Demethylating Reagent 5-Azacytidine Inhibits Telomerase Activity in Human Prostate Cancer Cells through Transcriptional Repression of *hTERT*

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ABSTRACT

Telomerase activation is thought to be a critical step in cellular immortality and oncogenesis. Several reagents including differentiation-inducing and antineoplastic agents are known to inhibit telomerase activity, although the molecular mechanisms through which they inhibit telomerase activity remain unclear. Demethylating reagents have recently been used as potential antineoplastic drugs for some types of cancers including those of the prostate. In the present study, we examined the effect of the demethylating reagent 5-azacytidine (5-aza-CR) on telomerase activity using cells of two prostate cancer cell lines, DU-145 and TSU-PR1. 5-aza-CR treatment significantly reduced telomerase activity in TSU-PR1 cells, but not in DU-145 cells, although growth inhibition was observed to a similar extent in both cell lines. Reverse transcription-PCR analyses revealed that inhibition of telomerase activity was accompanied by down-regulation of telomerase catalytic subunit (hTERT) mRNA expression. Transient expression assays showed that 5-aza-CR repressed the transcriptional activity of the hTERT promoter and that the E-box within the core promoter was responsible for this down-regulation. Western blot analyses revealed that 5-aza-CR reactivated p16 expression and repressed c-Myc expression in TSU-PR1 cells but not in DU-145 cells. Overexpression of p16 in TSU-PR1 cells led to significant repression of c-*Myc* transcription. These findings suggest that 5-aza-CR inhibits telomerase activity via transcriptional repression of *hTERT*, in which p16 and c-Myc may play a key role.

INTRODUCTION

Telomeres are essential units that stabilize the ends of eukaryotic chromosomes and prevent the loss of genetic information. In humans, they exhibit specific structures composed of 500-3000 repeats of the sequence TTAGGG (1). Human telomeres in somatic cells undergo progressive shortening with cell division through replication-dependent sequence loss at DNA termini (2). The shortening of telomeres results in chromosomal instability, which leads to cellular senescence. Telomerase, a specialized ribonucleoprotein polymerase containing an integral RNA with a short template element, directs the *de novo* synthesis of telomeric repeats at chromosome ends (3). Recent studies have revealed that telomerase activity is expressed in most malignant tumors and tumor-derived cell lines (4–7) but is repressed in normal somatic cells, suggesting that telomerase reactivation is a critical step in cellular immortality and carcinogenesis (8, 9). Telomerase is a regulated enzyme, and studies have shown that telomerase activity is strongly associated with the growth properties of cells (10, 11). In particular, telomerase is strongly up-regulated in proliferating cells such as hematopoietic progenitors and endometrial cells, whereas cell differentiation is accompanied by inhibition of telomerase activity (12, 13). However, the molecular mechanisms by which telomerase activity is regulated in concordance with cell growth properties remain unclear.

Recently, the three major subunits comprising the hTR[2] complex have been identified. The RNA component of hTR provides the template for telomeric repeat synthesis (14). Telomerase-associated protein (TP1) has also been identified and found to be associated with telomerase activity (15, 16). The most important component responsible for the enzymatic activity of telomerase is hTERT (17, 18). Expression of hTERT is observed at high levels in malignant tumors and cancer cell lines but not in normal tissues or telomerase-negative cell lines, and a strong correlation has been found between hTERT expression and telomerase activity in a variety of tumors (19–21). Introduction of hTERT cDNA into normal cells confers telomerase activity on these cells (22, 23). These findings suggest that hTERT is a catalytic subunit protein of hTR and that expression of hTERT might be a critical event in carcinogenesis. Thus, analysis of the mechanisms by which hTERT is activated is essential for understanding the molecular basis of telomerase activation and carcinogenesis. Recently, we cloned the 5′-flanking sequence of *hTERT* and identified the core promoter region essential for transcriptional activation in immortalized and cancer cells (24). It has been shown that Myc proteins are critical

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[2] The abbreviations used are: hTR, human telomerase; 5-aza-CR, 5-azacytidine; RT-PCR, reverse transcription-PCR; hTERT, human telomerase reverse transcriptase; LUC, luciferase; cdk, cyclin-dependent kinase.

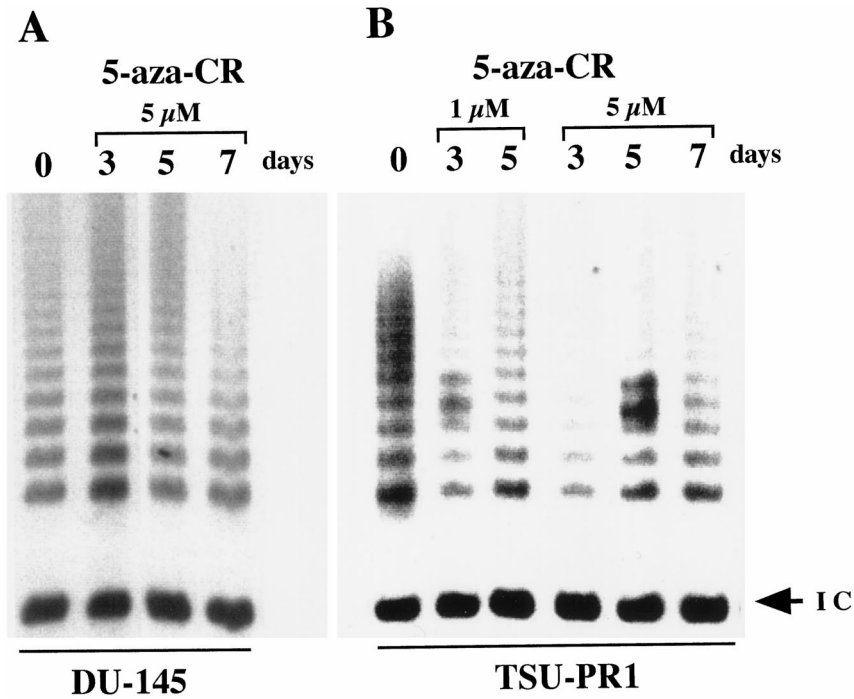

Fig. 1 Effect of 5-aza-CR on the level of telomerase activity in DU-145 (*A*) and TSU-PR1 (*B*) human prostate cancer cells. Cells were incubated in the absence or presence of 5-aza-CR (1 or 5 μM). On appropriate days of treatment, cell pellets were collected and subjected to stretch PCR assays. *IC*, internal control.

factors that directly activate *hTERT* transcription through the E-box located within the core promoter (25, 26).

5-aza-CR is a demethylating agent with which genes silenced by hypermethylation can be reactivated (27). The resulting DNA hypomethylation has been linked to the induction of cellular differentiation *in vitro* and altered expression of genes involved in tumor suppression (28–30). Recent studies have demonstrated that 5-aza-2′-deoxycytidine, a derivative of 5-aza-CR, inhibited the growth of human prostate cancer cells, and clinical trials using this reagent as an antineoplastic drug are in progress (31, 32). However, the mechanisms responsible for this reagent's inhibition of cell growth and its other biological effects remain unclear (32). In the present study, we examined the effect of 5-aza-CR on telomerase activity in prostate cancer cell lines and found that 5-aza-CR inhibits telomerase activity through transcriptional down-regulation of *hTERT* in one cell line but had no effect on the second cell line, although the growth of both cell lines was inhibited by the demethylating agent.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Human prostate carcinoma cell lines DU-145 and TSU-PR1 were derived from human metastatic prostatic cancer specimens and have been described previously (33, 34). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in the presence of 5% CO_2 at 37°C. After 24 h of seeding, the demethylating reagent 5-aza-CR (Sigma, St. Louis, MO) was added to the media at various concentrations. 5-aza-CR was subsequently added to the media every 48 h during observation.

Telomerase Activity. To quantify telomerase activity, stretch PCR was performed using the TeloChaser system according to the manufacturer's recommendations (Toyobo, Tokyo, Japan; Refs. 35 and 36). The PCR products were electrophoresed in 7% polyacrylamide gel and stained using SYBR Green I (FMC BioProducts, Rockland, ME). The staining intensities of the telomerase DNA ladders and internal controls were counted using picture analyzing software (NIH Image), and relative band intensities of telomere ladders were determined.

RT-PCR. Total RNA was isolated from cell lines using Isogen (Nippon Gene, Tokyo, Japan) and reverse transcribed by using random hexamers (TaKaRa, Tokyo, Japan). To amplify the cDNA, 1-μl aliquots of reverse-transcribed cDNA were subjected to PCR in 10 μl of reaction buffer containing 0.2 mM of each deoxynucleotide triphosphate and 0.6 unit of ExTaq DNA polymerase (TaKaRa). DMSO (Sigma) was used at a 7% final concentration for hTERT mRNA detection. The primer pairs used were 5′-CGGAAGAGTGTCTGGAGCAA-3′ and 5′-GGATGAAGCGGAGTCTGGA-3′ for hTERT mRNA (18), 5′-TGGAGCCTTCGGCTGAC-3′ and 5′-GGGACCTTCCGCGGCAT-3′ for p16 mRNA (37), and 5′-CTACTGATGGCAAGCGTGTC-3′ and 5′-CACTGTGGCTCAGAGCAGCTTG-3′ for cdc25A mRNA (38). The reaction conditions were as follows: (*a*) for hTERT, 20 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 30 s, and extension at 72°C for 90 s; and (*b*) for p16 and cdc25A, 20 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. PCR products were electrophoresed in 7% polyacrylamide gels and stained with SYBR Green I (FMC BioProducts) for hTERT and electrophoresed in 2% agarose gels and stained with ethidium bromide for p16. The efficiency of cDNA synthesis and amplification from each sample was estimated by PCR with β-actin-specific primers (39). For quantitative evaluation, band intensity was analyzed by NIH image software and normalized to that of β-actin products.

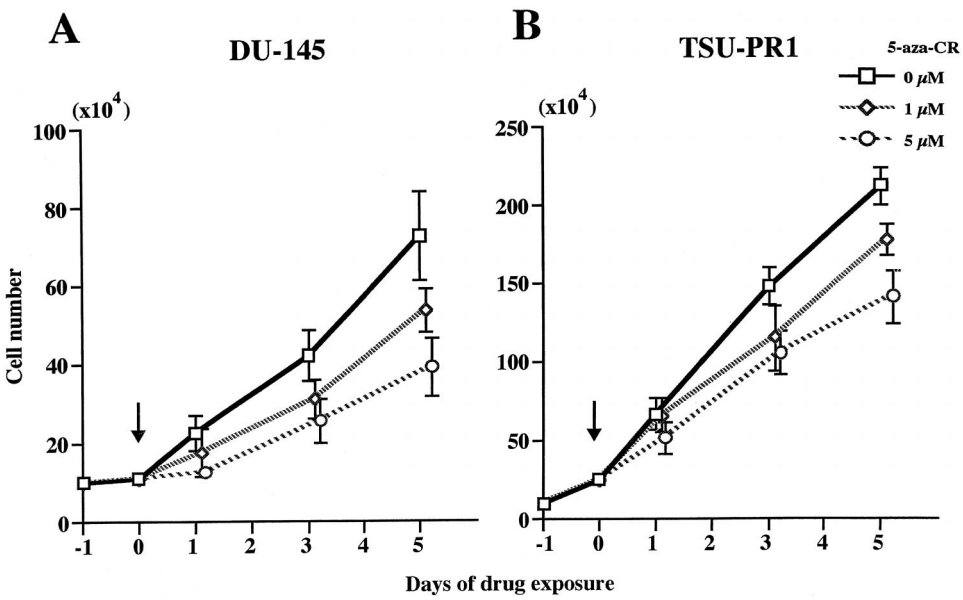

Fig. 2 Effect of 5-aza-CR on the growth of DU-145 (*A*) and TSU-PR1 (*B*) cells. Cells were incubated in the absence or presence of 5-aza-CR (1 or 5 μM) for 5 days. Cell numbers were counted on days 1, 3, and 5 of treatment. Media were replaced with fresh medium on days 2 and 4, and 5-aza-CR was added with the fresh medium. Values are expressed as means ± SE of quadruplicate well cultures. *Bars*, SD.

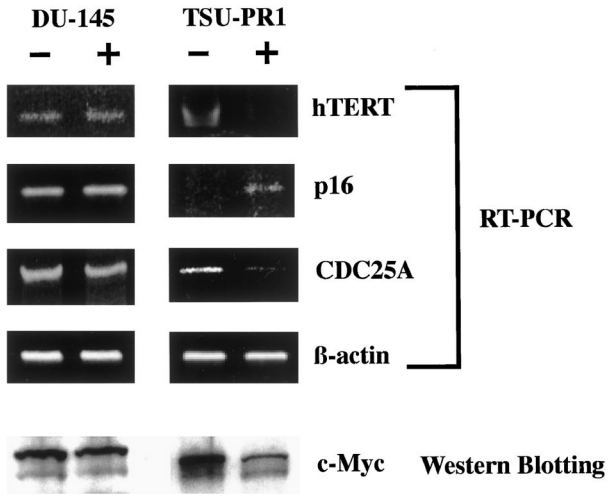

Fig. 3 Changes in expression of hTERT, p16, cdc25A mRNA, and c-Myc protein in DU-145 and TSU-PR1 cells during exposure to 5-aza-CR. Both types of cells were incubated in the absence (−) or presence (+) of 5-aza-CR (5 μM) for 5 days. Total RNA and nuclear proteins were extracted, and RT-PCR and Western blot analysis were performed (see "Materials and Methods").

Plasmid Construction. The structures of hTERT promoter-LUC plasmids are shown in Fig. 4. DNA fragments of various lengths upstream of the initiating ATG codon of the *hTERT* gene were PCR amplified and inserted into a LUC reporter plasmid (pGL3-Basic) lacking a promoter and enhancer (Promega, Madison, WI) in a sense orientation. pGL3-Control (Promega) driven by the SV40 enhancer/promoter was used as a positive control plasmid. The name of each reporter construct accorded with the 5′-end nucleotide numbers of inserted promoter sequences upstream (−) or downstream (+) of the transcriptional start site (24). For the construction of reporter plasmids containing substitution mutations in the E-boxes, site-specific mutagenesis was performed by a PCR-based protocol (40). A p16 expression vector, pSR-p16, was constructed by inserting the *Eco*RI-*Xho*I fragment of p16 cDNA (37) into pcDL-SRα296 (41) at the *Xho*I site. The c-Myc reporter plasmid, pmycplucAlu(+), was kindly provided by Drs T. Taira and H. Ariga (Hokkaido University, Sapporo, Japan; Ref. 42).

LUC Assay. Transient transfection of LUC reporter plasmids into DU-145 and TSU-PR1 cells was performed by the calcium phosphate precipitation method (43). LUC assays were performed using the Dual-LUC Reporter Assay System (Promega) according to the manufacturer's protcol, in which *Renilla* LUC plasmids are cotransfected as a control plasmid to standardize transcription efficiency. All experiments were performed at least three times for each plasmid, and mean LUC activities were determined.

Western Blot Analysis. For Western blot analysis for c-Myc expression, cell extracts from cell lines were prepared by the method of Schreiber *et al.* (44). Proteins (20 μg) were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride membranes. Filters were incubated with specific antibodies against c-Myc (N-262; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by reaction with horseradish peroxidase-linked antirabbit IgG. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL) as suggested by the manufacturer. For quantitative evaluation, band intensity was analyzed by NIH image software and normalized to that of glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

To examine the effects of 5-aza-CR on telomerase activity, two representative prostate cancer cell lines, DU-145 and TSU-PR1, were cultured in the absence or presence of 1–5 μM 5-aza-CR, and telomerase activity was measured by quantitative stretch PCR assay after 3, 5, and 7 days of exposure to 5-aza-CR

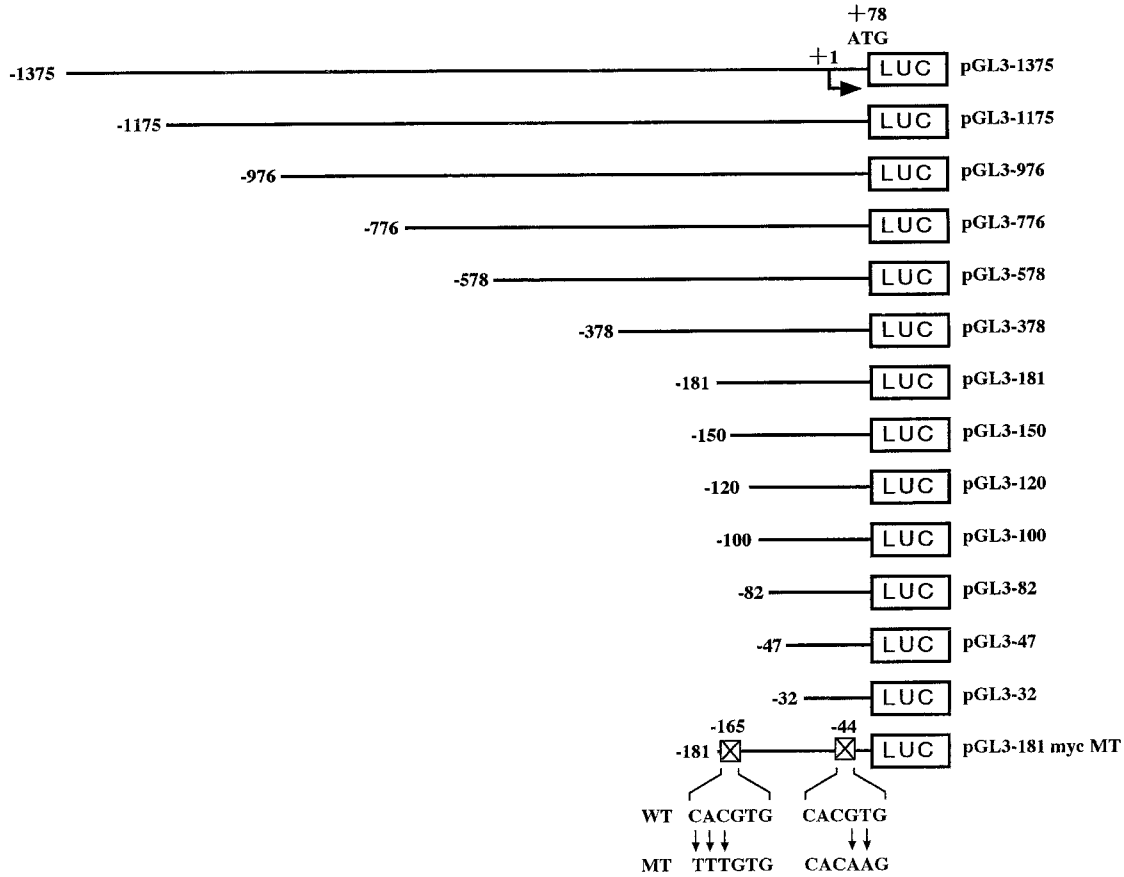

Fig. 4 LUC reporter plasmids used in the LUC assay. The 1.4-kb hTERT promoter sequences (*pGL3-1375*) upstream of the initiating ATG as well as various lengths of 5′-truncated fragments were cloned upstream of the LUC gene (*LUC*) in a sense orientation. *Arrow*, the transcription start site. ☒, E-boxes in which substitution mutations were introduced. *WT*, wild type; *MT*, mutation.

(Fig. 1). Significant reduction of telomerase activity in TSU-PR1 cells was observed with 5-aza-CR treatment, with maximal repression on day 3 at 5 μM 5-aza-CR. In contrast, telomerase activity was not altered in DU-145 cells during the time course observed. Thus, the effect of 5-aza-CR on telomerase activity differed by cell type. We next examined the effect of 5-aza-CR on cell growth. DU-145 and TSU-PR1 cells were grown in the absence or presence of 5-aza-CR, and the cell number was counted (Fig. 2). As shown in Fig. 2, 5-aza-CR inhibited the growth of both types of cells in a dose-dependent manner. Moreover, no significant cell death or apoptosis as detected by the terminal deoxynucleotidyl transferase-mediated nick end labeling method was observed with 5-aza-CR treatment (data not shown), suggesting that the telomerase repression observed in TSU-PR1 cells was not due to lethal cell damage. The findings observed for DU-145 cells indicate that cell growth properties are not always correlated with the status of telomerase activity in some types of cells.

Of the components comprising telomerase, hTERT is a critical determinant of the enzymatic activity of telomerase. We therefore examined changes in hTERT expression on treatment with 5-aza-CR using the RT-PCR assay (Fig. 3). In DU-145 cells, expression of hTERT mRNA was not affected by treatment with 5-aza-CR (Fig. 3). In contrast, hTERT mRNA expression was decreased in TSU-PR1 cells by treatment with 5-aza-CR, and only faint expression of hTERT mRNA (16% of control) was observed after 5 days of exposure to 5 μM 5-aza-CR (Fig. 3). Thus, telomerase repression by 5-aza-CR was associated with down-regulation of hTERT mRNA in TSU-PR1 cells. To examine whether this down-regulation was due to transcriptional repression, LUC assays were performed with reporter plasmids containing the 1.4-kb promoter sequences of *hTERT* gene that we had cloned previously (Ref. 24; Fig. 4). As shown in Fig. 5*A*, a 1.4-kb promoter (*pGL3-1375*) demonstrated significant transcriptional activity in both DU-145 and TSU-PR1 cells [50–70% of control reporter plasmids (*pGL3-Control*)]. Treatment with 5-aza-CR resulted in a more than 50% reduction of transcriptional activity in TSU-PR1, but did not reduce transcriptional activity in DU-145. These findings suggest that repression of telomerase activity by 5-aza-CR was due to transcriptional down-regulation of *hTERT* in TSU-PR1 cells.

To identify the promoter elements responsible for transcriptional down-regulation, mutant reporter plasmids with serial deletions of promoter sequences were prepared (Fig. 4), and LUC assays were performed. In the absence of 5-aza-CR, a series of mutant reporter plasmids from pGL3-1375 to pGL3-181 exhibited

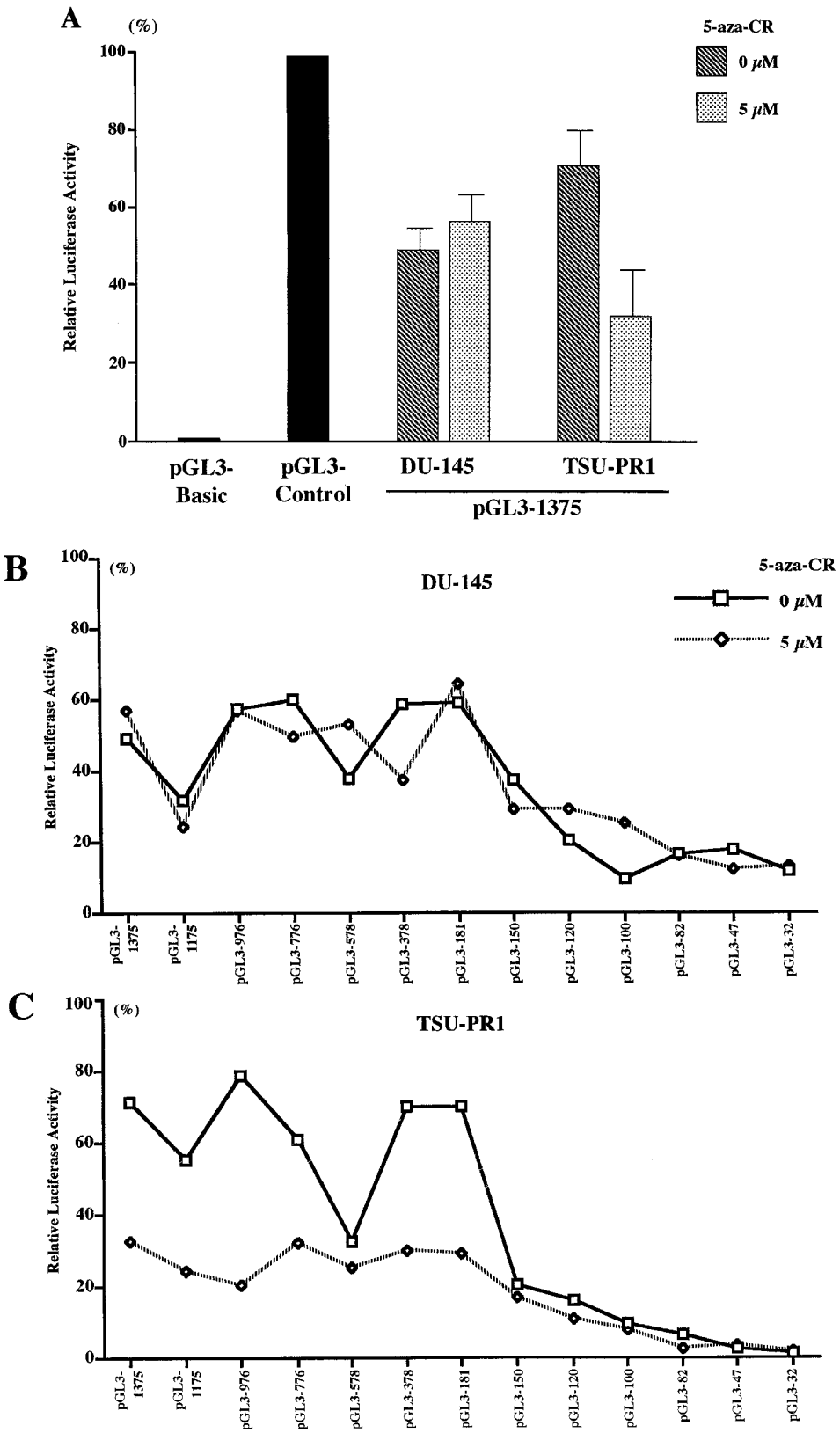

Fig. 5 Effect of 5-aza-CR on the transcriptional activity of *hTERT* in DU-145 and TSU-PR1 cells. Transcriptional activity of a 1.4-kb promoter (pGL3-1375; *A*) as well as 5′-deleted promoters (pGL3-1175 to pGL3-32) was examined in the absence or presence of 5-aza-CR (*B*, DU-145; *C*, TSU-PR1). Transcriptional activity is represented as the LUC activity relative to that of control samples transfected with pGL3-control. *Bars*, SD. *D*, involvement of the E-box in down-regulation of *hTERT* by 5-aza-CR. Wild-type pGL3-181 and mutant pGL3-181 in which E-boxes were mutated (pGL3-181 mycMT) were transfected in DU-145 and TSU-PR1 cells in the absence or presence of 5-aza-CR, and LUC assays were performed. *Bars*, SD.

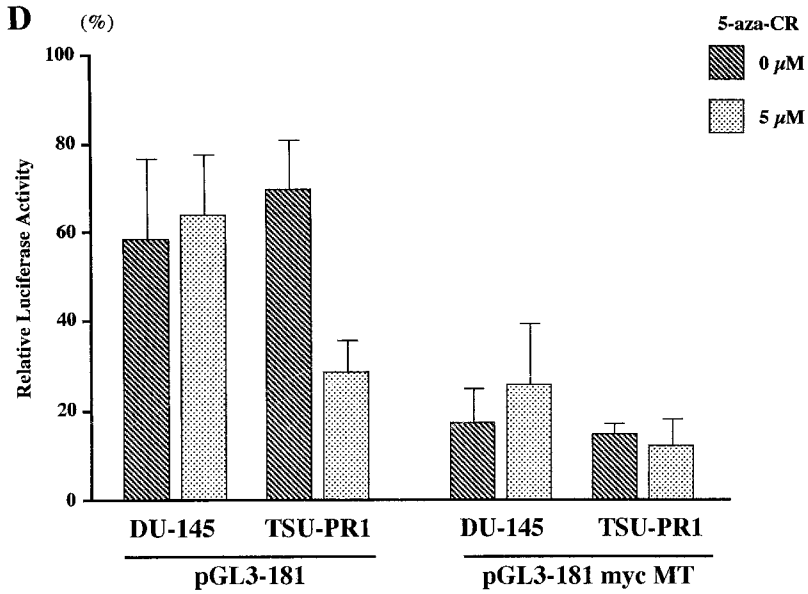

Fig. 5 Continued.

similar transcriptional activity, except for pGL3-578, which exhibited decreased activity (Fig. 5, *B* and *C*). The proximal 181-bp region (pGL3-181) retained strong transcriptional activity, whereas more extended deletions resulted in a stepwise reduction of transcriptional activity, suggesting that this region functions as the minimal core promoter essential for transcriptional activation (24). In the presence of 5-aza-CR, transcriptional activity was significantly inhibited with a series of reporter plasmids from pGL3-1375 to pGL3-181 in TSU-PR1 cells, but not in DU-145 cells (Fig. 5, *B* and *C*). However, the repression was abrogated in mutant reporter plasmid (pGL3-150) lacking the 5′, 31-bp region of the 181-bp core promoter and in mutants with more extended deletions. Interestingly, this 31-bp region contains an E-box (CACGTG) at −165 that is known to bind Myc and Max proteins. To examine the involvement of this E-box in down-regulation of transcriptional activity, a reporter plasmid with substitution mutations in both E-boxes at −165 and +44 (pGL3-181 mycMT) was prepared, and LUC assays were performed (Fig. 5*D*). In TSU-PR1 cells, 5-aza-CR significantly reduced the transcriptional activity of pGL3-181, but not that of pGL3-181 mycMT. These findings suggest that these E-boxes play essential roles in the down-regulation of *hTERT* by 5-aza-CR.

c-Myc is known to be a critical regulator of hTERT expression (24–26). We next examined the changes in c-Myc expression levels in DU-145 and TSU-PR1 cells during treatment with 5-aza-CR. Western blot analyses revealed that both types of cells constitutively expressed significant levels of c-Myc (Fig. 3). 5-aza-CR treatment decreased the levels of c-Myc expression in TSU-PR1 cells (74% reduction), but not in DU-145 cells. To confirm the biological significance of this repression, we examined whether a decreased level of c-Myc by 5-aza-CR leads to down-regulation of specific gene expression. We monitored the changes in expression of cdc25A mRNA, which is known to be a direct target of c-Myc (45), during exposure to 5-aza-CR. As expected, cdc 25A mRNA expression was decreased in TSU-PR1 cells 5 days after treatment with 5-aza-CR (61% reduction) but was not decreased in DU-145 cells (Fig. 3). Evidence revealed that c-Myc expression is affected by cell cycle regulator p16 (46–48), which is silenced by hypermethylation in a variety of cell types (29). Because 5-aza-CR is demethylating reagent, it is possible that treatment with 5-aza-CR alters the levels of p16 expression. We therefore examined the changes in p16 mRNA expression level during exposure to 5-aza-CR. RT-PCR assays revealed that expression of p16 was not detectable in TSU-PR1 cells (Fig. 3) but was reactivated by treatment with 5-aza-CR (Fig. 3). In contrast, p16 mRNA was constitutively expressed in DU-145 cells, and the levels of p16 mRNA expression were not altered by treatment with 5-aza-CR (Fig. 3). We next examined the effects of p16 on c-Myc transcription in TSU-PR1 cells. p16 expression vectors (pSR-p16) were cotransfected with c-Myc promoter reporter plasmids [*pmycplucAlu(+)*] in TSU-PR1 cells, and the LUC assays were performed. As expected, overexpression of p16 significantly inhibited the promoter activity of c-*Myc* (Fig. 6).

DISCUSSION

Several studies have found that telomerase activity in cancer cells was inhibited by differentiation-inducing and antineoplastic agents (12, 13, 49, 50) and that telomerase activity was useful as a novel marker of efficacy of chemotherapy (49, 50), although the molecular mechanisms by which these agents exert inhibitory effects on telomerase activity remain unclear. In the present study, we demonstrated that 5-aza-CR inhibits telomerase activity in human prostate cancer cells (TSU-PR1) through transcriptional down-regulation of *hTERT in vivo*.

Telomerase repression by 5-aza-CR accompanied down-regulation of hTERT mRNA expression. Transient expression assays using hTERT promoter reporter plasmids revealed that 5-aza-CR suppressed the transcriptional activity of *hTERT* genes. Down-regulation was observed in reporter plasmids containing an E-box at −165 in the core promoter but not in

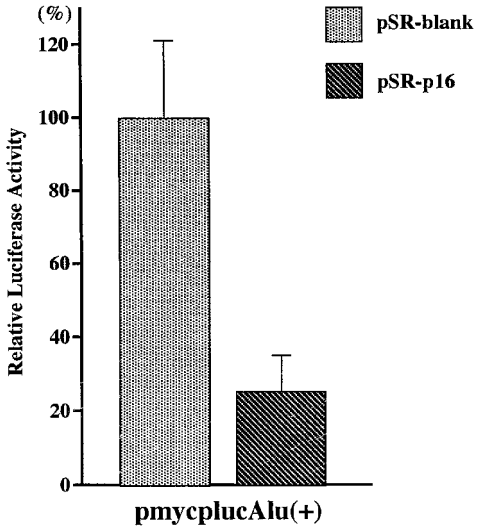

Fig. 6 Effect of p16 on the transcriptional activities of c-*Myc* promoters in TSU-PR1 cells. p16 expression vectors (*pSR-p16*) were cotransfected with c-Myc reporter plasmid [*pmycplucAlu(+)*] in TSU-PR1 cells, and LUC assays were performed. *Bars*, SD.

deletion mutants lacking this site, suggesting a role for the E-box in this regulation. c-Myc is the most representative E-box binding transcription factor. Wang *et al.* (51) demonstrated that expression of human papillomavirus E6 protein induces c-Myc expression in mammary epithelial cells, leading to enhanced expression of telomerase activity in these cells. Introduction of antisense oligonucleotides against c-Myc decreased telomerase activity in human leukemia cell lines (52). Most recently, studies have suggested that c-Myc binds directly to the E-box at −165 in the hTERT promoter and activates *hTERT* transcription (25, 26). These findings suggest that Myc proteins play critical roles in telomerase activation through up-regulation of *hTERT* transcription. In the present study, we showed that c-Myc expression was repressed by 5-aza-CR in TSU-PR1 cells. This may be one mechanism by which 5-aza-CR represses *hTERT* transcription.

An interesting question is why c-Myc expression was repressed by 5-aza-CR. p16 is a G_1-phase-specific cell cycle regulator and inhibits cdk4 and cdk6 (37). Inhibition of cdks blocks phosphorylation of the Rb protein, preventing the release of functionally active E2F transcription factor from the pRb-E2F complex (46). It has been suggested that the c-Myc promoter contains an E2F site and that E2F can activate c-*Myc* transcription (47). This evidence prompted us to examine the effect of p16 on c-Myc expression. p16 expression was reactivated by treatment with 5-aza-CR, and transient expression assays revealed that p16 represses the promoter activity of c-Myc. Previous studies have also demonstrated that overexpression of p16 and cyclin-cdk4 complexes posttranscriptionally block c-Myc function (48). These findings indicate that up-regulation of p16 and subsequent down-regulation of c-Myc are major pathways for hTERT repression by 5-aza-CR. However, because a variety of genes are reactivated by demethylating reagents, such as androgen receptor and prostate-specific antigen (53), other factors may regulate Myc expression in response to 5-aza-CR. In contrast to findings for TSU-PR1 cells, p16 and c-Myc were constitutively expressed in DU-145 cells, and no significant change in expression was observed on treatment with 5-aza-CR. One possible explanation for this is that the *p16* gene is not hypermethylated in this cell type and is therefore not affected by treatment with 5-aza-CR.

In the present study, we demonstrated that the demethylating reagent 5-aza-CR represses telomerase activity through down-regulation of *hTERT* transcription. c-Myc was found to play a key role in this regulation. Our findings may provide insights into one mechanism through which 5-aza-CR exerts growth-inhibitory effects on prostate cancers.

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