Sp1 and Sp3 Recruit Histone Deacetylase to Repress Transcription of Human Telomerase Reverse Transcriptase (hTERT) Promoter in Normal Human Somatic Cells*

Jaejoon Won‡§, Jeongbin Yim‡§, and Tae Kook Kim§¶**

From the ‡National Creative Research Initiative Center for Genetic Reprogramming, Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea, the §Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea, and the ¶Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Activation of telomerase is crucial for cells to gain immortality. In human cells, telomerase activity is tightly regulated by the expression of its catalytic subunit, human telomerase reverse transcriptase (hTERT). In most normal human somatic cells, hTERT is not expressed, and its suppression acts as an important gatekeeper against tumorigenesis. Here we describe the systematic analyses of hTERT promoter to understand the transcriptional repression mechanism of the hTERT gene in normal human somatic cells. Through the serial deletion analysis of hTERT promoter in normal human fibroblasts, we identified a critical repressive element on the hTERT promoter. The repressive element formed DNA-protein complexes with Sp1 and Sp3 in nuclear extracts. Using formaldehyde cross-linked chromatin immunoprecipitation analysis, we found that Sp1 and Sp3 were associated with the endogenously repressed hTERT promoter in human fibroblasts. Furthermore, Sp1 and Sp3 interacted with histone deacetylase (HDAC) in these cells. Overexpression of dominant-negative mutants of Sp1 and Sp3, which contained mainly the HDAC2-binding domain, relieved the HDAC-mediated repression of the hTERT promoter. Taken together, these results suggest that Sp1 and Sp3 associate with the hTERT promoter, recruiting HDAC for the localized deacetylation of nucleosomal histones and transcriptional silencing of the hTERT gene in normal human somatic cells.

All tumor cells have functional telomere stabilization mechanisms, and most of them employ the enzyme telomerase to achieve this requirement (1–3). By catalyzing the addition of telomeric TTAGGG repeats onto the chromosome ends, telomerase stabilizes the telomere, and thus maintains the stability and integrity of linear chromosomes. In contrast, most normal human somatic cells lack the mechanisms to maintain telomeric DNA, and the progressive loss of telomeres during DNA replication limits the life span of these cells both in vivo and in vitro (4–6). Furthermore, abrogation of the telomerase activity in tumor cells has been reported to induce cell growth arrest and apoptosis (7).

Human telomerase is a complex composed of an RNA component that provides the template for the addition of new telomeric repeats, a catalytic subunit known as human telomerase reverse transcriptase (hTERT), and some additional associated proteins (8–10). The RNA component of human telomerase is expressed constitutively in most human tissues, whereas hTERT is not expressed in most normal human somatic tissues. Thus, in normal human somatic cells, telomerase activity is tightly regulated by the repression of the hTERT gene. In tumor cells, telomerase activity is closely correlated with the expression and derepression of hTERT. These findings indicate that derepression of the hTERT promoter might be an important mechanism leading to activation of the hTERT gene and telomerase enzyme in the cells during tumorigenesis.

Although hTERT gene expression has been well established as essential for the progression and maintenance of the human tumors, its activation/derepression mechanism in the course of tumorigenesis is not well understood. In the course of tumorigenesis many mutations occur, and some of these mutations are thought to contribute to the activation and derepression of hTERT. Several studies including ours have shown that one of the oncogenes deregulated in various human tumors, c-myc, can contribute to the transcriptional activation of the hTERT gene in tumor cells (11–13). In addition, amplification of the hTERT locus observed in some human tumors probably contributes to the misregulation of hTERT gene expression (14).

Genetic complementation approaches have suggested that the hTERT repression mechanism is dominant over the activation/derepression mechanism (15–17). Furthermore, hTERT expression in normal human somatic cells is tightly repressed even below the limit of detection by PCR. Thus, it is critical to understand the transcriptional repression mechanism of the hTERT gene in normal human somatic cells. Some of the transcriptional repressors of the hTERT gene have been identified, including WT1 and Mad from our studies (18–21). However, these repressors seem to be either tissue-specific or relatively minor contributors. The understanding of the general mechanism of transcriptional repression of the hTERT gene in normal cells is still limited. Experiments in a wide variety of normal cells are needed to develop this understanding.

*This work was supported by the Brain Korea 21 Project of the Korean Ministry of Education, Molecular Medicine Research Group Program M1-0106-00-0117, and the Creative Research Initiatives of the Korean Ministry of Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’.

†To whom correspondence may be addressed: National Creative Research Initiative Center for Genetic Reprogramming, Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea.

‡To whom correspondence may be addressed: Institute of Chemistry and Cell Biology, Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA. Tel.: 617-432-4954; Fax: 617-432-3702; E-mail: TK_Kim@hms.harvard.edu.

1 The abbreviations used are: hTERT, human telomerase reverse transcriptase; TSA, trichostatin A; HDAC, histone deacetylase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; HA, hemagglutinin.
Sp1 and Sp3 Recruit HDAC to Repress hTERT Promoter

Formaldehyde Cross-linked Chromatin Immunoprecipitation (X-ChIP) Assay—X-ChIP assays were performed using the ChIP assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. Cells were fixed in normal culture medium with formaldehyde at a final concentration of 1% for 10 min at 37 °C. Sonication was performed to achieve an average DNA length of 500 bp. The following antibodies were used for the immunoprecipitation of the cross-linked chromatin: anti-Mad (65396E, Pharmingen), anti-Sp1 (sc-59, Santa Cruz Biotechnology), anti-Sp3 (07-107, Upstate Biotechnology), and anti-HA (71-5500, Zymed Laboratories Inc.). A ~160-bp fragment in the hTERT proximal promoter was amplified using the primers 5′-TGGCCTCTGCCTGAGGAGCGTG-3′ and 5′-TGGCCTCTGCCTGAGGAGCGTG-3′. The amplified DNA was separated on 1% agarose gel and visualized by ethidium bromide.

Co-immunoprecipitation—Co-immunoprecipitation was performed as described (27) with minor modifications. Whole cell extracts were prepared by resuspending IMR90 or HFF cells in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and Boehringer Complete protease inhibitor mixture, and incubated on ice for 30 min. After centrifugation of the lysates at 16,000 × g for 5 min at 4 °C, 1 mg of the supernatants (cell extracts) were precleared with 50 μl of protein A-agarose bead suspension (Santa Cruz Biotechnology), the mixture was further incubated with rotation for 4 h at 4 °C. After three washes with extraction buffer, the beads were resuspended in 50 μl of extraction buffer. For immunoprecipitation of the endogenous proteins, anti-Mad (65396E, Pharmingen), anti-Sp1 (sc-59, Santa Cruz Biotechnology), and anti-Sp3 (07-107, Upstate Biotechnology) antibodies were used. Anti-HA antibody (71-5500, Zymed Laboratories Inc.) was used as a negative control for the immunoprecipitation of the endogenous proteins. For immunoprecipitation of the HA-tagged proteins, anti-HA antibody (sc-7392, Santa Cruz Biotechnology) was used.

Western Blot—Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 5% nonfat milk and probed with anti-HDAC2 antibody (51-5100, Zymed Laboratories Inc.). Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (sc-2054, Santa Cruz Biotechnology) and visualized using the ECL system (Amersham Biosciences).

Plasmids—The construction of p-2396 and p-1003 was described previously (12, 20). A series of deletions of the 3396-bp hTERT promoter region was generated from p-3396 by unidirectional 5′–deletion using Erase-a-Base (Promega). pGL3-Promoter-RE was constructed by the insertion of the sequences between −168 to −3 of the hTERT promoter into the upstream of the SV40 promoter in pGL3-Promoter (Promega) using the BamHI/BgII sites in polylinker region. To obtain p-188-Sp-m1, p-188-Sp-m2, p-188-Sp-m3, p-188-Sp-m4, and p-188-Sp-m5, site-directed mutagenesis was performed with p-188 essentially as described (20). CMV-Sp1 and CMV-Sp3 were generous gifts from M. W. Hur (28). Ccineo-HA-Sp1 (1–621) and Ccineo-HA-Sp3 (622–788) were kindly provided by C. Seiser (27). g55-luc, pm-Sp1, pm-Sp3, pm-Sp3-1 (398), and pm were generous gifts from T. Sakai (29). pm-Sp1 contains Sp1 with its N-terminal 82 amino acids deleted (29). To prepare pm-Sp1-Δ(38–511), pm-Sp1 was digested with BamHI/BgII, and then three stop codons were inserted. For the construction of HA-Sp1, HA-Sp3, HA-Sp3-Δ(398–654), the DNA inserts were obtained by PCR using CMV-Sp1 or CMV-Sp3 as the template, and the resultant products were cloned into the HA tagging plasmid Ccineo-HA.

RESULTS

Identification of the DNA Element Critical for the Transcriptional Repression of hTERT—To identify the DNA element within the hTERT promoter critical for its transcriptional repression, we generated a series of 5′–deletion mutant constructs from the 3396-bp hTERT promoter (Fig. 1). We cloned the firefly luciferase reporter plasmid (Fig. 1A). These constructs were transiently transfected into normal human lung fibroblasts (IMR90), where the expression of the hTERT gene was undetectable and tightly repressed. Subsequently, luciferase assays were performed. Serial deletions from −3396 up to −275 did not affect the hTERT promoter activity (Fig. 1A).

Human somatic cells with trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC), suggest that histone deacetylation is essential to the transcriptional repression mechanism of the hTERT gene (22–24). In addition, TSA treatment has been shown to significantly up-regulate hTERT promoter activity and the corresponding telomerase activity to a level comparable with that in human tumor cells. Thus, HDAC-mediated repression could be the major, universal transcriptional repression mechanism of the hTERT gene in normal human somatic cells.

In this study, we carried out systematic analyses of hTERT promoter to identify such repressive DNA elements and interacting regulatory factors. Through a serial deletion analysis of the hTERT promoter in cultured human fibroblasts, we identified a TSA-responsive repressive element. This element formed complexes with Sp1 and Sp3 proteins in nuclear extracts derived from normal human somatic cells. Moreover, the endogenous hTERT promoter in the cells was found to be tightly associated with Sp1 and Sp3. Sp1 and Sp3 were found to have the intrinsic ability to interact with HDAC, and through this interaction HDAC is recruited onto the hTERT promoter. Thus, we show that Sp1 and Sp3 are involved in the HDAC-mediated transcriptional repression of the hTERT gene in normal human somatic cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Luciferase Assay—IMR90 (normal human lung fibroblast), WI38 (normal human lung fibroblast), and HFF (normal human foreskin fibroblast) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 120 μg/ml penicillin, and 200 μg/ml streptomycin. Transfection was carried out with LipofectAMINE reagent (Invitrogen). Luciferase assays were performed 30–48 h after transfection using the reagents from Promega according to the manufacturer’s instructions. TSA (Sigma) was dissolved in Me2SO and added to the culture medium at a final concentration of 50–200 μM. A corresponding volume of Me2SO was added to control cells not treated with TSA.

RNA Extraction and Quantitative Reverse Transcriptase-PCR—Total RNA was isolated with the Trizol reagent (Invitrogen) in accordance with the manufacturer’s instructions. The cDNA synthesis, reverse transcription, and quantitative PCR were performed essentially as previously described (25). The amplified DNA was separated on 1% agarose gel and stained with ethidium bromide.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared essentially as previously described (26) with minor modifications. Briefly, cultured cells were collected, washed with phosphate-buffered saline, and pelleted by centrifugation at 1500 × g for 3 min. The pellet was resuspended in 5 packs of pellet volume of ice-cold buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, and Boehringer Complete protease inhibitor mixture (Roche Molecular Biochemicals). The cells were allowed to swell on ice for 15 min, after which ½ packed pellet volume of a 10% Nonidet P-40 was added. The tube was vigorously vortexed for 10 s, and the homogenate was centrifuged at 20,000 × g for 30 s. After removal of the supernatant, the nuclear pellet was resuspended in 2 packed pellet volumes of ice-cold buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 10 mM KCl, 1.5 mM MgCl2, 25% glycerol, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, and Boehringer Complete protease inhibitor mixture. The tube was vigorously rocked at 4 °C for 30 min on a shaking platform. The nuclear extract was centrifuged at 20,000 × g for 5 min at 4 °C, and the supernatant was frozen in aliquots at −70 °C.

Portions of nuclear extract (5 μg) were incubated with 0.5 μg of poly(dIdC) in the absence or presence of specific antibodies at room temperature for 20 min in a 15-μl reaction volume containing 10 mM Tris-HCl (pH 7.5), 4 mM HEPES (pH 7.9), 50 mM NaCl, 20 mM KCl, 1 mM MgCl2, 0.54 mM EDTA, 0.6 mM dithiothreitol, and 8% glycerol. Antibodies used for the supershift assay were anti-Sp1 (sc-59, Santa Cruz Biotechnology) and anti-Sp3 (07-107, Upstate Biotechnology). Following incubation, the 22P-end-labeled double-stranded oligonucleotide probe (upper strand sequence: 5′-CTTCCAGCTCGCCCTCCGC- CCGCGGGA-3′) was added and incubated at room temperature for an additional 20 min. These reaction mixtures were subjected to autoradiography in a 6% polyacrylamide gel in 0.25× TBE buffer, dried, and subjected to autoradiography.
However, further deletion from $-274$ to $-189$ caused a slight increase in promoter activity, consistent with our previous report that the E-box within this region acts as the repressive element through interaction with the Mad-Max complex in normal human somatic cells (19). Interestingly, further deletion from $-188$ to $-180$ increased the promoter activity even more significantly. These findings suggest that a repressive regulatory element is within or extends into the region between $-188$ and $-180$ of the hTERT promoter.

We next investigated whether the abrupt increase in the hTERT promoter activity after deletion of the region from $-188$ to $-180$ can be observed in other normal human cells. The deletion constructs p-188 and p-179, which differ only by the “9 base pairs (bp)” between $-188$ and $-180$ of the hTERT promoter, were transfected into three different kinds of normal human fibroblasts, and then the promoter activities of the two hTERT promoter constructs were compared (Fig. 1B). Promoter activity of p-179 was significantly higher than that of p-188 in all of these cells. Thus, it is quite possible that the region of sequence between $-188$ and $-180$ may act as a repressive element in many types of normal human cells.

The Identified Repressive Element Responds to TSA, but Only in the Context of the hTERT Promoter—Because histone deacetylation has been proposed as one transcriptional repression mechanism for the hTERT gene (22), we examined whether the identified repressive element might involve this mechanism. To this end, we exploited the effects of TSA, which specifically inhibits histone deacetylases (30). As a preliminary experiment, we treated IMR90 cells with increasing amounts of TSA and examined the changes in the hTERT mRNA levels by quantitative reverse transcriptase-PCR with total RNA isolated from these cells 24 h after TSA treatment. TSA at 200 nM
Identified repressive element is TSA-responsive, but requires the hTERT promoter context. A. TSA induced both hTERT mRNA expression and hTERT promoter activity in a dose-dependent manner in IMR90 cells. Quantitative reverse transcriptase-PCR for hTERT or β-actin was performed with total RNA prepared from IMR90 cells treated with TSA (0, 50, 100, 150, and 200 nM) for 24 h (bottom panel). At each concentration of TSA, the corresponding luciferase activity of p-3396 is also shown (top panel). In this case, TSA treatment was given 6 h after transient transfection. B, deletion construct p-179 exhibits lower promoter activity than p-188 in IMR90, WI38, and HFF cells in the absence of TSA. At 6 h after transfection, cells were treated with TSA (200 nM), and luciferase assays were performed 24 h thereafter. For each of the 6 groups (3 cell types + TSA), the luciferase activity of p-188 was set to 100, and the relative luciferase activity of p-179 is presented. TSA up-regulated the reporter activity of p-188 −62, −84, and −52-fold in IMR90, WI38, and HFF cells, respectively. Results shown are the average of three experiments, and bars indicate standard deviations. C, the isolated TSA-responsive repressive element of the hTERT promoter does not function within a heterologous (SV40) promoter. The sequences around the identified repressive element (−195 to −180 of the hTERT promoter) were cloned upstream of the SV40 promoter. Then the activity of the SV40 promoter was measured by firefly luciferase reporter expression in IMR90, WI38, and HFF cells (Fig. 2C). The promoter activities of pGL3-Promoter in these normal human somatic cells were normalized to compare them with those of pGL3-Promoter-RE in the absence or presence of TSA treatment. In IMR90 cells, this introduced element decreased SV40 promoter activity in the absence of TSA treatment. In WI38 and HFF cells, this element did not significantly down-regulate the SV40 promoter activity. In addition, this isolated element supported the TSA-mediated induction of the SV40 promoter in IMR90 slightly, but not in WI38 and HFF cells. Taken together, these results imply that the repressive function of this element in normal human somatic cells requires the promoter context of the hTERT gene.

The TSA-responsive Repressive Element of the hTERT Promoter Forms Complexes with Sp1 and Sp3—To identify the nuclear protein(s) binding to the identified repressive element of the hTERT promoter, we performed an EMSA with nuclear extracts from IMR90 and HFF cells probed with the repressive element (Fig. 3). As a preliminary experiment, we performed the competition assays with unlabeled oligonucleotides containing either consensus or mutant-binding sites for Sp1, AP2, Sp3, SP1 and Sp3 Recruit HDAC to Repress hTERT Promoter.
Egr, and E2F. Because the DNA sequence within the identified repressive element is GC-rich (see Fig. 4A), it was possible that the putative transcription factors (Sp1, AP2, Egr, and E2F) could interact with the DNA element. We found that the upper three DNA-protein complexes (I, II, and III in Fig. 3) were specifically competed by the oligonucleotides containing consensus binding sequences for Sp1, but not by the oligonucleotides containing the binding sequences for AP2, Egr, or E2F.  

Consistent with these results, when these DNA-protein complexes (I, II, and III) were challenged with anti-Sp1 and anti-Sp3 antibodies, we detected the presence of Sp1 in DNA-protein complexes (I, II, and III) were challenged with anti-Sp1 and anti-Sp3 antibodies, we detected the presence of Sp1 in DNA-protein complexes I, II, and III (Fig. 3). Under these conditions, we also found that antibodies against AP2, Egr, E2F1, and other Sp family members (e.g. Sp2 and Sp4) did not affect these specific band-shift patterns.  

We next investigated any changes in these DNA-protein complexes after treatment with TSA. Nuclear extracts from IMR90 and HFF cells were treated with or without TSA. We then compared band-shift patterns with the radiolabeled probe encompassing the repressive element. No additional band(s) appeared after treatment with TSA (Fig. 3). Furthermore, we failed to detect any significant alteration in the band-shift patterns. Notably, the DNA binding activities of neither Sp1 nor Sp3 changed after TSA treatment. These results imply that the induced DNA binding activities of Sp1 and Sp3 may not be involved in the up-regulation of the hTERT promoter activity after treatment with TSA.  

**Mutation of the Sp Sites in the Proximal hTERT Promoter Increases Promoter Activity and Decreases TSA Responsiveness**—Because the TSA-responsive repressive element (−188 to −180) can form complexes with Sp1 and Sp3 in nuclear extracts (Fig. 3), the Sp site within that region was assumed to be responsible for the HDAC-mediated repression of the hTERT promoter. To assess this, we mutated the core sequences of the Sp site within this TSA-responsive repressive element (Sp site 1 in Fig. 4A; p-188-Sp-m1 in Fig. 4B). Then we examined whether the mutation produced the same effects (increased promoter activity and decreased TSA responsiveness) as the deletion of the repressive element. The construct p-188-Sp-m1 showed enhanced promoter activity in comparison with its wild-type counterpart p-188 in all three cell types (Fig. 4B). This enhancement was similar to that observed by deletion of the repressive element (from −188 to −180) from p-188 in Fig. 1B. In addition, we investigated the TSA responsiveness of this Sp site by comparing the promoter activities of p-188 and p-188-Sp-m1 in these cells after treatment with TSA. The p-188-Sp-m1 showed less promoter activity in comparison with p-188 after treatment with TSA (Fig. 4B). Consistently, we found that a mutation in this Sp site produced similar results to those with the p-179 construct, relative to p-188.
Sp1 and Sp3 Recruit HDAC to Repress hTERT Promoter

The Endogenous hTERT Promoter Is Associated with Sp1 and Sp3—To directly address whether Sp1 and Sp3 are associated with the repressed hTERT promoter, we used formaldehyde cross-linking X-ChIP analysis (Fig. 3A). After formaldehyde cross-linking of the IMR90, WI38, and HFF cells, chromatin immunoprecipitation was performed with antibodies directed against Mad1, Sp1, Sp3, and HA. The precipitated DNA was subjected to PCR with the use of specific primers for the hTERT proximal promoter region. As expected from previous reports (19, 32), tight association of the hTERT promoter with Mad1 was observed in all three cell types (Fig. 3A). In addition, both Sp1 and Sp3 were found to be associated with the endogenous hTERT promoter. The tight association of Sp1 and Sp3 with the repressed hTERT promoter strongly suggested that Sp1 and Sp3 may play a functional role in transcriptional repression of the hTERT promoter. This idea is also consistent with our results in Fig. 3A showing that mutation of Sp sites increased the hTERT promoter activity in normal human somatic cells.

Examination of the DNA binding ability of Sp1 and Sp3 after treatment with TSA showed that there is no significant alteration in the DNA-protein complex formation with the repressive element of the hTERT promoter in the EMSA (Fig. 3B). We further examined these observations in normal human somatic cells with X-ChIP analysis after treatment with TSA (Fig. 3B). Sp1 and Sp3 play an important role in transcriptional repression of the hTERT promoter. This idea is also consistent with our results in Fig. 3B showing that mutation of Sp sites increased the hTERT promoter activity in normal human somatic cells.

Endogenous Sp1 and Sp3 Are Associated with HDAC in Human Normal Somatic Cells—Based on the association of Sp1 and Sp3 with the repressed hTERT promoter, we examined the physical interaction of Sp1 and Sp3 with HDAC through co-immunoprecipitation (Fig. 5). As a preliminary experiment, we performed a Western blot with IMR90 and HFF cell extracts using anti-HDAC1, anti-HDAC2, anti-HDAC3, anti-HDAC4, anti-HDAC5, and anti-HDAC6 antibodies to examine which HDAC family member is highly expressed in these cells. Because HDAC2 is the predominant form in these cells, we immunoprecipitated Sp1 and Sp3 protein complexes from IMR90 and HFF cell extracts with anti-Sp1 and anti-Sp3 antibodies, respectively, and then we performed Western blot with anti-HDAC2 antibody (Fig. 5A). As a positive control, we used pRB, which is known to interact with HDAC2 (33). Both Sp1 and Sp3 formed complexes with HDAC2 (Fig. 5A). Interestingly, we observed a reproducibly stronger interaction of Sp3 with HDAC2 under these conditions (Fig. 5A). The association of Sp1 and Sp3 with HDAC further supports our conclusion that Sp1 and Sp3 play an important role in transcriptional repression of the hTERT promoter through recruitment of HDAC in normal human somatic cells.

Ectopic Expression of HDAC-binding Domain of Sp1 and Sp3 Increases hTERT Promoter Activity and Decreases Its TSA Responsiveness—Lastly, we addressed whether the derepression of the hTERT promoter by mutations (Figs. 1, 2A, and 4B)
can also be obtained by blocking the protein-protein interactions between the HDAC and transcription factors Sp1 and Sp3. Our results from the analyses of TSA responsiveness (Figs. 2B and 4B) and HDAC interaction (Fig. 6) indicated that Sp1 and Sp3 may play a role in the transcriptional repression of the hTERT gene through interaction with HDAC and its recruitment onto the promoter. Thus, we designed a dominant-negative approach in which we overexpressed the deletion mutants of Sp1 and Sp3, which retain the region important for the HDAC interaction but lack the DNA-binding domain (zinc finger regions indicated in Fig. 7A).

We first examined the interaction of HDAC2 with the N-terminal and C-terminal domains of Sp1 and Sp3 in normal human somatic cells. To this end, HA-tagged Sp1, Sp3, and their truncated mutants were expressed in IMR90 cells (Fig. 7A). After transient expression in IMR90 cells, protein was immunoprecipitated with anti-HA antibody and assayed for the presence of HDAC2 by Western blot with anti-HDAC2 antibody (Fig. 7B). The expression of each HA-tagged Sp protein was normalized in transfected cells. Under these conditions, full-length Sp1 and Sp3 interacted with HDAC2. Interestingly, the N-terminal domain of Sp1 and Sp3 seemed to interact with HDAC2 more efficiently than the C-terminal DNA-binding domain. These results were reproducible, and suggest that the N-terminal domain of Sp1 and Sp3 can interact with HDAC2 in normal human somatic cells.

We next investigated the intrinsic TSA responsiveness of the N-terminal regions of Sp1 and Sp3. To this end, we took advantage of a GAL4 system in which the N-terminal domains of Sp1 and Sp3 were fused with the GAL4 DNA-binding domain (Fig. 7C). These GAL4 fusion constructs were co-transfected with pG5-luciferase reporter plasmids containing five consensus GAL4-binding sites into IMR90, WI38, and HFF cells in the absence or presence of TSA. The GAL4 DNA-binding domain itself supported TSA-mediated transcriptional activation slightly (by 4–6-fold). Under these conditions, the N-terminal 621-amino acid residues of Sp1 and Sp3 exhibited a dramatic increase in TSA responsiveness (Fig. 7C). Interestingly, consistent with stronger HDAC2 interaction of the N-terminal domain of Sp1, compared with full-length Sp1 (Fig. 7B), we detected more potentiated TSA responsiveness with the N-terminal domain of Sp1 fused with the GAL4 DNA-binding domain in comparison with full-length Sp1. Taken together with the results in Fig. 7B, these results suggest that the N-terminal domain of Sp1 and Sp3 can repress transcription through interaction with HDAC2.

Based on these results, we examined whether the N-terminal domain of Sp1 and Sp3 might function as a dominant-negative.

**FIG. 7. Involvement of the N-terminal domain of Sp1 and Sp3 in the HDAC-mediated repression of the hTERT promoter.** A, a schematic diagram of the HA-tagged proteins analyzed by immunoprecipitation with anti-HA antibody. Appropriate HA-tagged constructs derived from the pCIneoHA vector were used to express the proteins indicated. Bars A, B, C, and D indicate four regions that contribute to the transcriptional properties of Sp1, as defined by Courey and Tjian (50). B, physical interaction of Sp1, Sp3, and their N- or C-terminal regions with HDAC2 in IMR90 cells. IMR90 cells were transfected with the appropriate HA-tagged constructs or the pCIneoHA vector (negative control). Whole cell extracts prepared from these transfected cells were immunoprecipitated with anti-HA antibody, and Western blot was performed with anti-HDAC2 antibody. Whole cell extract prepared from IMR90 cells (control) served as a positive control for detection of HDAC2. C, TSA responsiveness of Sp1, Sp3, and their N-terminal regions in normal human somatic cells. IMR90, WI38, and HFF cells were cotransfected with 4 μg of a luciferase reporter plasmid containing five GAL4-binding sites (pG5-luc) and 2 μg of a plasmid expressing each of the GAL4 fusion proteins indicated. Six hours after transfection, cells were treated with TSA (200 nM for 24 h). Appropriate GAL4 constructs were used to express the proteins indicated. Results shown are the average of three experiments, and bars indicate standard deviations. Fold induction by TSA is shown above the graph in parentheses. D, dominant-negative Sp1 and dominant-negative Sp3, which retain the N-terminal 621 or 398 amino acids, respectively, but lack the DNA-binding domain, relieve the repression of the hTERT promoter in IMR90 cells. IMR90 cells were cotransfected using p-1003 in conjunction with pCIneoHA-Sp1-(1–621) or pCIneoHA-Sp3-(1–398), and luciferase assays were performed 36 h thereafter. E, dominant-negative Sp1 and dominant-negative Sp3 suppress the induction of the hTERT promoter activity in IMR90 cells in the presence of TSA. Transfections were performed as in D, but 6 h after transfection, cells were treated with TSA (200 nM). After incubation for 24 h, luciferase assays were performed.
mutant that specifically abrogates the HDAC-mediated repression of the hTERT promoter. In transient transfection experiments, these deletion mutants of Sp1 and Sp3 up-regulated the hTERT promoter activity in a dose-dependent manner in IMR90 cells (Fig. 7D). These results suggest that hTERT promoter activity may be derepressed through blocking the Sp1/Sp3 interaction with HDAC. In addition, we investigated the TSA responsiveness with overexpression of these deletion mutants after treatment with TSA (Fig. 7E). Consistent with the results obtained from the mutation of the repressive element (Fig. 2B and 4B), overexpression of the mutants inhibited the induction of the hTERT promoter activity in the presence of TSA (Fig. 7E). These results are consistent with the idea that Sp1 and Sp3 play a role in transcriptional repression of the hTERT promoter through recruitment of HDAC in normal human somatic cells.

**DISCUSSION**

Previous studies have implicated histone deacetylation in the transcriptional repression of the hTERT gene in normal human somatic cells (22–24). However, little is known about the mechanisms, including the identity of transcription factors that recruit HDAC to the hTERT promoter. Our present studies provided direct evidence that the hTERT promoter is tightly associated with Sp1 and Sp3 in normal human somatic cells, and these transcription factors in turn recruit HDAC, resulting in transcriptional silencing of the hTERT gene. In contrast to the demonstrated role of Sp3 as a transcriptional repressor (34–38), Sp1 has most often been described as a transcriptional activator. The ectopic overexpression of Sp1 was reported to induce transcription of the hTERT gene in tumor cells (21, 31). We also observed that transient transfection of Sp1 expression plasmids induced the hTERT promoter in normal human somatic cells. However, as demonstrated in many previous studies, these overexpression experiments could misidentify the normal function of the transcription factor in cells. Indeed, we have obtained a series of results indicating that Sp1, like Sp3, plays a role in the transcriptional repression of the hTERT promoter. Most importantly, our X-ChIP analysis demonstrates that the endogenous hTERT promoter was associated with Sp1 in the absence of ectopic Sp1 expression (Fig. 5). Furthermore, a dominant-negative deletion mutant of Sp1, which contains the N-terminal 621-amino acid residue encompassing the activation domains, was found to relieve the repression of the hTERT promoter (Fig. 7D). This dominant-negative experiment suggested that endogenous Sp1 may repress the hTERT promoter in normal human somatic cells. We would expect the down-regulation of hTERT promoter activity in this dominant-negative experiment, if endogenous Sp1 is an activator. In fact, in certain promoter contexts, Sp1 has been reported to be engaged in transcriptional repression (39–44). Moreover, several studies have indicated the potential interaction of Sp1 with HDAC (45–48). Given our consistent results, how can we explain the activation of the hTERT promoter over the repression of Sp1? Sp1 has the ability to interact with other Sp1 proteins to form multimeric complexes, which in turn activate transcription synergistically (49). Thus, excessive Sp1 protein could shift to form higher order Sp1 homomultimers, which might be nucleated by the Sp1 proteins already occupying the hTERT promoter. Related to this possibility, Sp3, which cannot form these multimeric complexes, failed to activate hTERT promoter upon its ectopic overexpression in normal human somatic cells. Taken together, our studies revealed the repressive function of Sp1 (and Sp3) in the hTERT promoter activity in normal human somatic cells.

TSA-mediated induction of the hTERT transcription was observed previously in various normal human somatic cells. Based on these results, an HDAC-mediated mechanism was proposed as the universal transcriptional repression mechanism of the hTERT gene (22–24). Our results demonstrated the involvement of Sp1 and Sp3 in the TSA-mediated induction of the hTERT promoter activity through recruitment of HDAC. Furthermore, we found that Sp1 and Sp3, the transcription factors binding to the identified TSA-responsive element, were not altered by TSA treatment; TSA did not alter their abundance in the nucleus, their DNA binding activities, or their occupation of the hTERT promoter (Figs. 2A and 3B). What then is responsible for the Sp1/Sp3-mediated activation/derepression of the hTERT promoter in response to TSA? We observed that Sp1 and Sp3 fused to the GAL4 DNA-binding domain were able to dramatically induce transcription after treatment with TSA. These results suggest that TSA may convert the Sp1 and Sp3 on the hTERT promoter from repressors into activators by the abrogation of the associated HDAC activity.

Activation of the hTERT gene is a crucial step during the immortalization and malignant transformation of human cells. However, it is poorly understood how the hTERT gene is activated. Multiple mechanisms are probably involved in transcriptional activation of the hTERT gene during tumorigenesis. For example, deregulated c-Myc overexpression might contribute to the up-regulation of the hTERT promoter in the course of tumorigenesis. The amplification of the hTERT locus during tumorigenesis probably contributes to the misregulation of hTERT transcription in some kinds of human tumors (14). In addition to these previously proposed mechanisms, our studies indicate that abrogation of the regulatory pathway(s) governing the functional interaction of Sp1 or Sp3 with HDAC could be an important mechanism for inducing hTERT gene expression during tumorigenesis. This newly proposed mechanism for the transcriptional derepression of the hTERT gene could provide the platform for the detailed analyses of the transcriptional regulation of the hTERT gene, and help to unveil possible therapeutic targets for the development of therapeutic drugs against cancer.

**REFERENCES**

1. Bryan, T. M., Engleman, A., Gupta, J., Bacchetti, S., and Reddel, R. R. (1995) *EMBO J.* **14**, 4240–4248.
2. Kim, N. W., Pietszynska, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Covello, G. M., Wright, E. W., Weinrich, S. L., and Shay, J. W. (1994) *Science* **266**, 2011–2015.
3. Shay, J. W. (1995) *Mol. Med. Today* **1**, 378–384.
4. Chiu, C. P., and Harley, C. B. (1997) *Proc. Soc. Exp. Biol. Med.* **214**, 99–106.
5. Klapper, W., Parwaresch, R., and Krupp, G. (2001) *Mech. Ageing Dev.* **122**, 765–772.
6. Levy, M. Z., Allopp, R. C., Futterer, A. B., Greider, C. W., and Harley, C. B. (1992) *J. Biol. Chem.* **267**, 9511–9515.
7. Zhang, X., Mar, V., Zhou, W., Harrington, L., and Robinson, M. O. (1999) *Genes Dev.* **13**, 2388–2399.
8. Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allopp, R. C., Yu, J. L., Le, S. W., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W., and Villeponteau, B. (1995) *Science* **269**, 1236–1241.
9. Harrington, L., McPhail, T., Mar, V., Zhou, W., Oulton, R., Bass, M. B., Arruda, L., and Robinson, M. O. (1997) *Science* **275**, 971–975.
10. Harrington, L., Zhou, W., McPhail, T., Oulton, R., Yeung, D. S., Mar, V., Bass, M. B., and Robinson, M. O. (1997) *Genes Dev.* **11**, 3109–3115.
11. Hirakawa, I., Cable, P. L., Ahsan, C., and Barrett, J. C. (1999) *Cancer Res.* **59**, 826–830.
12. Oh, S., Song, Y. H., Kim, U. J., Yim, J., and Kim, T. K. (1999) *Biochem. Biophys. Res. Commun.* **263**, 361–365.
13. Wu, K. J., Grandori, C., AmCKER, M., Simon-Vermot, N., Polack, A., Lingner, J., and Dalla-Favera, R. (1999) *Nat. Genet.* **21**, 220–224.
14. Zhang, A., Zheng, C., Lindvall, C., Hou, M., Ekedahl, J., Lewensohn, R., Yan, Z., Tung, X., Henriksson, M., Blennow, E., Nordenkajd, M., Zetterberg, A., Bjorklund, M., Gruber, A., and Xu, D. (2000) *Cancer Res.* **60**, 6230–6235.
15. Ishii, Y., Tsuayama, N., Maeda, S., Tahara, H., and Ide, T. (1999) *Mech. Ageing Dev.* **110**, 175–193.
16. Sedyj, J. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9078–9081.
17. Shay, J. W. (1999) *J. Natl. Cancer Inst.* **91**, 4–6.
18. Fujimoto, K., Kyo, S., Tskakura, M., Kanaya, T., Kitagawa, Y., Itoh, H., Takahashi, M., and Inoue, M. (2000) *Nucleic Acids Res.* **29**, 2557–2562.
19. Oh, S., Song, Y. H., Yim, J., and Kim, T. K. (2000) *Oncogene* **19**, 1485–1490.
20. Oh, S., Song, Y., Yim, J., and Kim, T. K. (1999) *J. Biol. Chem.* **274**, 37473–37478.
Sp1 and Sp3 Recruit HDAC to Repress hTERT Promoter

21. Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Peterson, C., Wiman, K. G., and Pisa, P. (2000) *Oncogene* **19**, 5123–5133
22. Cong, Y-S., and Bacchetti, S. (2000) *J. Biol. Chem.* **275**, 35665–35668
23. Takakura, M., Kyo, S., Sowa, Y., Wang, Z., Yatabe, N., Maida, Y., Tanaka, M., and Inoue, M. (2001) *Nucleic Acids Res.* **29**, 3006–3011
24. Xu, D., Popov, N., Hou, M., Wang, Q., Bjorkholm, M., Gruber, A., Menkel, A. R., and Henriksson, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3826–3831
25. Ulaner, G. A., Hu, J. F., Vu, T. H., Giudice, L. C., and Hoffman, A. R. (1998) *Cancer Res.* **58**, 4168–4172
26. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
27. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999) *Mol. Cell. Biol.* **19**, 5504–5511
28. Kwon, H. S., Kim, M. S., Edenberg, H. J., and Hur, M. W. (1999) *J. Biol. Chem.* **274**, 20–28
29. Sowa, Y., Orita, T., Minamikawa-Hiranabe, S., Mizuno, T., Nomura, H., and Sakai, T. (1999) *Cancer Res.* **59**, 4266–4270
30. Yoshida, M., Horinouchi, S., and Beppu, T. (1995) *Bioessays* **17**, 423–430
31. Kyo, S., Takakura, M., Takahiro, T., Kanaya, T., Itoh, H., Yutsudo, H., Ariga, H., and Inoue, M. (2000) *Nucleic Acids Res.* **28**, 669–677
32. Gunes, C., Lichtsteiner, S., Vasserot, A. P., and Engler, C. (2000) *Cancer Res.* **60**, 2116–2121
33. Dahiya, A., Gavin, M. R., Luo, R. X., and Dean, D. C. (2000) *Mol. Cell. Biol.* **20**, 6799–6805
34. Conn, K. J., Rich, C. B., Jensen, D. E., Fontanilla, M. R., Bashir, M. M., Rosenbloom, J., and Foster, J. A. (1996) *J. Biol. Chem.* **271**, 28853–28860
35. De Luca, P., Majello, B., and Lania, L. (1996) *J. Biol. Chem.* **271**, 8533–8536
36. Majello, B., De Luca, P., and Lania, L. (1997) *J. Biol. Chem.* **272**, 4021–4026
37. Sridhar, P., Liu, Y., Chin, L. D., Berja, C. E., Mann, M., Skopicki, H. A., and Freter, R. R. (1999) *Mol. Cell. Biol.* **19**, 4219–4230
38. Vines, C. R., and Weigent, D. A. (2000) *Endocrinology* **141**, 938–946
39. Dean, G., Young, D. A., Edwards, D. R., and Clark, I. M. (2000) *J. Biol. Chem.* **275**, 32664–32671
40. Li, J., and Ou, J. H. (2001) *J. Virol.* **75**, 8400–8406
41. Li, R., Hodny, Z., Luciakova, K., Barath, P., and Nelson, B. D. (1996) *J. Biol. Chem.* **271**, 18925–18930
42. Pagliuca, A., Cannada-Bartoli, P., and Lania, L. (1998) *J. Biol. Chem.* **273**, 7668–7674
43. Shou, Y., Barron, S., and Ponec, M. (1998) *J. Biol. Chem.* **273**, 5716–5726
44. Zaid, A., Li, R., Luciakova, K., Barath, P., Nery, S., and Nelson, B. D. (1999) *J. Bioenerg. Biomembr.* **31**, 129–135
45. Hodny, Z., Li, R., Barath, P., and Nelson, B. D. (2000) *Biochem. J.* **346**, 93–97
46. Huang, L., Sowa, Y., Sakai, T., and Pardee, A. B. (2000) *Oncogene* **19**, 5712–5719
47. Walker, G. E., Wilson, E. M., Powell, D., and Oh, Y. (2001) *Endocrinology* **142**, 3817–3827
48. Yang, J., Kawai, Y., Hanson, R. W., and Arinze, I. J. (2001) *J. Biol. Chem.* **276**, 25742–25752
49. Pascal, E., and Tjian, R. (1989) *Genes Dev.* **5**, 1646–1656
50. Courey, A. J., and Tjian, R. (1988) *Cell* **55**, 887–898
Sp1 and Sp3 Recruit Histone Deacetylase to Repress Transcription of Human Telomerase Reverse Transcriptase (hTERT) Promoter in Normal Human Somatic Cells
Jaejoon Won, Jeongbin Yim and Tae Kook Kim

J. Biol. Chem. 2002, 277:38230-38238.
doi: 10.1074/jbc.M206064200 originally published online July 31, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206064200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 50 references, 28 of which can be accessed free at
http://www.jbc.org/content/277/41/38230.full.html#ref-list-1