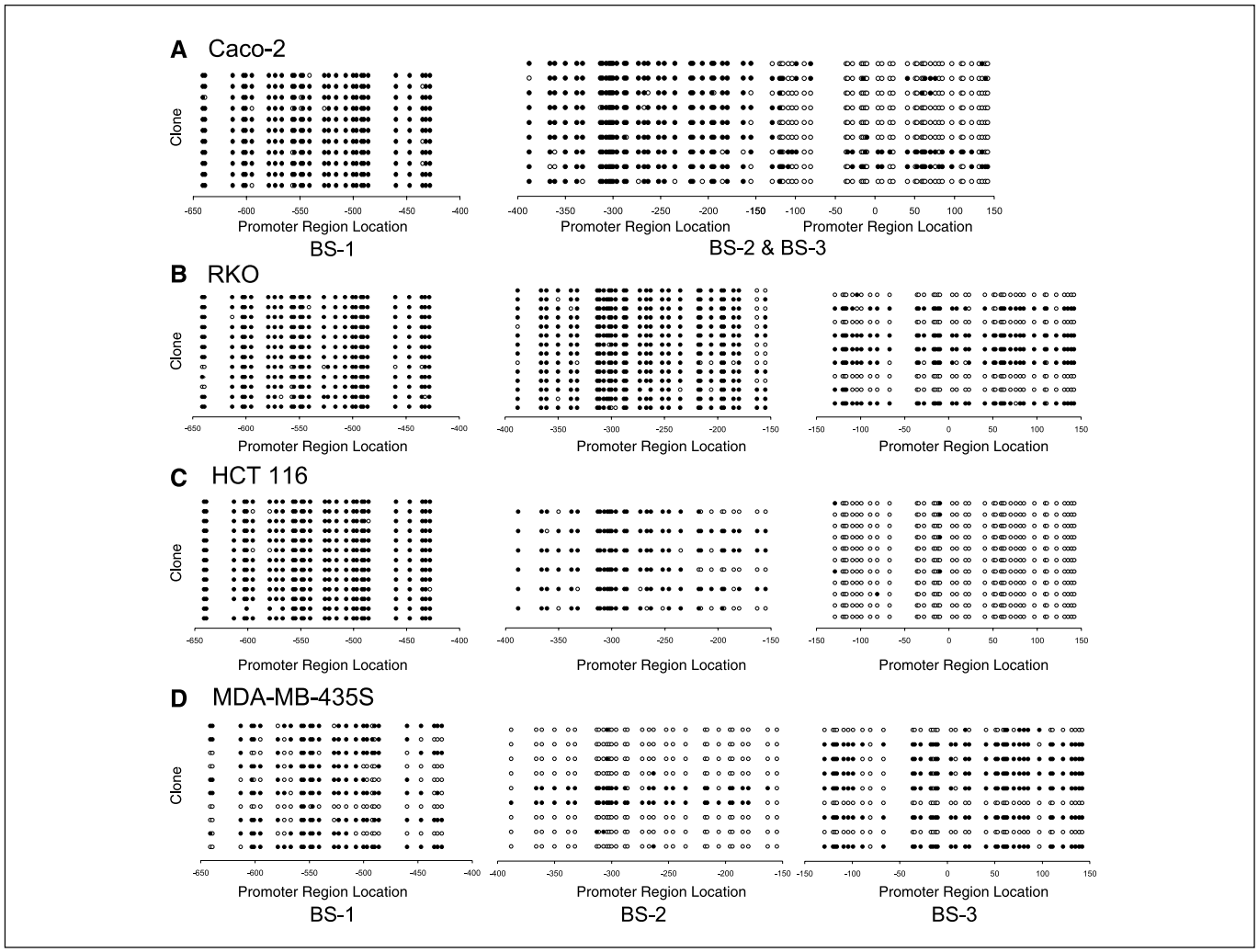

Figure 2. Bisulfite sequencing of cancer cell lines. Bisulfite-treated DNA was PCR amplified using three primer sets (BS-1, BS-2, and BS-3; Fig. 1*A*) spanning the promoter region of *hTERT*. PCR products were cloned, and multiple clones were sequenced and analyzed for their methylation status. Results are shown for (*A*) Caco-2, (*B*) RKO, (*C*) HCT 116, and (*D*) MDA-MB-435S cancer cell lines. *Circle,* CpG site. ●, methylated CpG sites; ○, unmethylated CpG sites. The CpG sites are mapped in reference to the transcription start site. For Caco-2, the BS-2 forward and BS-3 reverse primers were used and the results are combined into one graph.

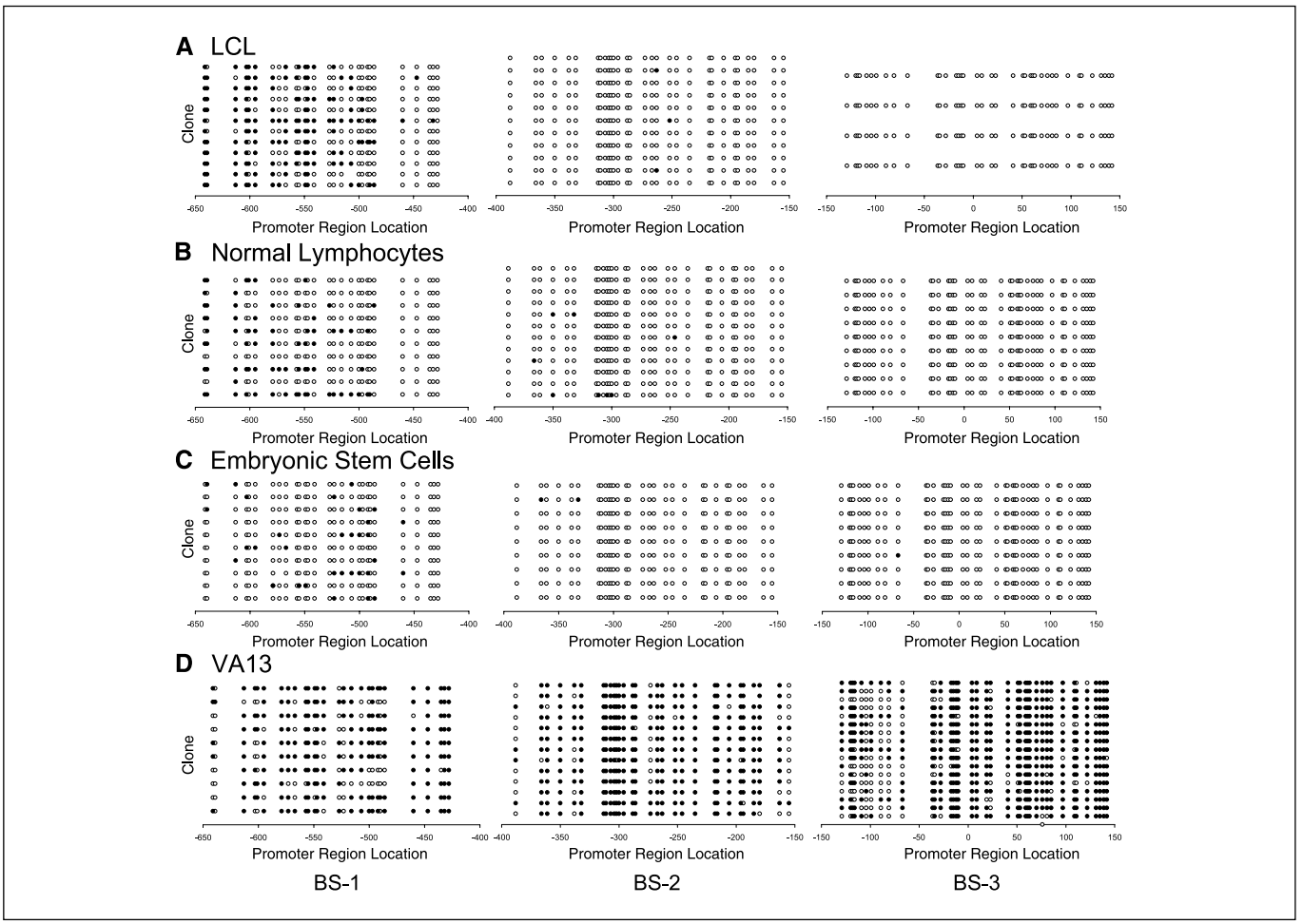

Figure 3. Bisulfite sequencing of immortalized and nontransformed cells. Results are shown for (*A*) LCL, (*B*) normal lymphocytes (NL), (*C*) embryonic stem cells (ES), and (*D*) VA13 cells using bisulfite sequencing primers (BS-1, BS-2, and BS-3) shown in Fig. 1*A*.

from cells when they were 50% to 80% confluent. Limiting dilutions were done in 96-well plates to obtain single-cell clones. Plates were scored after 4 days for wells that contained only a single colony of cells. All wells with more than one visible colony were excluded. Several single-cell clones for each cell line were obtained and grown in 50% conditioned medium for several passages. Isolation of DNA and RNA was done after four or five passages when cell number was sufficient for analysis.

Chromatin immunoprecipitation. Cross-linking was done as described previously (9). Chromatin immunoprecipitation (ChIP) was done using the ChIP Assay kit (Upstate, Charlottesville, VA) with some modifications to the protocol. Briefly, ~100 μg chromatin per antibody was resuspended in SDS lysis buffer and sonicated (Branson Sonifier, Danbury, CT) with twenty 10-s pulses to shear DNA to 200 to 800 bp. Modifications to the protocol were that no preclear was done and a 1:1 mixture of protein A to G agarose beads was used. Antibodies for acetyl-H3K9, dimethyl-H3K4, trimethyl-H3K9, and trimethyl-H3K27 were all purchased from Upstate Chemicon and used to capture protein-DNA complexes. DNA was recovered by using QIAprep Spin Miniprep kit columns and solutions. Briefly, 5 volumes of PB buffer were added to DNA and applied to columns and washed with PE buffer. DNA was eluted in 100 μL EB buffer. ChIP PCR analysis was done by using 2 to 3 μL of ChIP DNA and primers spanning the region −500 to +150 of *hTERT*. PCR products were analyzed by PAGE and quantified using Kodak (Rochester, NY) Digital Science 1D Image Analysis software. Enrichment was calculated by taking the ratio of the net intensity of bound (immunoprecipitated) DNA over input (unimmunoprecipitated) DNA. These values were calculated for at least two independent ChIP experiments and averaged. Control PCRs for each antibody immunoprecipitation were done using primers for *GAPDH* (24) and *SFRP1* (25). *hTERT* primer sequences and conditions are provided in Supplementary Table S1 and their location is shown in Fig. 1*A*.

ChIP-MSP. Approximately 50 μL of the 100 μL ChIP product were bisulfite treated as described above. MSP was done using the *hTERT* MSP downstream primers near the transcription start site or using primers for p16 (26).

Results

For an initial assessment of the methylation status of the *hTERT* promoter region, MSP (using primers shown in Fig. 1*A*) was done on colon, breast, and lung cancer cell lines, leukemia cell lines, immortalized cell lines, and normal cells. All cancer cell lines and immortalized cell lines were either completely or partially methylated in the region −600 bp upstream of the transcription start site (MSP up primer set), whereas the region around the transcription start site (MSP down primer set) was either partially methylated or completely unmethylated (Fig. 1*B* and *C*). DKO, which are HCT 116 cells with *DNMT1* and *DNMT3b* knocked out, showed no methylation in either region as expected because these cells lose >90% of their methylation (27). Human mammary epithelial cells (HMEC), which are normal somatic cells, also showed no methylation in either region. VA13, which is a SV40 immortalized lung fibroblast that maintains telomeres through the alternative lengthening of telomeres pathway, showed partial

methylation in the upstream region and complete methylation in the downstream region (Fig. 1*C*).

To study the methylation patterns in more detail, we did bisulfite sequencing of cloned alleles over a region −650 to +150 of the *hTERT* promoter using three different primer sets (Fig. 1*A*). Bisulfite sequencing confirmed that the *hTERT* promoter region, particularly the most 5′ region, was densely methylated in all cancer cell lines. Caco-2 (Fig. 2*A*) and RKO (Fig. 2*B*) colon cancer cells were densely methylated from −650 to approximately −150 from the transcription start (regions BS-1 and BS-2) but remained only partially methylated in the region −150 to +150 (BS-3). This demarcation between completely methylated and unmethylated regions was most clearly observed for Caco-2 cells, where primer sets for regions BS-2 and BS-3 were combined (Fig. 2*A*). A similar pattern was also observed for SW480 (colon) and MCF7 (breast) cancer cells (Supplementary Fig. S1). HCT 116 colon cancer cells (Fig. 2*C*) and H209 lung cancer cells (Supplementary Fig. S1) had very similar patterns to those observed in Caco-2 and RKO cells, except that the region −150 to +150 (BS-3) was completely unmethylated, matching the findings by MSP. MDA-MB-231 breast cancer cells also had a similar pattern but seemed to have fewer unmethylated alleles in the region −150 to +150 (Supplementary Fig. S1). MDA-MB-435 (breast; Fig. 2*D*) and H82 (lung; Supplementary Fig. S1) had dense methylation limited to the BS-1 region, little methylation in the BS-2 region, and partial methylation in the BS-3 region around the transcription start.

We also examined the immortalized cell line, LCL, to determine its methylation patterns compared with cancer cell lines. This cell line had some DNA methylation in the far upstream region (BS-1), but it was less dense than that observed in the cancer cell lines (Fig. 3*A*). No methylation was observed in regions BS-2 and BS-3. We also examined methylation patterns in normal lymphocytes (Fig. 3*B*) and embryonic stem cells (Fig. 3*C*). Both of these had a few methylated sites in the far upstream region (BS-1) and little or no methylation in other regions. Finally, we examined VA13 cells, which were densely methylated in all regions and showed no completely unmethylated alleles in the region containing the transcription start site (Fig. 3*D*).

We next determined the relationship between DNA methylation patterns and expression of *hTERT*. Real-time RT-PCR in colon, breast, and lung cancer cell lines revealed that all cancer cell lines expressed *hTERT* as expected despite being densely methylated in the *hTERT* promoter region (Fig. 4). However, expression levels were variable and lower in cancer cell lines than in either LCL or ES cells, which expressed the highest levels of *hTERT*. HMEC and VA13 served as negative controls and did not express *hTERT* as expected (Fig. 4).

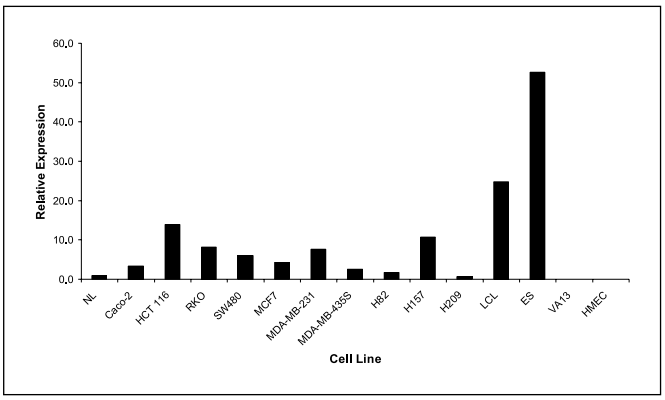

Figure 4. Quantitative expression of *hTERT* in cancer cell lines. Real-time RT-PCR was done on cell lines, and *hTERT* expression was normalized to *GAPDH* and calculated relative to normal lymphocytes.

The patterns of partial methylation in the region −150 to +150 raised two possibilities for the cause of this heterogeneity: (*a*) the methylation patterns found in the cancer cell lines were due to mixed populations of cells, where some cells were *hTERT* positive and unmethylated and some were *hTERT* negative and methylated, or (*b*) the methylation patterns were due to heterogeneous alleles that exist within each cell. To determine which of these possibilities produced the partial methylation patterns observed, we did single-cell dilution cloning using SW480 and MDA-MB-231 cells, both of which had mixed methylation patterns in the BS-3 region −150 to +150. Several single-cell clones were obtained for each cell line, and MSP, bisulfite sequencing, and real-time RT-PCR were done. MSP (Fig. 5*A*) and bisulfite sequencing (Fig. 5*B*) in the region −150 to +150 around the transcription start site confirmed that all of the clones maintained the same heterogeneous methylation patterns as originally observed in the parent lines. Quantitative RT-PCR also showed that all clones expressed *hTERT* (data not shown). Collectively, these results suggest that the heterogeneous methylation patterns exist within individual cells.

This raised the possibility that the expression of *hTERT* was due to allele-specific patterns of DNA methylation and chromatin marks. To explore this, we first did ChIP in RKO, SW480, HCT 116, MCF7, and VA13 cells on the *hTERT* promoter region using several primer sets spanning the region of interest (Fig. 1*A*) and antibodies for both transcriptionally active (acetyl-H3K9 and dimethyl-H3K4) and inactive (trimethyl-H3K9 and trimethyl-H3K27) marks of chromatin. In RKO, SW480, HCT 116, and MCF7, ChIP analysis showed that both active and inactive marks of chromatin were present across the region analyzed (data not shown), a finding consistent with the heterogeneous DNA methylation patterns. All cancer cell lines, however, show a modest enrichment of active marks (acetyl-H3K9 and dimethyl-H3K4) compared with the *hTERT*-negative VA13 cell line (Fig. 6*A*). Although the inactive mark of trimethyl-H3K9 was present in the cancer cell lines, the enrichment was lower than in the *hTERT*-negative VA13 cells. Similarly, trimethyl-H3K27 was depleted in the cancer cell lines when compared with VA13 but to a lesser extent than trimethyl-H3K9.

The varied patterns of DNA methylation and chromatin marks provided a unique ability to determine the association of DNA methylation with chromatin marks. We examined how the active and inactive chromatin were distributed with respect to the DNA methylation patterns of individual alleles for the *hTERT* promoter in the region −150 to +150 by combining ChIP and MSP into a novel approach we called ChIP-MSP. After doing ChIP, the isolated DNA was bisulfite treated and analyzed by MSP in the region −150 to +150 where differential methylation was observed (Fig. 1*A*). To validate this technique, we first examined *p16* in HCT 116 cells, which has been previously shown to have one unmethylated allele that is mutated and expressed and one methylated allele that is silent (26). ChIP using either acetyl-H3K9 or dimethyl-H3K4 antibodies precipitated predominantly unmethylated DNA by MSP analysis. Conversely, ChIP using antibodies for inactive marks (trimethylated H3K9 or H3K27) enriched for methylated DNA (Fig. 6*B, top*). This analysis confirmed that, for the *p16* promoter region in HCT 116 cells, active chromatin marks are associated with unmethylated DNA, whereas inactive chromatin marks are associated with methylated DNA.

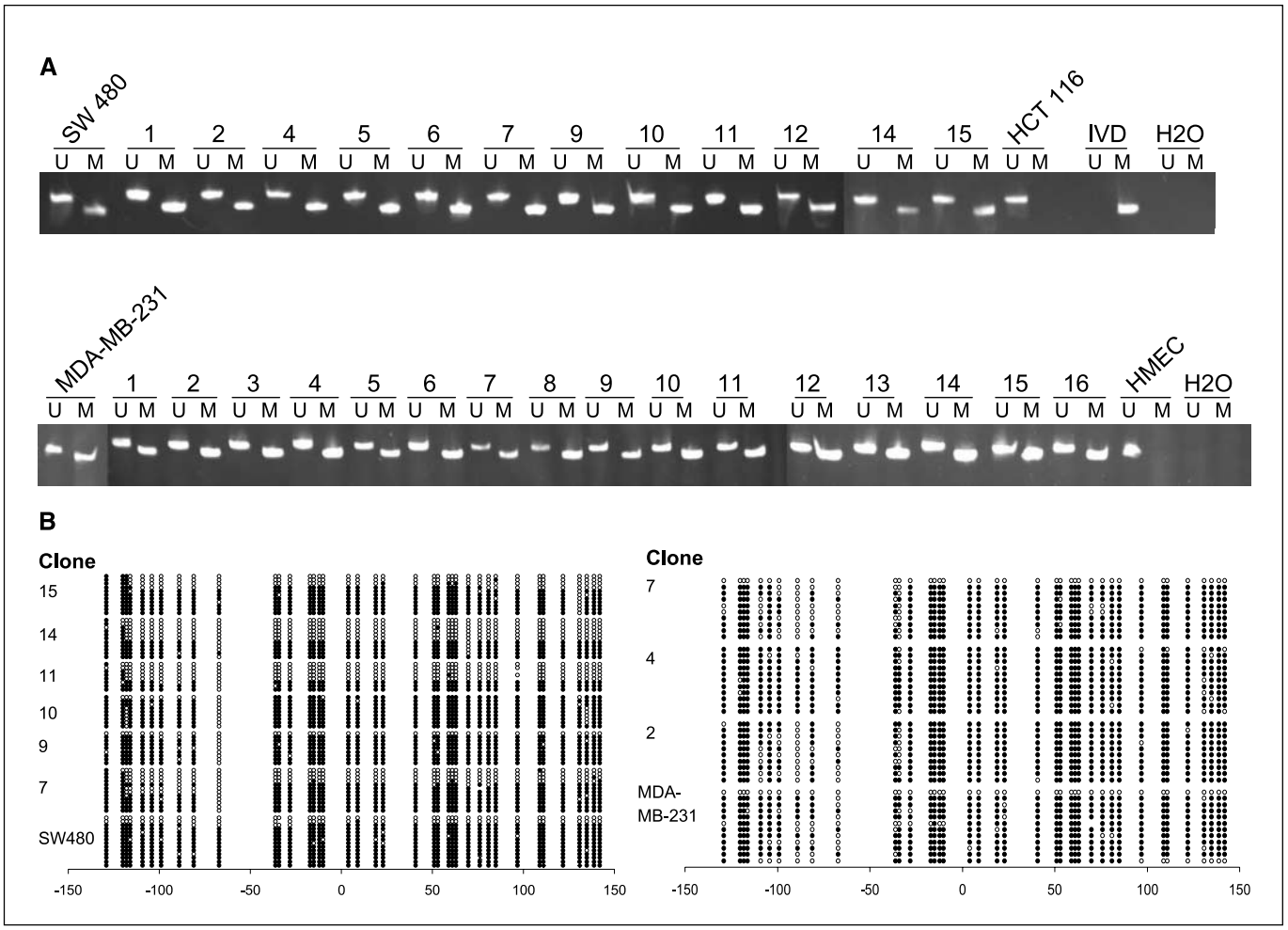

Figure 5. The DNA methylation patterns of *hTERT* observed in SW480 and MDA-MB-231 are retained in single-cell clones. *A,* MSP using the MSP down primer set (Fig. 1*A*) done on the SW480 (*top*) and MDA-MB-231 (*bottom*) single-cell clones shows they are partially methylated in this region. *B,* bisulfite sequencing of SW480 and MDA-MB-231 single-cell clones using BS-3 primer set (Fig. 1*A*) shows that they have similar methylation patterns to the parental line. *Left,* individual clone numbers.

ChIP-MSP analysis of *hTERT* in HCT 116 cells, where the promoter is completely unmethylated in the region −150 to +150, showed that acetyl-H3K9 and dimethyl-H3K4 were primarily associated with the unmethylated DNA in this region, whereas trimethyl-H3K9 and trimethyl-H3K27 were not associated with unmethylated DNA in this region (Fig. 6*B, bottom*). We then did ChIP-MSP in RKO, SW480, and MCF7 cells, where the region −150 to +150 is partially methylated. Both acetyl-H3K9 and dimethyl-H3K4 associated primarily with unmethylated DNA in this region in both RKO and SW480 (Fig. 6*C*). The same was also observed in MCF7 cells (data not shown). ChIP-MSP on DNA immunoprecipitated with trimethyl-H3K9 or trimethyl-H3K27 antibodies, in contrast, revealed that these inactive marks were primarily associated with methylated DNA (Fig. 6*C*) and were only occasionally associated with unmethylated DNA (data not shown). This suggests that the presence of active chromatin marks on unmethylated DNA allows for the continued expression of *hTERT*.

Discussion

The present study of the relationship between *hTERT* promoter region methylation and gene expression clarifies several conflicting studies in the literature. *hTERT* does indeed have, in most cancer cell lines, dense hypermethylation in the promoter region as reported previously (15–19). However, unlike some tumor suppressor genes where dense methylation extends throughout the CpG island, including the transcriptional start site (*p16* and *hMLH1*), *hTERT* methylation can be heterogeneous and some alleles lack methylation around the transcription start site. In this regard, *hTERT* maintains an unmethylated region in at least some of the alleles in *hTERT*-expressing cancer cells, which maps to −150 to +150 around the transcription start site. Single-cell dilution cloning of SW480 and MDA-MB-231 cells confirms that the mixed methylation patterns we observed in the parent cell lines exist within individual cells, which further suggests that the region surrounding the transcription start site must remain unmethylated for *hTERT* to be expressed. These spared alleles seem responsible for continued expression of *hTERT* despite dense methylation throughout the promoter. MDA-MB-231 breast cancer cells, which have very few alleles unmethylated in this region, still maintain some expression of *hTERT*. When this region is completely methylated, as in VA13 cells, expression of *hTERT* is absent. The idea that this region is important to remain at least partially unmethylated for expression to occur is also supported by

Devereux et al. (16) who showed that the cell line SUSM-1 is completely methylated in the region around the transcription start and does not express *hTERT*. Treatment of this cell line with the DNA demethylating agent 5-aza-2′-deoxycytidine resulted in restored expression of *hTERT*.

Expression of *hTERT* in cancer cells seems to be lower than in immortalized cell lines and embryonic stem cells. Consistent with this, the promoter region DNA methylation in these latter cell types seems to be much less prominent, further suggesting that, when DNA methylation is throughout the entire CpG island, it may lead to down-regulation of expression. Therefore, *hTERT* may be similar to genes that can become silenced in tumor cells, where dense and complete methylation of the promoter region does lead to repressive chromatin and gene silencing. However, it is important to note that, for *hTERT*, as for these other genes, DNA methylation in the promoter region seems to be an abnormal change from patterns maintained in normal stem cells and adult cells. Thus, both normal lymphocytes and embryonic stem cells show little or no DNA methylation of the promoter.

Chromatin analysis of the *hTERT* promoter in cancer cells further provides an explanation for the complicated DNA methylation and gene expression patterns. Although both active and inactive marks of chromatin are present throughout the *hTERT* promoter region, active marks are enriched in *hTERT*-positive cancer cells when

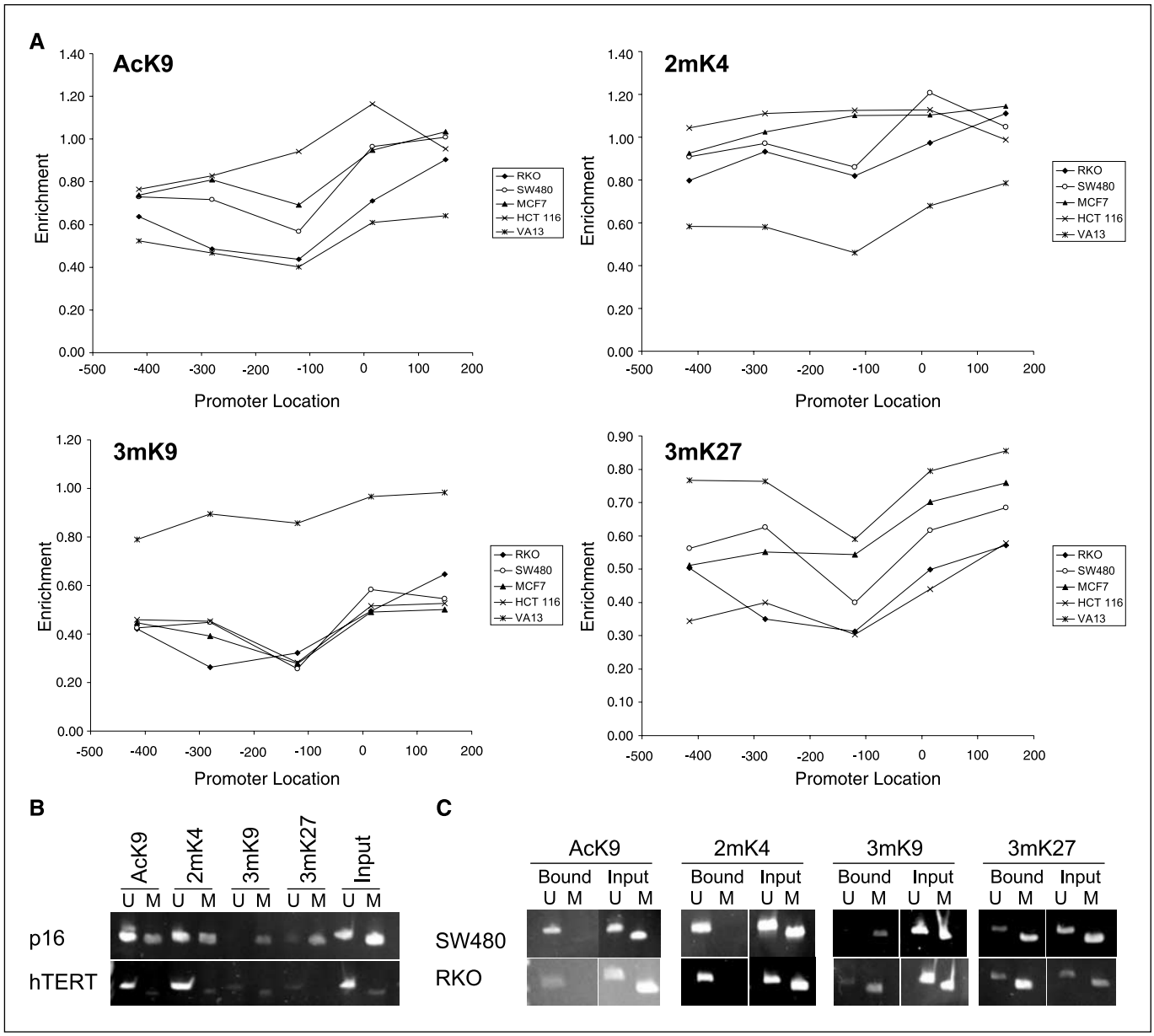

Figure 6. ChIP and associated DNA methylation patterns of the *hTERT* promoter region. *A,* ChIP PCR was done using the five primer sets shown in Fig. 1*A* in RKO, SW480, MCF7, HCT 116, and VA13 cell lines. Occupancy of each of the chromatin marks: acetyl-H3K9 (*AcK9*), dimethyl-H3K4 (*2mK4*), trimethyl-H3K9 (*3mK9*), and trimethyl-H3K27 (*3mK27*). *B,* ChIP-MSP analysis of *p16* (*top*) in HCT 116 cells shows that active marks of chromatin are associated with unmethylated DNA, whereas inactive marks of chromatin are associated with methylated DNA. Input serves as both the unmethylated and methylated control. ChIP-MSP analysis of *hTERT* downstream region in HCT 116 cells shows that the fully unmethylated input DNA primarily associates with active chromatin marks. *C,* ChIP-MSP analysis around the transcription start site of *hTERT* in RKO and SW480 cells shows that acetyl-H3K9 and dimethyl-H3K4 are primarily associated with unmethylated DNA, whereas trimethyl-H3K9 and trimethyl-H3K27 are primarily associated with methylated DNA.

compared with the *hTERT*-negative VA13 cell line. Conversely, inactive marks are depleted when compared with the VA13 cell line. However, acetyl-H3K9 and trimethylated H3K27 do not show as much of a difference between the *hTERT*-positive and *hTERT*-negative cell lines than the differences observed for dimethyl-H3K4 and trimethylated H3K9. This may suggest that, at least for *hTERT*, the dimethyl-H3K4 mark may be more important in determining its expression, whereas the trimethyl-H3K9 mark may be more important for its repression.

Furthermore, ChIP-MSP analysis in cell lines where *hTERT* is partially methylated shows that acetyl-H3K9 and dimethyl-H3K4 are predominantly associated with alleles having unmethylated DNA. Trimethyl-H3K9 and trimethyl-H3K27 are generally associated with methylated DNA, but this pattern is less distinct than the active marks. This could be a technical problem related to the resolution of ChIP and the complex methylation pattern of *hTERT*. For example, depending on the sonicated DNA fragment size, the trimethyl-H3K9 or trimethyl-H3K27 antibodies may have bound to the methylated DNA in more 5′ regions and pulled down unmethylated DNA near the transcription start site. Another possibility is that these marks do not only associate with methylated DNA but perhaps they can associate with unmethylated DNA as well. McGarvey et al. (28) have recently shown that when a silenced tumor suppressor gene is treated with 5-aza-2′-deoxycytidine, although expression is restored and the gene becomes demethylated, trimethyl-H3K9 and trimethyl-H3K27 remain associated with the gene promoter. However, our results clearly show that the active marks (acetyl-H3K9 and dimethyl-H3K4) are present almost exclusively with unmethylated DNA in the *hTERT* promoter region −150 to +150, suggesting that transcription is occurring from these alleles and not from the completely methylated *hTERT* alleles.

These studies were done in cell lines because they allow for a comprehensive analysis of DNA methylation, expression, and chromatin changes in the same cells, which would not be possible in primary tumors. Furthermore, in primary tumor samples, mixed populations of normal and neoplastic cells make it difficult to determine the origin of methylation. However, Guilleret et al. (18) reported partial methylation in the *hTERT* promoter region for several types of *hTERT*-positive tumor tissues, including breast, lung, and colon, suggesting that similar patterns exist in primary tumors. Our results show that the expression and DNA methylation patterns for *hTERT* do not constitute a complete contradiction to the well-established association of promoter region methylation with gene silencing as suggested by previous reports. These findings suggest that, in order to maintain replication capacity by continued expression of *hTERT*, cancer cells have pressure to maintain alleles of *hTERT* that are protected from DNA methylation and must balance forces leading to DNA methylation with the need to keep *hTERT* expressed. Finally, we show the use of combining two techniques (ChIP and MSP) into a novel approach that can be used to examine the association of certain chromatin marks or transcription factors with methylated or unmethylated DNA.

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Rebekah L. Zinn, Kevin Pruitt, Sayaka Eguchi, et al.

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