Histone Deacetylation Is Involved in the Transcriptional Repression of hTERT in Normal Human Cells*

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Transcriptional regulation of the human telomerase reverse transcriptase (hTERT) gene, encoding the catalytic protein of human telomerase, plays a critical role in the activation of the enzyme during cell immortalization and tumorigenesis. However, the molecular mechanisms involved in the regulation of hTERT expression are still not fully understood. We have previously cloned and characterized the genomic sequences and promoter of the hTERT gene. Here, we provide evidence that histone deacetylation is involved in the repression of hTERT in human cells. Inhibition of histone deacetylases by trichostatin A in telomerase-negative cells resulted in activation of telomerase activity and up-regulation of hTERT mRNA. Transient transfection experiments with a reporter under control of the hTERT promoter indicated that this promoter can be activated by trichostatin A. Finally, our results show that repression of the hTERT promoter by the Mad protein requires histone deacetylase activity, whereas de-repression by trichostatin A is independent of the E-boxes located in its core region.

Eucaryotic chromosomes are capped by specialized structures, the telomeres, that are composed of tandemly repeated telomeric DNA and its associated proteins (1). Telomeres are essential for the complete replication of chromosomes and for their stability. Because of the inability of conventional DNA polymerases to fully replicate the 3’ end of the lagging strand of linear molecules, telomeric sequences are progressively lost with cell division. This loss, which results in telomere shortening, is thought to act as a molecular clock that controls the replicative capacity of cells and their entry into senescence (2). One mechanism to overcome this control is the activation of telomerase, an RNA-dependent DNA polymerase that synthesizes telomeric DNA de novo and thus compensates for the sequence loss occurring during semi-conservative DNA replication (1). In human, telomerase is active during embryonic development, in adult germ line tissues, and in essentially all malignancies, but with the exception of stem cells, it is not expressed in somatic cells (3).

The human telomerase core enzyme consists of an essential structural RNA (hTER) with a template domain for telomeric DNA synthesis and of a catalytic protein (hTERT) with reverse transcriptase activity (3, 4). The telomerase-associated protein, TEP1, is also a component of the telomerase complex, but its function remains unclear. hTER and TEP1 are constitutively expressed in all cells and are therefore not likely to be involved in the regulation of telomerase activity (3, 5). On the other hand, expression of hTERT parallels that of telomerase activity. In vitro and in vivo reconstitution of telomerase has demonstrated that hTERT is the rate-limiting determinant of enzymatic activity (6–9). In addition, ectopic expression of hTERT in normal cells results in telomere maintenance and life-span extension (8, 9), whereas inhibition of the enzyme in immortal cells is lethal (10–12). Together, these observations support a key role for hTERT in cell immortalization and tumorigenesis.

Although recent studies indicate that the regulation of telomerase activity is multifactorial in mammalian cells (5), transcriptional regulation of hTERT appears to be the primary mode of control. The recent cloning and characterization of the hTERT gene and its promoter has provided essential reagents to study the molecular mechanisms of telomerase regulation (13–16). Sequence analysis has revealed that the hTERT promoter is highly GC-rich, lacks TATA and CAAT boxes, but contains binding sites for a numbers of transcription factors including the Myc/Mad binding site (E-box) and estrogen response elements. Although the GC content of the promoter suggested that methylation could be involved in promoter repression in normal cells, recent reports (17, 18) and our unpublished data do not support this hypothesis. We and others have recently shown that estrogen and its receptor activate telomerase in estrogen-responsive cells through the estrogen response element sites in the hTERT promoter (19, 20). It has been also demonstrated that the Myc and Mad proteins, respectively, can activate or repress the hTERT promoter through their interaction with the E-boxes (21–23). These findings, and the abundance of potentially regulatory motifs in the hTERT promoter, are compatible with the possibility that this promoter may be subject to multiple levels of control and may even be regulated by different factors in different cellular contexts.

Recent studies have provided molecular evidence that modifications of chromatin structure are of fundamental importance in gene regulation. Histone acetyltransferases and deacetylases are known to interact with components of the transcription machinery, causing promoter-specific alteration of chromatin (24). A number of transcription factors can associate with histone acetyltransferases, which stimulate transcription by acetylating histone and disrupting nucleosome structure. Conversely, several other factors have been shown to interact with histone deacetylases, whose activity leads to nucleosome formation and promoter repression (25–28). In this study, we provide evidence that histone deacetylases are involved in repression of the hTERT promoter through at least

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The abbreviations used are: hTER, human telomerase RNA; hTERT, human telomerase reverse transcriptase; TSA, trichostatin A; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; HER, human embryonic kidney; bp, base pair(s).
two mechanisms: one is mediated by Mad, and a second is independent of the presence of E-boxes in the promoter sequences.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human fibroblasts WI-38, MRC-5, and BJ and human embryonic kidney (HEK) cells are telomerase-negative normal cells (8, 9, 13). HA1 are SV40-transformed HEK cells that do not express telomerase activity prior to immortalization but become telomerase-positive after immortalization (HA1-IM) (29). HT1080 and HeLa cells are telomerase-positive tumor-derived cell lines. All cells were cultured in α-MEM (minimal essential medium) with 10% fetal calf serum. For experimental purposes, trichostatin A (TSA, Sigma) dissolved in Me2SO was added to the culture medium at a final concentration of 100 ng/ml for 24 h; a corresponding volume of Me2SO was added to control cultures. Plasmids and Transfections—p5996del was generated from p5996 (13) by deletion of the 325 bp of the hTERT coding region present in the latter plasmid. Plasmid p181, containing the core promoter of hTERT, and its derivatives Myc-MT1 and Myc-MT2 (with individually mutated E-boxes) and Sp1-MT1-5 (with mutation of 5 Sp1 sites) (30) were gifts from S. Kyo (Kanazawa University, Japan). Plasmid Myc MT1 +2 was generated by subcloning a SacII-BglII fragment from Myc-MT2 into the corresponding sites of Myc-MT1; Myc-MT1 +2 contains mutations in both E-boxes of the hTERT promoter. pSPMad and pSPMadpro were obtained from R. Eisenman (Fred Hutchinson Research Center, Seattle) and encode a wild type or a mutated inactive form of Mad (26). Cells were seeded at a density of 1.5 × 10⁵ cells/60-mm plate 1 day prior to transfection. Transfections were performed with LipofectAMINE (Life Technologies, Inc.) on triplicate cultures using 2 µg of each plasmid/plate and were repeated at least three times. When required, TSA was added 5 h after transfection. Transfected cells were harvested 24 h post-transfection, and cells extracts were prepared and assayed for luciferase activity using reagents and protocols from Promega. Enzymatic activity was normalized to amount of protein.

**Telomerase Assay**—Cell extracts were prepared by detergent lysis, and enzymatic activity was detected by the PCR-based telomerase repeat amplification protocol (TRAP) (31) and quantitated using an internal standard.

**RT-PCR Analysis of hTERT mRNA**—Total RNA was extracted with Trizol (Life Technologies, Inc.) and incubated with DNase I prior to cDNA synthesis. Briefly, a 30-µl reaction containing 2 µg of total RNA, 6 µl of 5X first-strand buffer, 2 units of DNase I, and 1 µl of RNasin (Life Technologies, Inc.) was incubated for 30 min at 37 °C and then heated at 65 °C for 10 min to inactivate the enzyme. Half of the treated RNA was used for RT-PCR analysis of hTERT mRNA using 200 ng of LT5 primer (32) and Superscript II (Life Technologies, Inc.) according to the manufacturer’s protocol. PCR amplification of 145 bp of hTERT cDNA was performed with primers and conditions described by Ulaner et al. (33). The remaining half of the RNA was used for RT-PCR of GAPDH mRNA (32). Amplified PCR products were electrophoresed in 2% agarose gel containing ethidium bromide (0.5 µg/ml) and visualized under UV light.

**RESULTS AND DISCUSSION**

The Histone Deacetylase Inhibitor, TSA, Induces Telomerase Activity and hTERT mRNA in Human Cells—To investigate whether histone deacetylation is involved in the repression of hTERT in human somatic cells, normal BJ fibroblasts, HEK cells, and SV40-transformed HA1 cells, all of which are telomerase-negative, were treated with TSA, a specific inhibitor of histone deacetylases. As shown in Fig. 1A, telomerase activity was readily detectable in all three cell types following TSA treatment. When used on telomerase-positive cells (Fig. 1B), TSA was unable to enhance telomerase activity in HT1080 or HeLa cells, both of which express abundant amounts of this enzyme. On the other hand, a reproducible increase in telomerase activity (3-4-fold) was detected in HA1-IM cells, which express constitutively low levels of telomerase (13, 34). These findings demonstrate that telomerase repression in normal cells can be reversed by inhibition of deacetylases and further indicate that a deacetylation-dependent degree of repression can persist in immortal cells.

The levels of hTERT mRNA were measured in TSA-treated and untreated cells by RT-PCR (Fig. 1C). The hTERT message was not detectable in human fibroblasts BJ, MRC5, and WI38, or in HEK and HA1 cells, and was weakly expressed in HA1-IM cells. Treatment with TSA up-regulated hTERT mRNA expression in all cell types. Although these results do not rule out the possibility that TSA indirectly enhances the stability of the hTERT mRNA, they strongly suggest that induction of telomerase by this compound may be caused by de-repression of hTERT transcription. This conclusion is fully supported by transient transfection experiments (see below) demonstrating...
that TSA enhances the transcriptional activity of the hTERT promoter.

**TSA Activates the hTERT Promoter**—We have previously shown that hTERT promoter activity is contained within a DNA fragment of 3996 bp upstream of the hTERT ATG (13). This promoter has a high GC content and lacks detectable TATA or CAAT boxes but contains an abundance of binding sites for several transcription factors (13). Core promoter activity has been mapped to a fragment of less than 300 bp upstream of the ATG, which contains two E-boxes and five Sp1 sites that are involved in promoter regulation (13–16). To further define the effects of histone deacetylation on hTERT repression, we transiently transfected HA1 and HA1-IM cells with p3996del, which encodes luciferase under control of the 3996 bp hTERT promoter (Fig. 2A). As previously shown (13), the activity of this promoter correlates with the presence of telomerase. Thus, only background levels of luciferase were detected in untreated HA1 cells, but treatment with TSA resulted in a 16-fold increase in activity (Fig. 2B). Similar results were observed with HEK, BJ, and MRC5 normal human cells (data not shown). As expected, luciferase was expressed in transfected HA1-IM cells even in the absence of TSA. Addition of the inhibitor, however, resulted in a 4.5-fold increase in activity (Fig. 2B), in agreement with the observed enhancement of telomerase activity and of hTERT mRNA. These results further indicate that active repression of hTERT requires histone deacetylase activity.

**TSA Relieves Mad-mediated Repression of the hTERT Promoter**—Recent studies have shown that Mad represses the hTERT promoter by competing with Myc for binding to E-boxes (22, 23). The members of the Myc network, which are central to the control of cell growth and development, regulate diverse processes such as cell transformation, differentiation, and apoptosis. Myc and Mad can form heterodimers with Max, which also binds to E-boxes. Whereas the Myc:Max complex activates transcription and promotes cell proliferation and transformation, Mad:Max heterodimers repress transcription and block Myc-mediated cell transformation (25–28). Repression by the Mad:Max complex is thought to be mediated by chromatin condensation through the recruitment of histone deacetylases (28). We reasoned that in such case, inhibition of these enzymes could block the Mad-dependent repression of the hTERT promoter. As shown in Fig. 3, treatment with TSA was again found to enhance luciferase activity (here, by about 5-fold) in HA1-IM cells transfected with p3996del. Cotransfection of the Mad expressing plasmid pSPMad resulted in repression of promoter activity. In turn, this repression was significantly alleviated by treatment with TSA. As expected, cotransfection of pSPMadpro encoding mutant Mad did not repress the hTERT promoter. Interestingly, we repeatedly observed that induction of luciferase activity by TSA was much more pronounced in cells co-transfected with the Mad mutant than in those receiving wild type Mad. The reasons for this effect are not clear at present. Our data confirm those of others (22, 23) showing that Mad represses the hTERT promoter, and they further show that this repression requires the activity of histone deacetylases, which may be recruited by Mad repressor complexes bound to the E-boxes within the promoter.

**FIG. 2.** Effects of TSA on the hTERT promoter activity. A, schematic map of p3996del. hTERT regulatory sequences, extending to −3996 bp relative to the hTERT ATG, were cloned into the pGL2 vector (Stratagene) upstream of the ATG of the luciferase reporter. Transcription factor binding sites relevant to our study and contained within the core promoter are shown. B, the empty vector pGL2 and the hTERT promoter construct p3996del were transiently transfected into telomerase-negative HA1 cells or telomerase-positive HA1-IM cells. Luciferase activity was normalized by protein concentration. Fold induction in response to TSA treatment, calculated relative to the activity of each construct in the absence of TSA, is indicated numerically above the respective histograms. The mean ± SD from three independent experiments is shown.

**FIG. 3.** TSA blocks Mad repression of the hTERT promoter. The indicated plasmids were transiently transfected into HA1-IM cells; luciferase activity in cell extracts was measured and normalized as described for Fig. 3.

**FIG. 4.** The de-repression of the hTERT promoter by TSA is independent of E-boxes. The indicated plasmids were transiently transfected into telomerase-negative HA1 cells; luciferase activity in cell extracts was measured and normalized as described for Fig. 3.
Repression by Histone Deacetylation Can Be Independent of E-boxes—We queried whether, in addition to E-boxes, there are other cis-elements in the hTERT promoter that are able to recruit histone deacetylases. To this end, we transiently transfected wild type and mutant hTERT promoter/reporter constructs (30) into HA1 cells. As previously shown for the larger promoter in p3996del, expression of luciferase under control of the hTERT core promoter (p181, containing 258 bp of hTERT regulatory sequences upstream of the ATG, Fig. 2A) was still capable of responding to TSA treatment to the same extent as the wild type promoter. This result suggests that histone deacetylases may indeed be tethered to the hTERT promoter by cis-regulatory elements other than E-boxes. Potential candidates would be Sp1 sites, which have recently been shown to interact with deacetylases to repress transcription (35). There are in fact five Sp1 sites between the two E-boxes of the core hTERT promoter (Fig. 2A (30)); however, mutations of these sites abolish promoter activity (data not shown (30)). Microcell fusion experiments have identified several human chromosome loci capable of repressing telomerase activity in diverse cellular contexts (36). Our findings that histone deacetylases are involved in the silencing of the hTERT promoter, via a mechanism that is independent of E-boxes and Mad, leaves open the possibility that these enzymes may be recruited to this promoter by one or more of the still uncharacterized hTERT repressors.

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