Regulation of the human telomerase reverse transcriptase (hTERT) gene is the primary determinant for telomerase enzyme activity, which is found in tumor cells but is largely absent from normal somatic cells. Recent studies have shown that Myc protein can transcriptionally activate the hTERT gene. However, little is known about the repression mechanism of the hTERT gene and telomerase enzyme. Here, we developed an expression cloning strategy to identify cDNAs whose products can repress hTERT promoter activity in telomerase-positive immortal cells. Using this screen, we isolated the Wilms’ tumor suppressor gene (WT1). WT1 can repress hTERT promoter activity in 293 kidney cells. The WT1 binding site on the hTERT promoter was identified by deletional analysis. Alteration of the WT1 binding site markedly derepresses transcription from an isolated hTERT promoter by inhibiting interaction of WT1 with DNA. These specific repression effects of WT1 were not observed in HeLa cells, which express no endogenous WT1. Furthermore, we show that WT1 can repress the endogenous hTERT promoter and telomerase enzyme activities. These results suggest that WT1 may be a transcriptional repressor of the hTERT gene, at least in some specific cells.

Telomerase is a ribonucleoprotein that synthesizes repeat sequences at chromosome ends (reviewed in Refs. 1–4). In the telomere hypothesis of replicative senescence, the lack of telomerase in normal somatic cells permits a shortening of telomeres with each cell division until the cells reach a point, termed mortality control point M1, at which they cease to divide. This limit can be overcome by inactivation of tumor suppressor genes (e.g., p53 and Rb), and the cells will again replicate and further shorten their telomeres until they reach a second barrier, the mortality control point M2. At this point, there is much genomic instability and most cells die, but in a rare cell the activation of telomerase can stabilize the chromosomes, and cell proliferation resumes. Thus, the maintenance of telomere length by the activation of telomerase is frequently associated with cellular immortality and may constitute a key step in the development of human cancer.

The cloning of the gene encoding the catalytic subunit of human telomerase reverse transcriptase (hTERT) has allowed a direct test of the telomere hypothesis. Expression of hTERT is sufficient to reconstitute telomerase activity in normal human cells and facilitate immortalization of some normal somatic cells (8, 9). Consistent with its role in immortalization, hTERT mRNA is detected in telomerase-positive tumor cells but not in normal somatic cells known to lack enzymatic activity (5–7). Furthermore, it is induced upon telomerase activation during cellular immortalization and is down-regulated in correlation with telomerase activity during induced differentiation (5). All of these data strongly suggest that hTERT mRNA expression is the rate-limiting determinant of telomerase activity (5–7). Thus, derepression of the hTERT gene may be directly linked to the activation of telomerase in tumor cells.

Recently, the Myc oncogene product was shown to be involved in hTERT gene activation (10–13). However, little is known about the mechanism that can repress the hTERT gene or reduce the activity of telomerase. The existence of repressors for telomerase has been implicated by several studies (reviewed in Ref. 14). To identify such repressors, we developed an expression cloning approach. This method identifies cDNAs whose expression can repress hTERT promoter activity in telomerase-positive immortal cells. We found that expression of the WT1 tumor suppressor gene represses transcription of the hTERT gene in 293 cells.

**MATERIALS AND METHODS**

**hTERT Promoter-Reporter Constructs**—The BAC library of human sperm genomic DNA was screened with a PCR-generated 0.2-kilobase pair DNA fragment corresponding to the 5′-end of the human hTERT gene (15). A series of deletions of an isolated 3396 bp of the hTERT promoter region was generated with polymerase chain reaction and restriction enzyme digestion and then subcloned into the firefly luciferase reporter plasmid, pGL3-Basic (Promega) for reporter gene assays as described below. The WT1 binding sequence in the isolated hTERT promoter region was changed from AGCGCCCGCG through site-directed mutagenesis (16). The base changes were verified by DNA sequencing.

**Expression cDNA Library**—Double-stranded cDNA was prepared by the SuperScript Plasmid System (Life Technologies, Inc.). Briefly, NotI oligo(dT)-primed cDNA that had been synthesized from poly(A)+ RNA (5 μg) using human normal kidney tissues was size-selected by gel filtration and ligated to BamHI adaptors. This cDNA was cloned into the pcDNA1.1/Amp plasmid (Invitrogen), which contains the SV40 origin of replication. A portion of the ligation mixture was transformed into competent Escherichia coli DH10B cells (Life Technologies), making a directional cDNA library with more than 106 independent recombinants. The library was divided into pools of about 100 cDNAs per pool. Pools of plasmids were grown overnight in LB medium, and plasmid DNA was purified (Qiagen).
hTERT Repression by WT1

**RESULTS**

**Differential hTERT Promoter Activities in Mortal and Immortal Cells**—To understand hTERT gene regulation, we isolated a 3396-bp promoter fragment of the hTERT gene from a human BAC library (15). To assay hTERT promoter activity, we fused the hTERT promoter to a firefly luciferase reporter gene and transfected it into 293 and WI38 cells. 293 cells are a telomerase-positive immortal cell line that has detectable hTERT gene expression, whereas WI38 is a telomerase-negative mortal cell line strain that has no detectable hTERT gene expression (5). The 3396-bp hTERT promoter fragment failed to induce the reporter gene in WI38 cells (Fig. 1, lanes 2–4). In contrast, it supported high levels of reporter gene expression in 293 cells (Fig. 1, lanes 6–8). These differential hTERT promoter activities were also observed in other telomerase-positive immortal (HeLa, BHK, and HepG2) and telomerase-negative mortal (IMR90 and foreskin) cells.²

**Identification of WT1 as a Repressor of the hTERT Promoter in Expression Cloning Assays**—To understand the repression mechanism of the hTERT gene, we developed an expression cloning approach using the 3396-bp functional hTERT promoter. We prepared a cDNA library from normal human kidney tissues in which hTERT gene expression is repressed (5). The library was divided into pools of about 100 cDNAs each and cotransfected into the 293T kidney cell line with a firefly luciferase reporter gene under the control of the 3396-bp hTERT promoter. To control for variations in transfection efficiency, we also cotransfected a plasmid encoding Renilla luciferase.

Among 1200 pools (approximately 120,000 cDNAs screened in total), three pools (numbers 376, 727, and 1012) significantly reduced the normalized ratio of firefly to Renilla luciferase activities. Fig. 2A shows one transfection experiment that included pool 376. Pool 376 was subfractionated into 30 subpools of approximately 10 colonies each, and two positive subpools (numbers 12 and 20) were identified (Fig. 2B). We chose one of them (pool 12), and further fractionation steps yielded three pure cDNA clones (pools 72, 78, and 87; Fig. 2C). Partial sequence analyses indicated that all of them encoded the WT1 (–KTS) protein without alternative splicing.

WT1 has been shown to control genes involved in growth and differentiation of specific cells (e.g. kidney cells), and its inactivation may contribute to the tumorigenesis as a tumor suppressor gene (reviewed in Refs. 19–23). WT1 mRNA is known to undergo alternative splicing at two sites. The WT1 (+KTS) isoform is associated with subnuclear clusters and splicing factors, and its function is uncertain, while the WT1 (–KTS) variant is present in the nucleus and probably plays the primary role in transcriptional regulation (reviewed in Ref. 19).

**Deletional Analysis of the hTERT Promoter**—To address the direct role of WT1 in hTERT gene repression, we attempted to identify its regulatory DNA sequences in the hTERT promoter. A series of deletions was made in the hTERT promoter fused to the firefly luciferase reporter. We transiently transfected these plasmids into 293 kidney cells, which express low levels of an endogenous WT1 protein. Hence, by analyzing the hTERT promoter in these cells, we eliminated the possible spurious effects of ectopic overexpression of WT1 (19). Deletion construct P-307, containing the 307 bp upstream of the hTERT ATG, produced a high level of promoter activity, comparable with that induced by P-3396 (Fig. 3). However, the level of promoter activity was reduced with a further 113-bp deletion (construct P-194), consistent with the requirement of the Myc site within that region for high levels of the hTERT promoter activation (10–13, 15). Interestingly, the addition of 116 bp of additional upstream sequences to P-307 (construct P-423) significantly and reproducibly decreased reporter gene expression from the hTERT promoter (see also Ref. 24), suggesting that this 116-bp region may be involved in the transcriptional repression of the hTERT gene in 293 cells.

**Importance of WT1 Binding Sequences for Transcriptional Repression**—DNA sequence analysis of the hTERT promoter revealed a potential binding site for the WT1 transcription factor between −307 and −423 (Fig. 3). This WT1 binding site (GGCGGGGCG) was also identified in the hTERT promoter sequences reported by other groups (Refs. 10–13; accession numbers AF097365, AF128893, and AB018788). To find whether the identified WT1 site is functional, we made a site-directed mutation of the putative WT1 binding sequence in the hTERT promoter. 293 cells were transfected with luciferase

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² S. Oh and T. K. Kim, unpublished data.
reporter constructs containing wild-type (wt) or mutated (mt) WT1 binding sites. Immortalized 293 cells supported high levels of hTERT promoter activity (Fig. 4, compare lane 1 with lanes 2 and 3). Under these conditions, mutation of the WT1 binding site, without any alteration in the length of the hTERT promoter, dramatically further induced reporter gene expression (Fig. 4, compare lanes 2 and 3 with lanes 4 and 5). Importantly, this stimulatory effect was observed under the physiologically relevant setting of 293 cells, in the absence of ectopic WT1. These results suggested that the putative WT1 binding site is required for transcriptional repression of the hTERT gene in 293 cells.

**Direct Interaction of WT1 with the hTERT Promoter**—Next, we directly tested whether WT1 can bind to the identified regulatory DNA sequence in the hTERT promoter. We expressed the DNA-binding domain of WT1 (KTS) in bacteria and purified the recombinant protein from the lysate. Purified WT1 protein efficiently interacted with the putative WT1 regulatory sequence from the hTERT promoter in an electrophoretic mobility shift assay (Fig. 5). This WT1-DNA interaction was specifically inhibited by competitor oligonucleotides containing the wild-type (wt), but not mutated (mt), WT1 binding sequence (Fig. 5, lanes 3 and 4). Consistently, this WT1-DNA complex formation was abrogated by the WT1 site mutation (Fig. 5, lane 7), which was shown to markedly potentiate hTERT promoter activity in 293 cells (Fig. 4). Thus, WT1 can specifically interact with the hTERT promoter, and the WT1 binding sequence is required for maximal transcriptional repression of the hTERT gene.

**WT1 Transcriptional Repression of the hTERT Gene in 293, but Not HeLa, Cells**—WT1 protein is expressed in the kidney, gonad, and spleen (19–23). WT1 protein can be de-
tested in the 293 kidney cell line, but it is undetectable in the HeLa cervical cell line. Thus, we expected that the mutant WT1 binding site would have no effect in HeLa cells. HeLa cells were transfected with the luciferase reporter constructs containing wild-type (wt; AGCGCCGGCGGCGGGGG) and mutant (mt; AGCGGAAGCCTGGCGGG) WT1 binding site.

To further address the specific effects of WT1, we analyzed the response of the hTERT promoter to the ectopic expression of the WT1 protein in 293 and HeLa cells. As expected, the hTERT gene was negatively regulated by ectopic expression of WT1 in HeLa cells (Figs. 2–4). In contrast, this mutant WT1 binding site had dramatic promoter effects from inhibition of the DNA binding of the endogenous WT1 protein in 293 cells (Fig. 4).

Repression of Endogenous hTERT Expression by WT1—We showed that the WT1 gene can be transcriptionally repressed by endogenous WT1 in 293 and HeLa cells. To assess this repression by WT1, the activity of the endogenous hTERT promoter was next examined in 293 cells (293-WT1), which stably overexpress the WT1 (−KTS) isoform (25). This established cell line was shown to overexpress WT1 at a moderate and physiological level, comparable with that of endogenous WT1 in HL60 cells (25). Total RNA was isolated from 293-WT1 and 293-Control cells, and RNase protection was used to compare endogenous hTERT mRNA levels. A larger amount of WT1 protein was associated with a markedly lower level of hTERT mRNA in 293-WT1 cells (Fig. 7A). We also assayed the telomerase enzyme activities in these cells. Concomitant with repression of the endogenous hTERT promoter, telomerase enzyme activity was sharply reduced in the 293-WT1 cells compared with that in 293-Control cells (Fig. 7B). Thus, WT1 can repress the activity of the endogenous hTERT promoter as well as telomerase enzyme in 293 cells.

![Image](http://www.jbc.org/)

**Fig. 5.** Direct interaction of WT1 with the hTERT promoter. The interaction of purified recombinant WT1 protein with its putative binding sequence was analyzed by an electrophoretic mobility shift assay in the absence or presence of a large molar excess of competitors. The competitors were double-stranded oligonucleotides of the wild-type (wt; AGCGCCGGCGGCGGGGG) and mutant (mt; AGCGGAAGCCTGGCGGG) WT1 binding site.

![Image](http://www.jbc.org/)

**Fig. 6.** Specific effect of WT1 on transcriptional repression of the hTERT gene in 293 but not HeLa cells. Lysates from transfected cells were prepared and assayed for firefly luciferase activities from pGL3-hTERT promoter plasmids. The transfection efficiencies were normalized by the Renilla luciferase activity from cotransfected pRL-CMV control plasmid. A, HeLa cells were transfected with increasing amounts (0.4 μg in lanes 1 and 3, 0.8 μg in lanes 2 and 4) of the 1003-bp pGL3-hTERT promoter plasmid containing wild-type (wt) or mutant (mt) WT1 sites, as described in the legend to Fig. 4. B, 293 and HeLa cells were cotransfected with increasing amounts (0.4 μg in lanes 2 and 5; 0.8 μg in lanes 3 and 6) of WT1 expression plasmid together with 0.8 μg of the 1003-bp pGL3-hTERT promoter plasmid.

**DISCUSSION**

In the present study, we developed an expression cloning approach to identify regulators that mediate repression of the hTERT gene. WT1 was isolated as a potential repressor of the hTERT gene from a cDNA library, which was prepared from normal human kidney tissues. When WT1 was stably overexpressed in the 293 immortal kidney cells, it dramatically repressed the activity of an endogenous hTERT promoter and reduced telomerase enzyme activity. The same results were obtained with several other stable cell lines overexpressing WT1, discarding the possible involvement of clonal variation in the reduced hTERT mRNA levels. Importantly, this repression effect was specifically detected, even in the absence of ectopic WT1 expression, in 293 cells containing some endogenous WT1 but not in HeLa cells expressing no WT1 protein. Mutation of the WT1 binding site showed almost no effect on the hTERT promoter activity in HeLa cells, in contrast to its marked stimulatory effect in 293 cells. This specific repression by endogenous WT1 in kidney cells (e.g., 293, COS, and BHK)

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3 T. K. Kim, unpublished data.
Heat treatment abolished telomerase activity in the TRAP assay. IC were assayed in lysates (20 ng) from 293-Control and 293-WT1 cells. RNA (10 μg) from these cell lines (center panel). Actin mRNA was used to normalize the amounts of RNA (lower panel). Telomerase activities were assayed in lysates (20 ng) from 293-Control and 293-WT1 cells. Heat treatment abolished telomerase activity in the TRAP assay. IC indicates an internal control.

allows us to eliminate the spurious effects from the overexpression of WT1 that could be observed in the transfection experiments. Taken together, these results suggest that WT1 may be a transcriptional repressor of the hTERT gene in a cell type-specific manner.

The possible involvement of WT1 in the repression of hTERT (and telomerase) is also consistent with previous results showing an inverse correlation between WT1 and hTERT/telomerase activity in differentiating cells. WT1 can function as a tumor suppressor gene that regulates transcription of genes involved in differentiation and growth of cells (reviewed in Refs. 19–23). Hence, WT1 plays an important role in the differentiation of kidney and gonad cells. On the other hand, telomerase, in cooperation with other oncogene products, facilitates the tumorigenic transformation of human cells (26). Furthermore, telomerase activity is down-regulated during differentiation, whereas it is induced in highly proliferating cells, including tumor cells (reviewed in Refs. 1 and 2). Thus, WT1 may repress the hTERT gene during the differentiation of certain cells, and the loss of functional WT1 might contribute to the deregulation of its target genes during tumor formation. Interestingly, WT1 mutants activated rather than repressed expression of its target genes including the early growth response-1 gene (27). Furthermore, WT1 mutations with high levels of induced telomerase activities have been detected in many tumors including Wilms’ tumor (28, 29). However, it remains to be determined whether inactivation of WT1 tumor suppressor gene directly facilitates telomerase activation by derepressing the hTERT gene during tumorigenesis.

It seems likely that WT1 is not the sole determinant of hTERT gene activity. Several pieces of evidence suggest that there are inducers and additional repressors. First, microcell fusion studies identified multiple chromosomal loci of potential regulators involved in the mortality control, including repressors for telomerase (30–35). Second, the WT1 gene is expressed in specific cell types (e.g. kidney, gonad, and spleen); thus, other tissues probably contain other regulators to repress the hTERT promoter. Third, 293 kidney cells still induced some hTERT mRNA despite the presence of endogenous WT1 protein, although its DNA binding site is required for maximal repression of the hTERT gene; thus, some potent inducers (e.g. Myc) may be responsible for increased overall levels of hTERT mRNA in these cells and/or some other repressors may also be required for the efficient silencing of hTERT gene activity. And fourth, we identified additional repressors during our cDNA expression screen.3

Thus, in future studies, we will analyze the mechanisms by which multiple regulatory factors, including WT1, functionally interact to tightly maintain the repression state of the hTERT promoter in mortal cells. Furthermore, it will be important to analyze the mechanisms of how inactivation of repressors and/or activation of inducers can lead to telomerase activation in tumor cells. It is quite possible that complex patterns of hTERT gene regulation may occur in a cell type-specific manner, as suggested by the present study.

Since telomerase activity is detected in most tumors, it may be possible to develop therapies that inhibit telomerase activity and thus inhibit the growth of many types of cancer. Combined with conventional treatments including surgery, radiotherapy, and chemotherapy, anti-telomerase agents would limit the proliferative capacity of the rare surviving tumor cells and potentially prevent cancer recurrence. In addition, telomerase inhibitors could also be used as chemopreventive agents in persons with high cancer susceptibility or in early stage cancer to prevent overgrowth of metastatic cells. Thus, it is critical to elucidate the multiple signaling pathways (36) by which telomerase enzyme activity can be repressed and derepressed in cells. The present study provides some initial sights into one likely mechanism of hTERT gene repression by identifying WT1 as a specific repressor. In addition, the developed expression cloning approach will provide relevant targets that can be used for the specific modulation of the hTERT gene and telomerase activity for therapeutic purposes.

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REFERENCES
The Wilms' Tumor 1 Tumor Suppressor Gene Represses Transcription of the Human Telomerase Reverse Transcriptase Gene
Sangtaek Oh, Younghwa Song, Jeongbin Yim and Tae Kook Kim

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