

Distinct and Temporal Roles of Nucleosomal Remodeling and Histone Deacetylation in the Repression of the *hTERT* Gene

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hTERT, the human telomerase reverse transcriptase, is highly expressed in stem cells and embryonic tissues but undetectable in most adult somatic cells. To understand its repression mechanisms in somatic cells, we investigated the endogenous *hTERT* gene regulation during differentiation of human leukemic HL60 cells. Our study revealed that silencing of the *hTERT* promoter was a biphasic process. Within 24 h after initiation of differentiation, *hTERT* mRNA expression decreased dramatically, accompanied by increased expression of *Mad1* gene and disappearance of a nucleosome-free region at the *hTERT* core promoter. Subsequent to this early repression, nucleosomal remodeling continued at the promoter and downstream region for several days, as demonstrated by micrococcal nuclease and restriction enzyme accessibility assays. This later nucleosomal remodeling correlated with stable silencing of the *hTERT* promoter. Progressive changes of core histone modifications occurred throughout the entire differentiation process. Surprisingly, inhibition of histone deacetylation at the *hTERT* promoter did not prevent *hTERT* repression or nucleosomal deposition, indicating that nucleosomal deposition at the core promoter, but not histone deacetylation, was the cause of transcriptional repression. Our data also suggested that succeeding nucleosomal remodeling and histone deacetylation worked in parallel to establish the stable repressive status of *hTERT* gene in human somatic cells.

INTRODUCTION

A central mechanism of cell specification and differentiation during development is transcription programming, the selective activation and silencing of specific genes (Muller and Leutz, 2001). This programming is largely achieved through highly regulated modulation of chromatin structures that package the eukaryotic genome. An important issue to resolve is how patterns of gene expression are established and stably maintained through subsequent cell divisions. On the one hand, activation of tissue-specific gene expression during cell differentiation has been studied in several models, which often involves temporal actions of transcription factors and chromatin-modifying complexes for covalent modifications of core histones and ATP-dependent nucleosomal remodeling at the promoters (de la Serna *et al.*, 2006; Kaeser and Emerson, 2006). On the other hand, distinct and redundant chromatin modifications such as histone deacetylation and nucleosomal remodeling have been implicated in the transcriptional repression of several yeast genes (Zhang and Reese, 2004). However, little

is known about the temporal processes of transcriptional repression during cell differentiation.

Telomeres are specialized nucleoprotein complexes capping chromosomal ends, and they play a key role in cellular aging and cancer (Morin, 1989; Kim *et al.*, 1994). In stem cells and cancer cells, telomeres are replenished by telomerase, a ribonucleoprotein polymerase that maintains telomere ends by the addition of telomere repeat TTAGGG (Sharpless and DePinho, 2004). Telomerase consists of a catalytic reverse transcriptase subunit, encoded by the human telomerase reverse transcriptase (*hTERT*) gene, and an RNA component that serves as the template for telomere repeats. In most cells, the *hTERT* mRNA level parallels telomerase activity (Aisner *et al.*, 2002). Both *hTERT* transcription and telomerase activity are down-regulated during cellular differentiation (Xu *et al.*, 1999; Günes *et al.*, 2000). Therefore, the *hTERT* gene is normally repressed in most postnatal somatic cells, resulting in progressive shortening of telomeres and proliferative senescence (Wright *et al.*, 1996).

Studies of *hTERT* transcriptional regulation have largely been focused on factors that bind to the *hTERT* promoter. By binding to the E-box consensus sites in the *hTERT* promoter region, transcription factor c-Myc played a crucial role in the regulation of *hTERT* gene in both normal cells and cancer cells (Wu *et al.*, 1999). It was shown that a switch of promoter occupancy by Myc/Max in proliferating cells to Mad/Max in differentiated cells correlated with *hTERT* repression during differentiation of HL60 cells (Xu *et al.*, 2001). The mechanisms of transcriptional regulation by Myc/Max/Mad network proteins have been studied extensively (Eisenman, 2001; Luscher, 2001). It was shown previously that the activation domain of c-Myc protein bound to complexes containing histone acetylase (HAT) activities, such as TRRAP,

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Abbreviations used: ChIP, chromatin immunoprecipitation; *CRR9*, cisplatin-resistance-related gene 9; DHS, DNase I hypersensitive site; DMSO, dimethyl sulfoxide; HDAC, histone deacetylase; *hTERT*, human telomerase reverse transcriptase; Pol II, RNA polymerase II; TSA, trichostatin A; TSS, transcription start site.

p300/CBP, and TIP60 (Ikura *et al.*, 2000; McMahon *et al.*, 2000; Vervoorts *et al.*, 2003). At the same time, c-Myc also associated with the SWI/SNF nucleosomal remodeling complex and the ATPase/helicases TIP48 and TIP49 (Cheng *et al.*, 1999; Wood *et al.*, 2000). Thus, recruitment of these two types of complexes is thought to be the mechanisms of transcriptional activation by c-Myc (Hooker and Hurlin, 2006). Conversely, Mad family proteins recruit mammalian Sin3 proteins (mSin3A and mSin3B) (Ayer *et al.*, 1995), which are scaffold proteins that tether histone deacetylases (HDACs) (Knoepfler and Eisenman, 1999; David *et al.*, 2008). In addition, histone modifications affect gene transcription by directly altering local nucleosomal architecture or through the recruitment of *trans*-acting factors that recognize specific histone modifications (Berger, 2002). ATP-dependent chromatin remodeling modifies histone-DNA contacts and results in insertion, eviction, and sliding nucleosomes along DNA, which change accessibility of DNA to transcription factors (Cairns, 2007). Hence, covalent modifications of core histones and ATP-dependent nucleosomal remodeling modulated by hTERT promoter binding proteins, such as Myc/Max/Mad network proteins, may play critical roles in the transcriptional control of the *hTERT* gene.

To understand the mechanisms of *hTERT* gene repression, we examined the chromatin structures of endogenous hTERT promoter during the differentiation of human HL60 cells. Upon stimulation by dimethyl sulfoxide (DMSO), HL60 cells underwent differentiation to granulocytic cells (Harris and Ralph, 1985), accompanied by a rapid and marked down-regulation of hTERT transcription. Our data revealed a dynamic process of transcriptional repression, as manifested by nucleosomal insertion and remodeling as well as core histone modifications. Interestingly, the initial cessation of hTERT transcription involved the deposition of a nucleosome and loss of c-Myc binding, but not histone deacetylation, at the core promoter region. Subsequent nucleosomal remodeling and histone deacetylation eventually led to a stably silenced state of hTERT promoter in fully differentiated cells. Therefore, our results demonstrated that nucleosomal remodeling/positioning and histone deacetylation played distinct roles in the establishment and maintenance of the stably repressive state of *hTERT* gene.

MATERIALS AND METHODS

Cell Culture and Differentiation

HL60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. To induce differentiation, 2.5% DMSO was added to HL60 cells at a density of 0.5×10^6 /ml. Cells were harvested at different time points after addition of DMSO.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated by TRIzol from 2×10^6 HL60 cells. cDNA was synthesized from 1 μ g of total RNA, and PCR reactions were performed as described previously (Wang and Zhu, 2004). Real-time PCR was performed in triplicates using an ABI StepOnePlus system (Applied Biosystems, Foster City, CA). All experiments were repeated at least once. PCR primers and TaqMan probes are summarized in Supplemental Table 1.

Chromatin Immunoprecipitation (ChIP) Assays

ChIPs were performed as described previously (Wang *et al.*, 2007). In brief, cells were cross-linked with 1% formaldehyde for 10 min at room temperature and harvested after quenching of formaldehyde with 125 mM glycine. After sonication, chromatin fragments of 200–800 base pairs were immunoprecipitated overnight at 4°C by using 0.5 μ g of antibodies and amplified by PCR. For the hTERT core promoter region, TaqMan assays were used to quantify the ChIP results. Primers, probe, and a list of antibodies for ChIP are summarized in Supplemental Tables 1 and 2, respectively.

Nuclease Accessibility Assays

Nuclei preparation was performed as described previously (Wang and Zhu, 2003, 2004). In brief, nuclei were prepared from 1×10^8 cells and incubated at 37°C with DNase I for 20 min, micrococcal nuclease (MNase) for 10 min, or MspI for 30 min. The nuclease digestion reactions were terminated by the addition of EDTA, and genomic DNAs were extracted. Genomic DNAs (10 μ g) were digested with EcoRI and SphI (for DNase I assays) or with DraI (for MNase and MspI assays), followed by Southern blotting and indirect end labeling. For DNase I assays, probes *a* (–3.9 kb) and *b* (+1.7 kb) were used for 5' and 3' ends, respectively, as described previously (Wang and Zhu, 2003). For MNase and MspI assays, genomic fragments XbaI-DraIII (–1.3 to –0.9 kb, relative to transcription start site [TSS]) and PstI-PstI (+2.1 to +2.5 kb) were used as 5' and 3' probes, respectively.

RESULTS

Expression of hTERT and Its Transcriptional Regulators during Cell Differentiation

Differentiation of human promyelocytic leukemia HL60 cells was accompanied by the repression of telomerase expression (Xu *et al.*, 1999) and therefore investigated as a model of telomerase repression during cell differentiation. To determine the kinetics of repression, the hTERT mRNA level was examined by RT-PCR. Because the cisplatin-resistance-related gene 9 (*CRR9*), located immediately upstream of the *hTERT* gene, was constitutively expressed in all cell types examined and during cell differentiation (Wang and Zhu, 2003; Wang *et al.*, 2004, 2007), its mRNA, and also 18S rRNA, were used as normalizing controls. hTERT mRNA was readily detected in proliferating cells but was barely detectable after 24 h of differentiation (Figure 1A). The reduction of hTert mRNA was subsequently determined quantita-

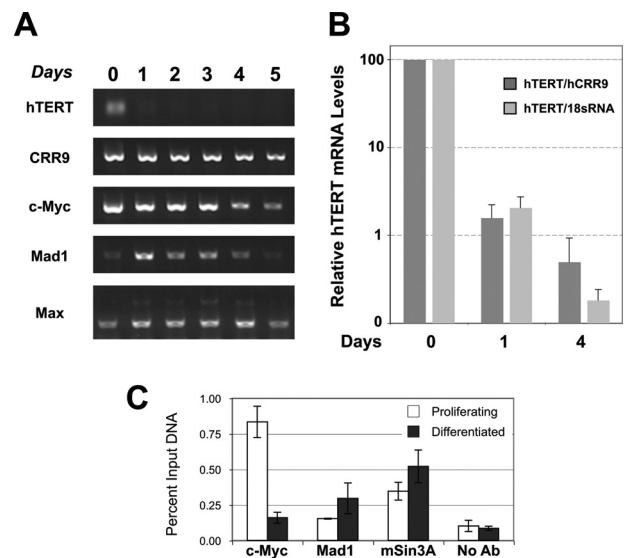


Figure 1. The gene expression profiles of hTERT and myc-family proteins and their bindings to the hTERT core promoter during HL60 cell differentiation. (A) mRNA levels of *hTERT* and Myc family genes in HL60 cells at various times of differentiation induced by 2.5% DMSO, as determined by RT-PCR and visualized on agarose gels. (B) hTERT mRNA levels in HL60 cells at days 0, 1, and 4 of differentiation, measured by real-time PCR analysis and normalized to the level of either *CRR9* mRNA or 18S rRNA. (C) Quantitative ChIP analysis of binding of c-Myc, Mad1, and mSin3A to the endogenous hTERT core promoter in HL60 cells treated without (proliferating cells) or with 2.5% DMSO for 4 d (differentiated cells). Immunoprecipitated genomic fragments were subjected to real-time PCR analysis (TaqMan assay). Data were normalized to input chromatin fragments and shown as percentages of input DNA. No Ab, no antibody controls.

tively by real-time PCR analysis. As shown in Figure 1B, the hTERT mRNA level decreased by 50- and 60-fold within 24 h of differentiation when normalized to 18S rRNA and CRR9 mRNAs, respectively. Four days after differentiation, the hTERT mRNA level decreased further to <100th of that in proliferating HL60 cells. Because it took at least 4 d for HL60 cells to completely differentiate into granulocytes (Birnie, 1988), these data indicated that down-regulation of hTERT expression was an early event of differentiation.

Myc/Max/Mad network transcription factors have been implicated in the regulation of *hTERT* gene, through binding to the E-box sites at the core promoter (Xu *et al.*, 2001). Thus, the expression levels of c-Myc and Mad1 during cell differentiation were determined using semiquantitative RT-PCR analyses. As shown in Figure 1A, DMSO treatment of HL60 cells led to a gradual decrease of c-Myc mRNA during the differentiation process, consistent with the pattern of changes of the c-Myc protein, as determined by Western analysis (data not shown). This result indicated that the level of c-Myc expression did not correlate directly with that of hTERT expression during differentiation. Conversely, Mad1 expression showed a marked increase on the first day of differentiation and decreased gradually thereafter. In addition, the expression of Max, the binding partner of both c-Myc and Mad1, persisted throughout the differentiation process. These results suggested that increased Mad1 expression, but not the decrease of c-Myc expression, was crucial for the initiation of hTERT repression. Because the Mad1 mRNA level decreased during later phases of differentiation, its expression was unlikely to account for the stable maintenance of hTERT repression in fully differentiated cells.

Next, the physical associations of c-Myc and Mad1 proteins with the endogenous hTERT promoter during differentiation were examined by ChIP analysis. As shown in Figure 1C, the c-Myc protein was detected at the core promoter in proliferating HL60 cells. The binding of c-Myc to the core promoter decreased markedly after 4 d of differentiation. In contrast, binding of Mad1 protein to the core promoter increased in differentiated HL60 cells. Mad1 was shown previously to repress transcription through recruiting corepressor complexes such as mSin3A and HDACs to the chromatin (Rottmann and Luscher, 2006). Indeed, ChIP analysis showed that the association of mSin3A with the hTERT promoter, although detected in proliferating cells, was moderately increased in differentiated HL60 cells (Figure 1C). However, although this trend of increase was detected in multiple experiments, it was not statistically significant ($p = 0.05\sim 0.1$). In short, these data were consistent with an earlier report by Xu *et al.* (2001) that c-Myc binding to the hTERT core promoter was replaced by promoter occupation of Mad family proteins during HL60 cell differentiation. The promoter association of mSin3A in proliferating cells was also consistent with our earlier observation that the hTERT promoter was subjected to repression even in telomerase-positive cells (Wang and Zhu, 2004; Wang *et al.*, 2009).

Histone Modifications at the hTERT Promoter

To determine whether histone modifications were linked to the rapid reduction of the hTERT mRNA level that occurred after the initiation of differentiation, the time course of histone modifications was determined by quantitative ChIP analysis. As shown in Figure 2A, acetylation of histones H3 and H4 decreased progressively at the hTERT core promoter during differentiation. Similarly, levels of both di- and trimethylation of H3 lysine 4 (H3K4) at the core promoter were

also reduced in differentiating cells. Whereas the trimethylated H3K4 became essentially undetectable in differentiated cells, a significant level of dimethylation of this residue remained, consistent with the notion that trimethylation, but not dimethylation, of H3K4 was correlated with active transcription. As a control, neither acetylation nor methylation of core histones was changed at the upstream CRR9 promoter (data not shown), which was constitutively active in both proliferating and differentiated cells (Figure 1A). Interestingly, dimethylation of H3K4 at the hTERT core promoter showed a transient increase on day 2 of differentiation. Although this H3K4 dimethylation on day 2 was lower compared with day 0, it was reproducible in multiple experiments. Finally, low levels of trimethylation of H3 lysine 27 (H3K27) were also detected at the hTERT promoter and displayed a gradual increase during differentiation. Thus, covalent modifications of core histones at the hTERT promoter underwent gradual changes during HL60 cell differentiation, whereas the decrease of hTERT mRNA expression was more rapid.

The gradual epigenetic changes over the hTERT promoter region raised the possibility that the initial rapid reduction of hTERT mRNA level during differentiation might be a posttranscriptional event. To address this issue, transcription in proliferating and differentiating HL60 cells was inhibited by actinomycin D treatment. The stability of the hTERT message in these cells was assessed by determining the hTERT mRNA levels at various times after the treatment. As shown in Figure 2B, the half-life of the hTERT message was 30 min to 1 h in both proliferating cells and cells that were induced for differentiation for 3 or 6 h, indicating that the stability of the hTERT mRNA was not significantly changed during differentiation. Thus, down-regulation of the hTERT mRNA level upon differentiation was unlikely to be a posttranscriptional event. In addition, as shown in Figure 2A by quantitative ChIP analysis, the association of RNA polymerase II (Pol II) with the hTERT promoter, and thus the formation of preinitiation complex (PIC), was greatly reduced within the first day of differentiation, correlated with the decreased level of hTERT mRNA. Together, our results indicated that transcriptional initiation of hTERT ceased early during differentiation.

Decreased DNase I Sensitivity of the hTERT Promoter Correlated with hTERT Repression

DNase I sensitivity assays were performed to determine chromatin structural changes at the endogenous hTERT promoter during HL60 cell differentiation. Nuclei isolated from proliferating and differentiating cells were treated with increasing amounts of DNase I. Genomic DNAs were digested with restriction enzymes EcoRI and SphI, subjected to Southern blotting, and followed by indirect end labeling. The EcoRI-SphI full-length genomic fragment was shown in Figure 3 as a prominent 5.6-kb band. A 3.9-kb band was found in proliferating HL60 cells when the Southern blot was hybridized to a probe specific to the 5' end of the full-length genomic fragment (Figure 3A). The intensity of this band initially increased with DNase I addition and subsequently decreased at higher nuclease concentrations, a characteristic of DNase I hypersensitive bands. The size of this band mapped a major DNase I hypersensitive site (DHS) to a region near the TSS. As HL60 cells started to differentiate, the intensity of this band decreased significantly within 24 h and the weakened band also shifted upward.

A higher resolution of DHS map was obtained, and multiple hypersensitive bands were detected when the same

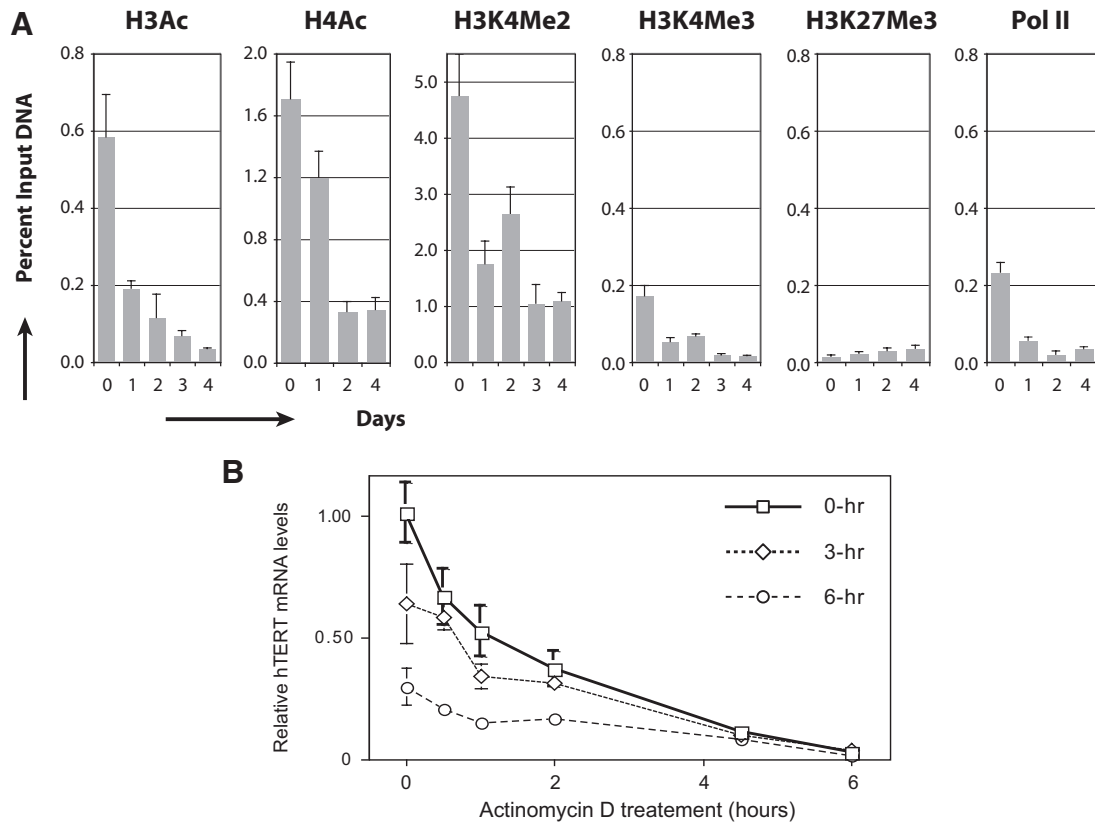


Figure 2. Covalent modifications of core histones at the hTERT promoter and stability analysis of hTERT mRNA during differentiation. (A) Time course of histone modifications at the proximal region of hTERT promoter. HL60 cells were induced to differentiate for 0, 1, 2, 3, and 4 d with 2.5% DMSO. Immunoprecipitated genomic fragments were subjected to real-time PCR. Data were normalized to input chromatin fragments and shown as percentages of input DNA. Specificities of antibodies: H3Ac and H4Ac, acetylated histones H3 and H4; H3K27Me3, trimethylated K27 of histone H3; H3K4Me2 and H3K4Me3, di- and trimethylated K4 of histone H3. (B) Stability of hTERT mRNA in differentiating HL60 cells induced by 2.5% DMSO. Actinomycin D (5 $\mu\text{g}/\text{ml}$) was added to proliferating cells (0 h) or cells at 3 and 6 h after initiation of differentiation. Total RNAs were harvested at 0, 0.5, 1, 2, 4.5, and 6 h after addition of actinomycin D. The levels of hTERT mRNA were determined by quantitative RT-PCR and normalized to 18S rRNA. Data shown are averages of triplicates.

blot was hybridized to a probe specific to the 3' end (Figure 3B). The first band is a diffused band around the position of 1.7 kb, corresponding to a major DHS at or immediately upstream of TSS. The intensity of this band decreased significantly within 24 h of differentiation. This DHS was probably the same as the DHS HS1 that we reported previously (Wang and Zhu, 2003). HS1 was found in all telomerase-expressing cells but undetectable in telomerase-negative cells (Wang and Zhu, 2004), indicating that HS1 was critical for hTERT transcription and probably a nucleosome-free region where Pol II and the transcription machinery were assembled. The rapid weakening of HS1 suggested that the nucleosome-free region was occupied by nucleosomes during an early stage of differentiation, correlating with the cessation of hTERT transcription. In addition, the 1.4- and 1.2-kb bands corresponded to DHSs in the first intron and the second exon of the *hTERT* gene, respectively. However, both of these DHSs appeared at higher concentrations of DNase I and were present throughout the differentiation process; therefore, they did not correlate with hTERT repression during cell differentiation.

Nucleosomal Remodeling at the hTERT Promoter Region

For a more detailed analysis of chromatin structure at the hTERT promoter, the nuclei were subjected to digestion by MNase, which preferentially cleaves linker DNA between

nucleosomes. Within the 1.6-kb region of -350 base pairs to $+1300$ base pairs relative to TSS, at least eight MNase cleavage sites were detected in both proliferating and differentiated HL60 cells when using a probe specific to the 5' end of the DraI fragment (Figure 4A). The majority of these cleavage sites were also detected when the same blot was hybridized to a probe specific to the 3' end (data not shown). In most cases, intervals between neighboring sites ranged from 150 to 250 base pairs. A similar pattern of cleavages was not obtained when naked genomic DNA was digested; therefore, these MNase cleavage sites were not intrinsic to the DNA sequences (Figure 4A, right). These features indicated that MNase sites were located at linker regions of nucleosomes and thus defined nucleosomal boundaries (Figure 4A, diagrams). A schematic interpretation of these data are shown in Figure 6. Although positions and relative intensities of most bands remained unchanged during differentiation, two clear differences of MNase digestion were identified between days 0 and 4. First, the region between two MNase sites within the first exon was also sensitive to the nuclease in proliferating cells (Figure 4A, left). This sensitivity (labeled by an open circle) persisted on days 0, 1, and 2 but not on day 4 (Figure 4A). The distance between these two MNase sites was <300 base pairs and was unlikely to accommodate two nucleosomes. Densitometric scanning of the cleavage profiles also confirmed increased MNase sen-

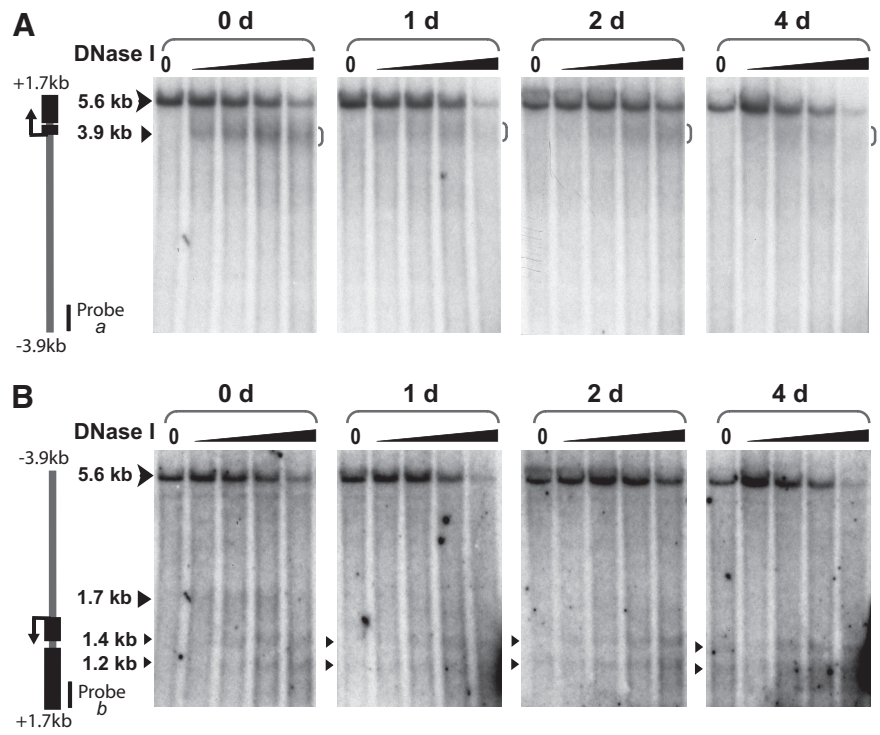
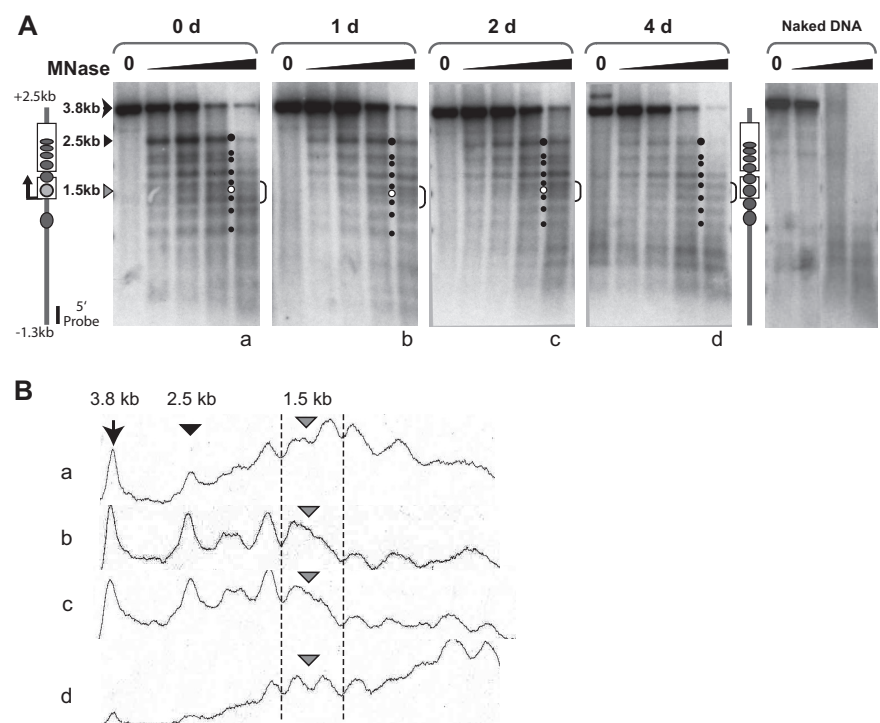


Figure 3. DNase I sensitivity of the hTERT promoter during cell differentiation. HL60 cells (1×10^8) were induced to differentiate with 2.5% DMSO for 0, 1, 2, and 4 d. Isolated nuclei were treated with 0, 2, 4, 8, and 16 U/ml DNase I at 37°C for 20 min. Genomic DNAs were isolated, digested with EcoRI and SphI, and analyzed on Southern blots, and followed by indirect end labeling. Diagrams of the hTERT promoter between the EcoRI (-3.9-kb, relative to TSS) and SphI (+1.7-kb) sites are displayed on the left of each figure. Rectangles depict the first and a part of second exon of the *hTERT* gene. Arrows indicate TSS and the direction of transcription. Southern blots were hybridized to upstream probe *a* (A) or downstream probe *b* (B) (Wang and Zhu, 2003), as indicated by short vertical bars in the diagrams. Full-length genomic DNA band and DHS bands are indicated by arrowheads and triangles, respectively.

sensitivity at this region on days 0–2 (Figure 4B, gray triangles at the 1.5-kb position). Because this region of the first exon was not hypersensitive to DNase I, it was likely that it was packaged into a nucleosome but somehow still sensitive to MNase. Second, a 2.5-kb band was dominant in proliferating

HL60 cells, corresponding to an MNase-hypersensitive cleavage site in the middle of exon 2. On differentiation, the band weakened progressively. A plausible interpretation is that an internucleosomal gap was present in the second exon in proliferating cells and this gap became smaller as HL60

Figure 4. Mapping of MNase cleavage sites in the hTERT promoter region. (A) MNase sensitivity assays of the hTERT promoter. HL60 cells were induced to differentiate for 0, 1, 2, or 4 d as shown above each panel. Nuclei were isolated and incubated with 0, 2, 4, 8, and 16 U/ml MNase for 10 min at 37°C. For naked genomic DNA, 0, 0.25, 0.5, and 1 U/ml MNase were used. Genomic DNAs were digested with DraI and subjected to Southern analysis, followed by indirect end labeling using a probe specific to the 5' end of DraI fragment. Schematic illustrations of hTERT promoter and nucleosomal positions are shown for proliferating cells (left) and differentiated cells (right). The short vertical bar in the left diagram indicates the 5' probe for Southern analysis. Ovals are deduced nucleosomal positions. MNase cleavage bands are indicated by closed and open circles, of which the open circles show the position of a 1.5-kb MNase band present in cells treated with DMSO for 0, 1, and 2 d but disappeared after 4 d of treatment. The brackets on the right of each Southern panel show the region of continuous nucleosomal remodeling in the first exon. The 3.8-kb full-length band and 2.5-kb MNase hypersensitive band are also marked. (B) Densitometric scanning of lanes a–d in A. Black arrow and arrowheads indicate the 3.8-kb full-length and the 2.5-kb MNase hypersensitive bands, respectively. Dashed lines outline the region indicated by brackets in A and triangles point to the positions where MNase sensitivity continued to change during later phases of differentiation.



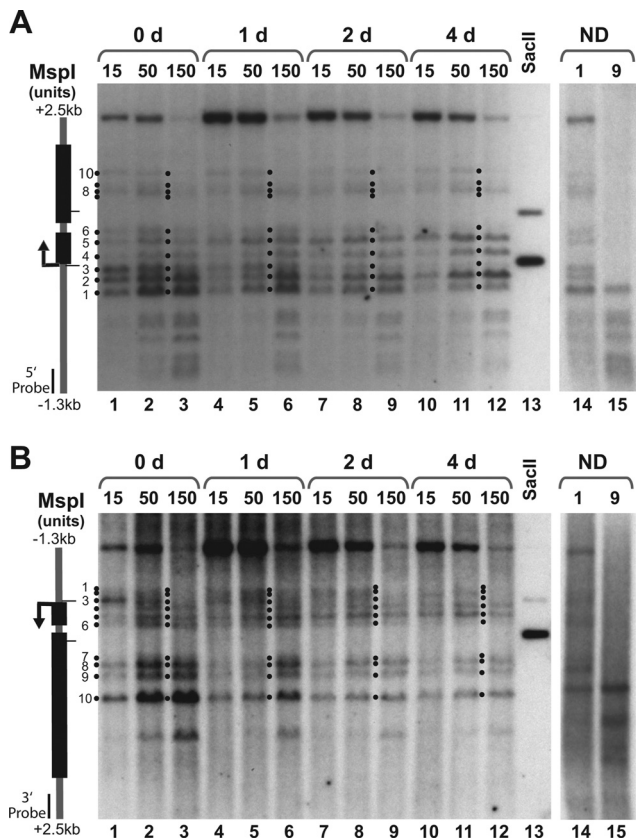


Figure 5. Restriction enzyme accessibility of the hTERT promoter region. HL60 cells were induced to differentiate for 0, 1, 2, or 4 d as shown above each panel. Nuclei were digested with 15, 50, and 150 U/ml MspI at 37°C for 30 min. Isolated genomic DNAs were digested with DraI and analyzed as in Figure 4. Schematic diagrams of the hTERT promoter region are shown on the left of each panel. The Southern blots were hybridized to the 5' end probe (A) and 3' end probe (B), as indicated by short vertical bars at the bottom of each diagram. The thin horizontal lines indicate the positions of two SacII sites. Ten MspI sites/clusters are depicted as black dots with numbers on the left. The SacII lane shows purified genomic DNA that was partially digested with SacII. ND, naked genomic DNA, which was digested with 1 or 9 U/ml MspI.

cells differentiated. Overall, the MNase experiment allowed us to predict the positions of 10 nucleosomes (−3 to +7) within the 1.6-kb region at the 5' end of the *hTERT* gene (see Figure 6).

To provide independent evidence about nucleosomal structures of the hTERT promoter, nuclei were also treated with restriction enzyme MspI. Within the 1.6-kb region, there are 10 single or clustered MspI sites (Figures 5 and 6). Due to the use of indirect end-labeling technique, the abundance of larger radiolabeled fragments was diminished by cleavages closer to the labeling end. Hence, cleavages were more accurately assessed at sites near the labeling end. In addition, relative sensitivities of adjacent cleavage sites could be compared. Three MspI clusters (1–3) are located within 300 base pairs upstream of TSS, a region that was hypersensitive to DNase I treatment (Figure 6). All three MspI clusters were accessible to the restriction enzyme in proliferating HL60 cells (Figure 5, lanes 1–3). Cluster 3, consisting of three MspI sites (−44 to −66 nt, relative to TSS; Figure 6), was especially sensitive to MspI digestion, and this hypersensitivity was not observed when naked genomic

DNA was used (Figure 5, A and B, lanes 14 and 15), suggesting that the hTERT core promoter was readily accessible in proliferating cells. The accessibility of cluster 3 decreased significantly within 24 h of differentiation (Figure 5, lanes 4–6), consistent with rapid incorporation of a nucleosome (−1) to the core promoter (Figure 6). This loss of accessibility also correlated with the cessation of hTERT transcription early during differentiation. MspI site 2, overlapping with an MNase site, remained sensitive to MspI throughout the differentiation process, suggesting that this site was in the linker region before and after differentiation. MspI cluster 1, in contrast, became progressively more resistant to MspI digestion as cells differentiated. Because cluster 1 was also in the DNase I hypersensitive region, it was possible that this site was in a nucleosome-free region in proliferating cells and a nucleosome was recruited to this region more slowly than the downstream (−1) nucleosome. Alternatively, MspI cluster 1 was loosely wrapped around a nucleosome (−2) in proliferating cells and the packaging became gradually more compact as cells differentiated (Figure 6).

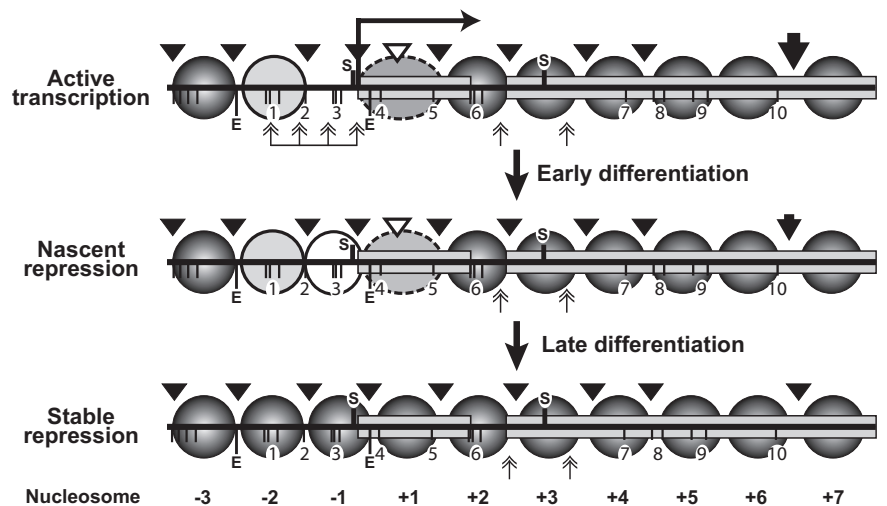
It was proposed that the first (+1) nucleosome downstream of TSS had a major impact on transcriptional regulation (Agalioti *et al.*, 2000; Chen and Yang, 2001). Our data indicated that the first 200 base pairs of exon 1 seemed to be packaged into a nucleosome (+1) in proliferating cells, because it was resistant to digestion by both DNase I and MspI (Figures 3 and 5). However, the same sequence was also sensitive to MNase cleavage, as indicated by an open circle in Figure 4 (also see Figure 6). These results suggested that the (+1) nucleosome was in a loose configuration and/or relatively mobile, covering an extended DNA segment. A similar feature was also found in the (+1) nucleosome downstream of the HPRT promoter, which was called “split nucleosome” (Lee and Garrard, 1991; Chen and Yang, 2001). Such an open nucleosomal configuration is probably crucial for transcription to occur. However, this open conformation persisted through the first 2 d of differentiation, long after hTERT transcription ceased. This phenomenon also coincided with increased accessibility of MspI sites 4 and 5 on day 4 (Figures 5A and 6), suggesting that the (+1) nucleosome underwent remodeling and became more compact at a late stage of differentiation and long after cessation of hTERT transcription.

In addition, site 10 was also hypersensitive to MspI and its sensitivity decreased as cells differentiated, consistent with its position in a nucleosomal gap in proliferating cells (Figures 5B and 6). Finally, nucleosomes became more evenly distributed over the hTERT promoter region 4 d after differentiation (Figure 4, 4d). This ordered nucleosomal array was probably present in most human somatic cells and important for stable repression of the hTERT promoter.

Remodeling of hTERT Promoter in the Early Phase of Differentiation

We have demonstrated that down-regulation of hTERT transcription occurred within the first 24 h of differentiation, whereas nucleosomal remodeling and histone modifications at the hTERT promoter continued throughout the entire differentiation process. In fact, the hTERT mRNA level was significantly decreased within 3–6 h after the induction of differentiation, as shown in Figure 2B. To further dissect the repression process in the early phase of differentiation, DNase I sensitivity of the hTERT promoter was determined at multiple time points within the first 24 h of differentiation. The experiment was performed in the same way as in Figure 3, except that HL60 cells were treated with DMSO for 0, 3, 6, and 24 h. When the blots were probed with the upstream

Figure 6. Model that summarizes the nucleosomal organization of the hTERT promoter in HL60 cells. Genomic DNA and hTERT exons are depicted by horizontal lines and gray rectangles, respectively. Active, nascently repressed, and stably repressed promoters are as designated on the left. Transcription is indicated by the horizontal arrow on the active promoter. Double arrows below each diagram show the positions of DHSs. Triangles above each diagram represent MNase cleavage sites and the downward arrows indicate an MNase hypersensitive site between nucleosomes (+6) and (+7). Predicted nucleosomal positions are shown as circles: gradient circles, regular nucleosomes; gray circle, nucleosome (-2) that is sensitive to MspI digestion; white circle, newly deposited nucleosome; dashed oval, nucleosome (+1) covering an extended region and was susceptible to intranucleosomal MNase I cleavage (white triangles). Thin short vertical lines and numbers indicate MspI site and clusters. S, SacII sites; E, E-boxes. The numbers at the bottom designate the positions of predicted nucleosomes relative to TSS.



probe *a*, the major DNase I hypersensitive band became weakened and shifted upward even at 3 h of differentiation (Figure 7A). Higher resolution of DNase I cleavage patterns at the hTERT promoter was revealed by hybridizing the same blots with the downstream probe *b* (Figure 7B). The 1.7-kb hypersensitive band corresponding to the HS1 at the hTERT core promoter virtually disappeared after 3 h of DMSO treatment, suggesting that the postulated nucleosomal deposition (the -1 nucleosome) at the core promoter occurred very early during differentiation. ChIP experiments showed that loss of c-Myc binding and dissociation of Pol II from the hTERT promoter also took place within this initial phase of differentiation (Figure 7C). Conversely, acetylation and methylation of histones decreased gradually (Figure 7C). Together, these results demonstrated that the initial phase of hTERT repression occurred almost immediately upon the initiation of differentiation and involved dissociation of c-Myc and Pol II from the core promoter and concurrent occupation by a de novo nucleosome.

Stable Silencing of the hTERT Promoter

Covalent modifications of histones and nucleosomal remodeling at the hTERT promoter seemed to be temporally distinct events during differentiation, leading to a stably silenced *hTERT* gene in differentiated cells. To determine when stable repression of the hTERT promoter was established, HL60 cells were treated with DMSO for various times, and hTERT expression was measured by real-time RT-PCR 24 h after DMSO removal. As shown in Figure 8A, the level of hTERT mRNA returned to ~70% of the pretreatment level after DMSO was withdrawn at 1 d of differentiation. Two days after the initiation of differentiation, reversal of hTERT repression still occurred but the recovered level was <20% of that in proliferating cells. After 3 and 4 d of differentiation, the hTERT transcriptional repression became essentially irreversible. Because c-Myc and Mad1 were probably involved in the transcriptional regulation of the *hTERT* gene, their expression levels were also determined by RT-PCR analysis. As shown in Figure 8B, DMSO withdrawal resulted in significant increases of c-Myc expression and marked decreases of the Mad1 mRNA level at all time points. Interestingly, the up-regulation of c-Myc and down-regulation of Mad1 did not lead to substantial increase of

hTERT expression at 3 or 4 d of differentiation. Therefore, these results indicated that repression of the hTERT promoter became stabilized on days 2–3 during differentiation, correlating with nucleosomal remodeling at the hTERT core promoter, especially the remodeling of +1 nucleosome.

Inhibition of Histone Deacetylation and hTERT Repression

Our results showed that deacetylation of core histones and nucleosomal remodeling occurred at the hTERT promoter in differentiating HL60 cells, suggesting that both processes contributed to hTERT repression. To determine whether the two processes were interdependent, HDACs were inhibited by trichostatin A (TSA) during HL60 cell differentiation. In this experiment, HL60 cells were only allowed to differentiate in the presence or absence of TSA for 24 h because longer TSA treatment resulted in increased cytotoxicity. Surprisingly, the hTERT mRNA level only slightly increased at higher concentrations of TSA (2 and 4 μ M) after 1.5 h of differentiation, but its level showed no difference with or without TSA treatment at 3 h of differentiation (Figure 9A). ChIP experiments showed that TSA treatment completely prevented deacetylation of histones H3 and H4 at the hTERT core promoter during the first 24 h of differentiation (Figure 9B). In addition, examination of the chromatin structures by DNase I analysis revealed that HDAC inhibition by TSA resulted in a significant increase of general sensitivity of the hTERT promoter region to DNase I digestion but did not prevent the disappearance of HS1 (Figure 7, A and B). A closer look at the chromatin structures of TSA treated cells at 24 h of differentiation confirmed that MspI clusters 1–3 in differentiating cells became much less accessible to MspI digestion than the control proliferating HL60 cells despite the lack of histone deacetylation (Figure 9C). Comparing with the proliferating cells, intranucleosomal MspI sites 4 and 6 in differentiating cells were also more resistant to MspI digestion, whereas the internucleosomal MspI 5 was unaffected, consistent with a more stable nucleosomal array in this region. These results demonstrated that histone deacetylation was dispensable for nucleosomal remodeling at the hTERT promoter during the early stage of cell differentiation. The cessation of hTERT transcription was more closely

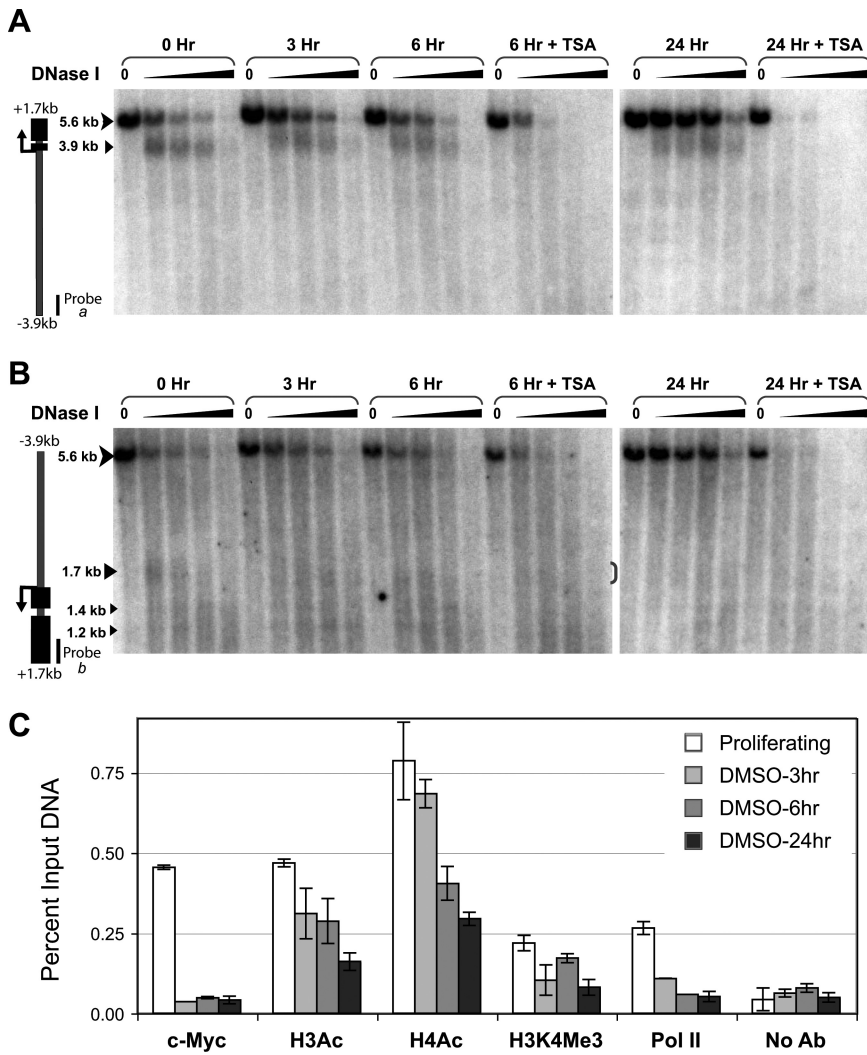


Figure 7. Analyses of the hTERT promoter during early phase of differentiation. (A and B) DNase I sensitivity of the hTERT promoter region. HL60 cells were induced to differentiate with 2.5% DMSO for 0, 3, 6, and 24 h in the presence or absence of 4 μ M TSA. The experiments were performed as those in Figure 3. (C) Quantitative ChIP analysis of the hTERT promoter. Experiments were carried out similarly as in Figure 2.

linked to nucleosomal remodeling than histone deacetylation at the hTERT promoter.

DISCUSSION

Telomerase activity and hTERT mRNA expression is undetectable in the majority of adult somatic cells (Wright *et al.*, 1996; Horikawa *et al.*, 2005). How the repression of *hTERT* gene is initiated and maintained in somatic cells remains to be an important and yet unresolved question. Here, using HL60 cells as a model and exploiting multiple nuclease mapping techniques, we delineated the chromatin structures of the hTERT promoter during its transcriptional repression in differentiating cells. Our results, for the first time, revealed that transcriptional repression of the *hTERT* gene underwent at least three different states: transcriptionally active, nascently repressed, and stably silenced states.

The active hTERT promoter in proliferating HL60 cells contained a strong DHS (HS1), most likely a nucleosome-free region, at the core promoter sequence immediately upstream of TSS, as revealed by its hypersensitivity to DNase I and restriction enzyme MspI. This nucleosome-free region was surrounded by an upstream (-2) and a downstream nucleosome (+1). Because intranucleosomal DNA sequences in both adjacent nucleosomes were sensitive to MNase

and/or MspI, the boundary nucleosomes were probably in modified and/or mobile configurations. Alternatively, these two nucleosomes might be absent in a subpopulation of cells, rendering the underlining sequence susceptible to nuclease cleavage. Although the exact status of these two nucleosomes remains to be elucidated, it was suggested previously that boundary nucleosomes were enriched with certain histone variants or covalent modifications, such as H3.3, H2A.Z, and/or ubiquitinated histones (Jin and Felsenfeld, 2007; Weake and Workman, 2008). The maintenance of this active transcriptional state probably involved binding of the c-Myc protein, or related transcription activators, to the hTERT promoter, recruiting HAT and ATP-dependent nucleosomal remodeling complexes (Vervoorts *et al.*, 2003), as the promoter region contained hyperacetylated histones H3 and H4.

Transcription of the *hTERT* gene ceased within the first few hours of differentiation. At this nascent repression state, the promoter contained a newly assembled nucleosome (-1) at the previously nucleosome-free region of the core promoter, as indicated by the loss of the major DHS HS1 as well as the decreased accessibility by MspI. However, the upstream (-2) and downstream (+1) nucleosomes remained largely unchanged at this time of differentiation. This nascent repression state coincided with the increase of expres-

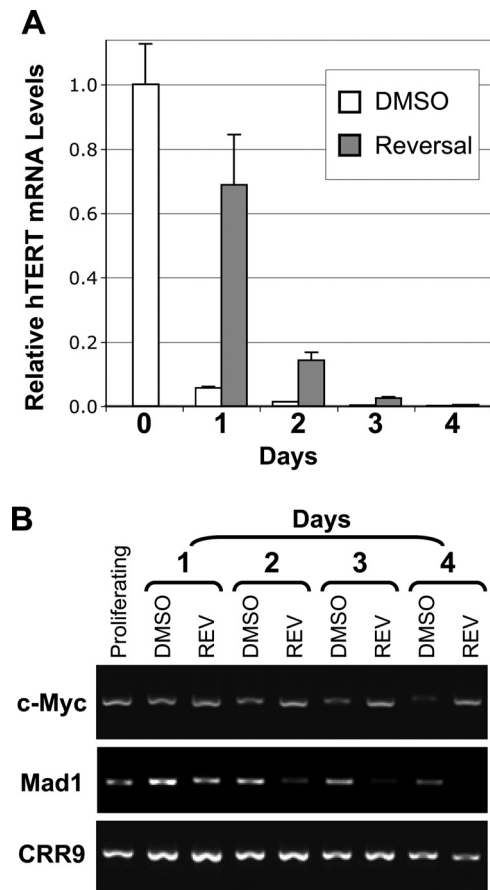


Figure 8. Stability of hTERT repression. (A) hTERT mRNA levels in differentiating HL60 cells upon DMSO withdrawal, as determined by real-time RT-PCR analysis. (B) The levels of c-Myc and Mad1 mRNAs as determined by semiquantitative RT-PCR analysis. HL60 cells were treated with 2.5% DMSO for 0, 1, 2, 3, or 4 d. At each day of differentiation, one of the duplicate plates was harvested for RNA (DMSO); the other plate was incubated in medium without DMSO for an additional 24 h (Reversal or REV). Levels of hTERT mRNA shown are averages of triplicates normalized to 18S rRNA. For semiquantitative PCR, equal amounts of cDNA were used in each PCR reaction and the CRR9 mRNA level was used as an internal control.

sion of Mad family genes (Figure 1A; data not shown) and the switch of promoter occupancy from c-Myc to Mad1 proteins, resulting in the recruitment of mSin3A-containing complexes with HDAC activity (Figure 1C; Xu *et al.*, 2001). In addition, recent data from the Xu laboratory showed that the histone demethylase RBP2 was also recruited to the hTERT promoter by Mad1 upon HL60 differentiation (Ge *et al.*, 2009). As a result, acetylation of core histones H3 and H4, as well as di- and trimethylation of lysine 4 of H3, decreased throughout the entire differentiation process. In spite of this, the conversion from the state of active transcription to the nascent repression state did not require histone deacetylation at the hTERT promoter (Figure 9). Thus, it was likely that removal of the c-Myc transcription factor and the associated nucleosomal remodeling complexes from the promoter resulted in the incorporation of a de novo nucleosome to the core promoter or vice versa. However, the repression at this nascent state remained fragile and reversible, because hTERT transcription was almost fully recovered upon DMSO withdrawal (Figure 8A).

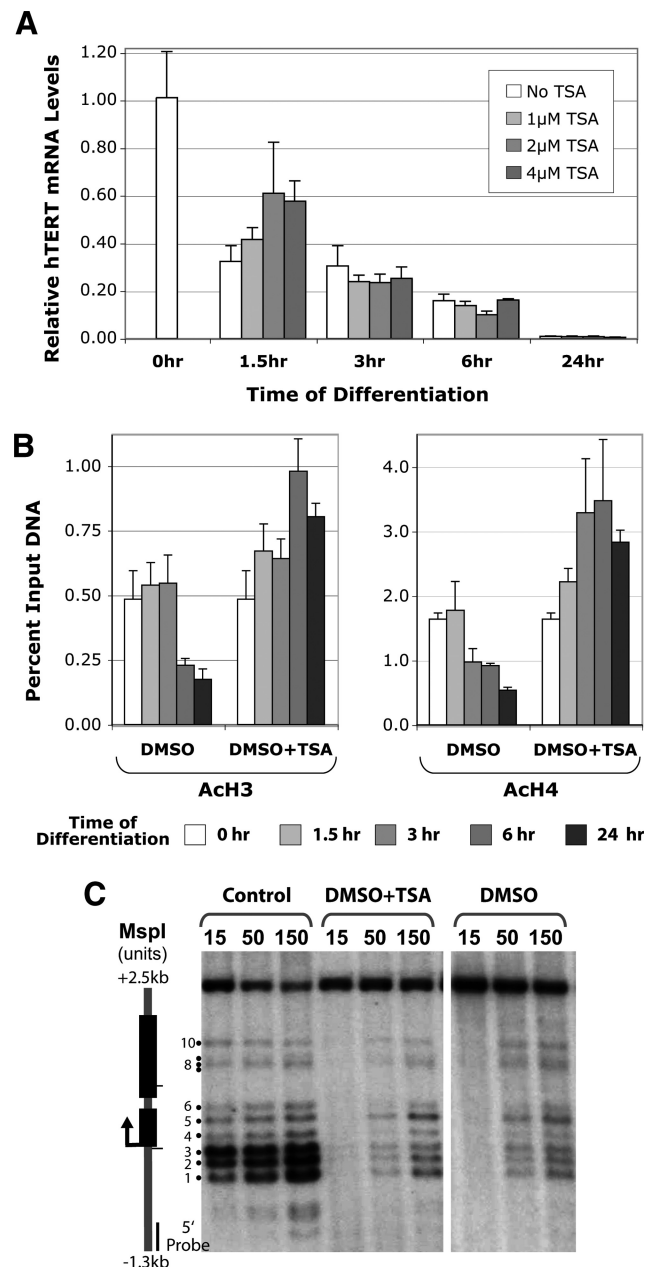


Figure 9. Histone deacetylation and repression of hTERT transcription. (A) Levels of hTERT mRNA at various time points during early phase of differentiation, as determined by real-time RT-PCR analysis and normalized to 18S rRNA. HL60 cells were treated with 2.5% DMSO in the presence or absence of HDAC inhibitor TSA. (B) Quantitative ChIP analysis of acetylations of H3 and H4 at the hTERT core promoter at various time points of differentiation in the absence or presence of 4 μM TSA. Experiments were carried out similarly as in Figure 2. Data shown in A and B were averages of triplicate samples. (C) Effect of histone deacetylation on MspI accessibility of the hTERT promoter. HL60 cells were treated with 2.5% DMSO for 24 h in the presence or absence of 4 μM TSA. Nuclei digestions with MspI, Southern analysis, and diagrams and labels were the same as described in Figure 5. Bands were detected by indirect end labeling using the 5' end probe.

During the later stages of differentiation (days 2–4), nucleosomes at the hTERT promoter continued to remodel and the repression ultimately became irreversible (Figure 8). In particular, nucleosomes (–2) and (–1), the two nucleosomes

overlapping with the hTERT core promoter, became more compact and stable, as indicated by the progressively inaccessible MspI clusters 1 and 3, respectively (Figure 5). In both proliferating and early differentiating cells (days 0, 1, and 2), nucleosome (+1) covered an extended region within the first exon and its DNA was susceptible to MNase attack (Figure 4), suggesting that this nucleosome was mobile and/or missing in a subpopulation of cells. Continuing remodeling resulted in stabilization of nucleosome (+1), as suggested by its decreased MNase sensitivity (Figure 4A, lane d, and Bd). This nucleosome was also more compact and occupied less DNA sequence, as indicated by increased accessibility of MspI sites 4 and 5 (Figures 5 and 6). It has been reported previously that the nucleosome (+1) also adopted such an altered configuration in the active, but not inactive, HPRT promoter (Chen and Yang, 2001). The molecular nature of this type of altered nucleosomal configuration remains to be determined, although it was proposed previously that incorporation of linker histones and histone variants, as well as covalent modifications of core histones, might exert a major influence on nucleosomal mobility and chromatin accessibility (Ura *et al.*, 1995; Jin and Felsenfeld, 2007; Weake and Workman, 2008). Our result indicated that remodeling of nucleosome (+1) was not required for the initiation of repression but might be important to lock the hTERT promoter into a stably repressive state. In this stably repressed state of the promoter, nucleosomes were orderly positioned over the 5' end of *hTERT* gene. Furthermore, the maintenance of this silenced state did not seem to depend on continued expression of Mad1, Mad3, and Mad4, which increased on day 1, peaked on days 1–3, and decreased after day 4 (Figure 1A; data not shown). In addition, in this silenced state, histones H3 and H4 at the core promoter were hypoacetylated. Although not required for the initial repression, histone deacetylation might be important for stable maintenance of the repressive state of the hTERT promoter.

Three parallel mechanisms of repression have been proposed from previous studies: nucleosome positioning, recruitment of HDACs, and direct interference with the general transcription machinery or activators. Using DNA damage-inducible genes, Zhang and Reese (2004) observed that genetically inactivating at least two of the three processes led to a corresponding increase in the expression of RNR3 and HUG1 in *Saccharomyces cerevisiae*. Although such genetic manipulations are difficult to achieve in a mammalian cell culture system, we believe that similar mechanisms are also important for repressing the hTERT promoter. Our experiments further demonstrated that nucleosome positioning and histone deacetylation might play temporally distinct roles in the course of repression of the *hTERT* gene.

Based on our experiments, transcription occurred at the hTERT promoter that contained a nucleosome-free region upstream of TSS. This nucleosome-free region, as revealed by HS1, has been detected in all telomerase-expressing cells (Wang and Zhu, 2004), indicating that it is essential for hTERT transcription. Maintaining this nucleosome-free region probably involves the binding of activators such as c-Myc protein, which recruits HAT and ATP-dependent nucleosome remodeling complexes. Although histone acetylation facilitates transcription factor binding and eases nucleosomal displacement, ATP-dependent nucleosomal remodeling complexes may be responsible for nucleosome removal. Indeed, moderate inhibition of histone acetylation by curcumin, which was shown to inhibit the p300 CBP (cAMP response element-binding protein binding protein) HAT (Balasubramanyam *et al.*, 2004), led to a significant reduction of hTERT transcription in proliferating cells (Sup-

plemental Figure S1). This inhibition also occurred in the presence of protein synthesis inhibitor cycloheximide, indicating a direct role of histone acetylation in maintaining the active state of the hTERT promoter (Supplemental Figure S1B). Therefore, during the initial phase of differentiation, binding of negative transcription factors such as Mad proteins to the hTERT promoter may indirectly result in nucleosomal deposition by displacing the activator c-Myc (Figure 7C) and thus its associated HATs and other chromatin modifiers. In addition, negative factors may also recruit nucleosome assembly factors/histone chaperones, which bind to histones and facilitate their assembly into nucleosomes (Akey and Luger, 2003). This nucleosomal deposition alone may be sufficient to shut down transcription because nucleosome structure is incompatible with the assembly of transcription machinery due to the large amount of DNA sequence directly contacted by the Pol II/PIC complexes (Kornberg and Lorch, 1999). As proposed by Zhao and colleagues, although histone acetylation is involved in nucleosomal eviction, nucleosomal deposition does not necessarily require histone deacetylation (Schones *et al.*, 2008). Instead, histone deacetylation may be more closely linked to chromatin stabilization after nucleosomal deposition.

Although histone deacetylation has been generally linked to gene repression, its precise role in transcriptional regulation remains unresolved. During HL60 cell differentiation, early repression of the hTERT promoter did not require histone deacetylation. Although surprising, this result was consistent with studies in other model systems. For example, repression of RNR3 remained when genes encoding histone deacetylases HDA1 and RPD3 were either deleted alone or together (Zhang and Reese, 2004). In contrast, significant derepression was observed when $\Delta hda1$ mutation was combined with another mutation $\Delta isw2$, which disrupted nucleosome positioning. However, histone deacetylation is probably essential for stable hTERT silencing, because HDAC inhibition led to hTERT transcription in many human somatic cells. The important role of histone deacetylation may lie in the subsequent remodeling of nucleosome (+1) and/or stabilization of the repressive state of the hTERT promoter in differentiated cells.

Several questions need to be addressed to further understand the mechanisms of hTERT silencing in somatic cells. First, besides the Myc/Max/Mad network proteins, other transcription factors, such as Sp1, E2F1, Ets family protein, and AP1 (activator protein-1), also have been implicated in hTERT regulation (Crowe *et al.*, 2001; Goueli and Janknecht, 2004; Takakura *et al.*, 2005; Wooten and Ogretmen, 2005). Thus, it is still not resolved whether the switch of promoter occupancy by the c-Myc protein to Mad repressors was sufficient for transcriptional repression. Second, factors that facilitate nucleosomal deposition and subsequent nucleosomal remodeling at the hTERT core promoter remain to be identified. Third, it is of significant interest to understand the detailed molecular characteristics of the boundary nucleosomes (–2) and (+1) before and after remodeling that eventually led to the stably repressed state of the hTERT promoter. Finally, although the current work provided significant insight into the mechanisms of hTERT repression in differentiating HL60 cells, future studies will be needed to determine whether these mechanisms are also involved in hTERT silencing in other somatic cells. To this end, our recent study also showed that hTERT repression was associated with decreased DNase I accessibility at the hTERT promoter during TPA (12-*O*-tetradecanoylphorbol-13-acetate)-induced U937 cell differentiation (Wang and Zhu, 2004; Wang *et al.*, 2009).

In summary, the current study identified several spatial and temporal steps of hTERT repression during the differentiation of HL60 cells. Our data demonstrated that the initial transcriptional repression of the hTERT promoter was independent of histone deacetylation but correlated with the deposition of a nucleosome to the core promoter. Subsequent remodeling of nucleosomes, in particular the nucleosome (+1) downstream of TSS, resulted in a stably silenced hTERT promoter.

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