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Down-regulation of Cdc6, a Cell Cycle Regulatory Gene, in Prostate Cancer*

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CDC6 plays a critical role in regulation of the onset of DNA replication in eukaryotic cells. We have found that Cdc6 expression is down-regulated in prostate cancer as detected by semiquantitative reverse transcriptase-PCR of prostate cell lines and laser-captured microdissected prostate tissues. This result was substantiated by immunohistochemical analysis of paraffin-embedded tissue sections and immunoblot analysis of benign (BPH-1) and adenocarcinomatous prostatic cells. Furthermore, a 100-fold reduction in the transcription efficiency of the Cdc6 promoter-luciferase construct was noted in the metastatic PC3 cells compared with that in BPH-1 cells. Concentration of the E2F and Oct1 transcription factors that have putative binding sites in the Cdc6 promoter was substantially low in PC3 cells compared with BPH cells. Mutagenesis of the two E2F binding sites on the Cdc6 promoter resulted in increased promoter activity in PC3 cells owing to elimination of the negative regulation by pRb/E2F complex but not to the level of that obtained in BPH cells. We conclude that an altered interaction of transcription factors may be responsible for the down-regulation of Cdc6 transcription in PC3 cells. Our study suggests a potential use of the lack of Cdc6 expression as an index of prostate cancer development.

Prostate cancer is a complex and heterogeneous disease, and it is the most prevalent form of cancer and the second leading cause of cancer death in men (1). Along with the classic methods of diagnosing and prognosticating prostate cancer, utilizing stage, grade, and serum prostate-specific antigen level, molecular markers of aggressive disease are currently being evaluated as adjuncts to improve identification of tumors associated with a poor outcome. Because abnormal regulation of the cell cycle is one of the major causes of tumorigenesis in recent years, major advances have been made to understand the molecular mechanisms of aberrant cell cycle control leading to the development and progression of tumors. Studies on the aberrant expression of cell cycle regulatory genes in prostate carcinoma have identified a cdk1 inhibitor p27kip1 (2) and p53 (3) as biomarkers. These markers show a strong correlation with a high, moderate, or low risk of occurrence of prostate cancer. Immunohistochemical analysis of other G1/S check point controlling proteins, including cyclins (4), the cdk inhibitor p16 (5), and retinoblastoma (6), have also been found to be helpful in identifying patients with aggressive prostate cancer.

One of the major underlying mechanisms of aberrant cell cycle progression is uncontrolled DNA replication irrespective of cellular signals as a result of G1/S checkpoint failure. DNA replication is a highly ordered event controlled by sequential binding to and release of a number of replication proteins from the DNA sequence close to the origin of DNA replication. This also restricts firing of the replication origin to once per cell cycle. The regulation of initiation of DNA replication has been studied extensively in Saccharomyces cerevisiae, whereas understanding of this regulation in the mammalian system has just begun. However, identification of human homologs of yeast replication proteins, such as origin recognition complex (7–10), CDC6 (11), and minichromosome maintenance proteins (MCM) (12, 13), suggests evolutionarily conserved replication machinery. Studies on DNA replication in S. cerevisiae and Xenopus laevis have established a critical role of CDC6 in the initiation of replication (14–16). In late mitosis, when mitotic cyclin (cyclin B) and cdk are inactive, CDC6 helps to establish the prereplicative complex (Pre-RC) on the chromatin-bound origin recognition complex, a six-subunit initiator protein (17), by recruiting MCM proteins and other factors (18–20). In middle to late G1, and at the G1/S transition, S-phase cyclins (cyclin E and cyclin A) and cdk2 are expressed (21, 22) and initiate DNA replication by phosphorylating CDC6, which causes it to dissociate from Pre-RC (23). Release of CDC6 from chromatin and its translocation to the cytoplasm triggers initiation of DNA replication (24). Another role of CDC6 in S. cerevisiae is to function as an inhibitor of the G2/M phase until the S phase is complete (25). This defines CDC6 as a unique and critical protein with a dual role in regulating both G1/S and G2/S checkpoints.

The regulation of CDC6 expression throughout the cell cycle in mammalian cells, however, is quite different than that observed in proliferating yeast. In yeast, most of the phosphorylated CDC6 at the G1/S boundary is degraded by ubiquitination, whereas in mammalian cells, a fraction of phosphorylated CDC6 remains bound to chromatin throughout the cell cycle.

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2 The abbreviations used are: cdk, cyclin-dependent kinase; RLU, relative luminescence unit; MCM, minichromosome maintenance protein; Pre-RC, prereplicative complex; RT, reverse transcriptase; AUC, area under the receiver operating characteristic curve; PIN, prostatic intraepithelial neoplasia.
and prevents reloading of MCM protein until the end of mitosis when degradation of mitotic cyclin occurs (26). Maintenance of a cytoplasmic pool of hypophosphorylated CDC6 has been proposed in a model for CDC6 regulation in mammalian cells (26), although its role in the reassembly of Pre-RC in the next round of DNA replication or in delaying the M phase is elusive. Being a key regulator of DNA replication and the G1/M checkpoint, CDC6 expression needs to be tightly regulated for normal cell cycle progression. Several lines of evidence indicate that a gain in expression of CDC6 leads to increased DNA synthesis (27–29), leading to the designation of CDC6 as a proliferation marker. On the other hand, loss of expression of CDC6 has been shown to cause chromosomal loss and a lack of G2/M checkpoint control resulting in abnormal daughter cells (11, 31). Although an important component of the cell cycle regulatory mechanism, potential involvement of CDC6 in carcinogenesis has been investigated only recently. Recent reports on the overexpression of CDC6 and MCM proteins in low grade and high grade squamous intraepithelial neoplasia of cervix and brain tumors suggested their possible use as proliferation markers for detecting abnormal precursor malignant cells in cervical smears and tumors in brain tissues (32, 33). These reports suggest a possible role for CDC6 in carcinogenesis and cancer progression.

Using differential display of mRNA and cDNA microarrays, we have identified altered expression of CDC6 in prostate cancer cell lines. Here we report loss of CDC6 transcription as an event toward acquiring a cancerous phenotype of prostatic epithelium as observed by immunohistochemistry of tissue sections and semiquantitative RT-PCR of laser-captured microdissected prostate tissues. We have used a cell line developed from benign prostate hyperplasia and a prostate adenocarcinoma cell line to elucidate the mechanism of down-regulation of CDC6 expression in a prostate cancer cell line. We provide evidence that the loss of CDC6 transcription is due to suboptimal transcription not mutation at the promoter sequence.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Three commercially available cell lines, LNCaP, DU145, and PC3 (ATCC) were cultured in RPMI 1640 (LNCaP and DU145) and in Ham’s F-12 (PC3) with 10% fetal bovine serum and penicillin/streptomycin. A benign prostate hyperplasia cell line, BPH-1 (a gift from P. Narayan, University of Florida, Gainesville, FL) (34), was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin. A metastatic cell line, MDAPCa2a (M.D. Anderson Cancer Center, Houston, TX) (35) was maintained in BRFF HPC1 (Biological Research Faculty and Facility, Inc. Jamesville, MD) medium containing cholera toxin, insulin, epidermal growth factor, and hydrocortisone. Total RNA was extracted from cell lines using a RNA extraction kit (RNAgent, Stratagene) according to the manufacturer’s instructions. Genomic DNA from BPH-1 and PC3 cells was extracted using a genomic DNA isolation kit (Puregene DNA Isolation kit, Gentra Systems) according to the manufacturer’s instructions.

**Tissue Samples and Laser Capture Microdissection**—Approximately 400–5000 cells were captured from prostate cancer or histologically normal prostatic epithelium using a Pix cell II laser capture microdissection system (Arcturus Engineering, Mountainview, CA). Frozen sections of prostate tissue from radical prostatectomy were used for microdissection of normal and cancerous areas on the basis of histologic features of normal or carcinomatous epithelia following staining with hematoxylin and eosin stain. Cells were microdissected rapidly from each frozen section in an RNase-free environment to avoid RNA degradation.

**Northern Hybridization and Semiquantitative RT-PCR**—Twenty micrograms of total RNA from BPH-1, LNCaP, and PC3 cell lines was used for Northern blot using a standard procedure. A 1.6-kb clone of human CDC6 cDNA was used as the probe for hybridization.

For semiquantitative RT-PCR, BPH-1, DU145, and MDAPCa2a cells and laser capture microdissected tissue samples obtained from radical prostatectomies were used. Total RNA was extracted using Stat 60 (Tel-Test Inc.) and used for RT reaction using oligo(dT) primer and 18S RNA primer pairs and their competitors (Quantum RNA, Ambion) and Superscript II reverse transcriptase (Invitrogen). The 18 S primers were used to yield a 489-bp fragment of 18 S rRNA as an internal control, and total RNA from BPH cells was used as the positive control. A CDC6 gene-specific forward primer of 5′-TTCGACCTTGCG-3′ and reverse primer of 5′-GAGCACGAGAAAGTAAAGGGC-3′ primer pair was used to yield an 811-bp fragment. Amplified fragments were visualized by Southern blot using CDC6 and 18 S cDNAs as probes. Positive signals were quantified in a PhosphorImager (Amer sham Biosciences) and normalized with the relative values of 18 S RNA signal.

**Cloning of CDC6 Promoter and Expression of Luciferase Gene**—For construction of promoter-luciferase constructs, the CDC6 promoter region spanning positions −1436 to +218 was amplified by genomic PCR using genomic DNA from BPH-1 cells as the template. Taq-Pf polymerase (Stratagene) (19:1 mixture), and primers (5′-GAGCGGATCCCTTCTT- CACCTTTC-3′ and 5′-GCCTTCTCTCCATCTACCTACCT-3′). The amplified product was cloned into pGL3 basic firefly luciferase vector (Promega) between SalI and HindIII sites and designated as construct C. Two other deletion constructs, construct B and construct A, containing the promoter fragments ranging from −794 to +218 and −391 to +218, respectively, were made. Fidelity of the amplified Cdc6 promoter sequence was confirmed by dyeoxy chain termination sequencing using an ABI 310 sequence analyzer (PE Biosystems), and putative transcription factor binding sites were identified using an online program (www.biomics.com). Five transcription factor binding sites were designated as D (NF-κB), E (C/EBP), F (NF-1), and G (Oct1) were made by PCR amplification to map the region between −1436 and −794 bases according to the putative transcription factor binding sites. A set of forward primers containing SalI restriction site (NFκB, 5′-GAGGCTCT- AAAACACTCAGC-3′; C/EBP, 5′-GAGTGGCGCTAATTATTATTTTT-GG-3′; NF1, 5′-GAGGCTGCGTGGGATGCGTAAATACG-3′; and Oct1, 5′-GAGGCTCCATCTTTGTTAAAGTGAATAAACA-3′) and a common reverse primer containing a HindIII restriction site (5′-AACAAGTCAGCC- AAGTGCCAAG-3′) were used for directional cloning. The construct C was used as the template.

A PCR-based mutagenesis method described in the QuikChange kit (Stratagene) was used to substitute nucleotides in two E2F binding sites at positions −43 to −36 and −8 to −1 from the transcription start site. The E2F binding motif TTTGCGG in both locations was converted to TTTGAAGG in construct C and used for transfection. Both BPH and PC3 cells were grown to 80% confluence and used for transient transfection with the pGL3 constructs and pRL-TK (Promega) as an internal control vector using LipofectAMINE (Life Technologies) according to the manufacturer’s specification. Transfected cells were collected at 16 h in the culture media and harvested between 48 and 72 h in 10% trichloroacetic acid and luciferase expression was monitored using a Dual Luciferase assay kit (Promega) according to the supplier’s protocol. Relative luminescence from the expressed luciferase was normalized with the expression of Renilla luciferase. Transfection efficiencies were calculated as the relative luminescence unit (RLU) ratio of pGL3 constructs over that of pGL3 basic vector.

**Preparation of Crude Cell, Cytoplasmic, and Nuclear Extract**—BPH-1 and PC3 cells were grown to 80% confluence and harvested using freeze-thaw cycle in a lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 2.5 mM EDTA, pH 8.0, 1, 0.1% phenylmethylsulfonyl fluoride, 0.4% Nonidet P-40, and 2 ml/ml of the protease inhibitor mixture Set III (Calbiochem)). Crude cell lysates were centrifuged at 16,000 × g for 10 min at 4 °C to remove cellular debris. For cytoplasmic and nuclear extract, 100 μl of packed cell volume of each cell and nuclear and cytoplasmic extraction reagents (NE-FER, Pierce) were used according to the manufacturer’s protocol. Cytoplasmic fractions in the supernatant were collected and stored at −80 °C until use. Nuclear pellets were collected and lysed by vortexing in a lysis buffer NER (Pierce), and soluble proteins were separated in the supernatant by centrifugation at 14,000 × g for 10 min at 4 °C. Supernatants were stored at −80 °C until use for Western blot analysis.

**Immunoblot and Immunochemistry**—Total cell extracts from asynchronous cultures of BPH-1 and PC3 were used for immunoblot analysis using a standard procedure. 50 μg of total protein and antibodies against CDC6 (a gift from Dr. Anindya Dutta, Harvard Medical School and Oncogene Research Projects, La Jolla, CA) and HER-2 (Santa Cruz Biotechnology Inc. Santa Cruz, CA) were used for Western blot detection (kite) was used to detect positive signals recognized by specific antibodies using either a goat anti-mouse or a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. For immunochemistry, paraffin-embedded tissue sections of normal and ma-
Cdc6 Expression in Prostate Cancer

Cdc6 Expression Is Down-regulated in Prostate Cancer Cells—In our effort to identify differentially expressed genes in prostate cancer using differential display RT-PCR and cDNA microarray technology, we identified Cdc6 as one of the candidate genes that are down-regulated in prostate cancer. A gradual loss of the steady-state Cdc6 mRNA concentration was detected by differential display RT-PCR in LNCaP and PC3 cells when compared with that in BPH-1 cells. This result was further confirmed by RNA blot analysis (Fig. 1A). In addition, a reduced concentration of Cdc6 transcript (ratio of expression: 31.2) in a metastatic prostate cancer cell line, MDAPCa2a, compared with a normal prostate cell line, PrEC, has been detected by hybridization to a cDNA microarray of 18,376 non-redundant clones (data not shown). Down-regulation of Cdc6 was also evident from semiquantitative RT-PCR followed by densitometric analysis of Cdc6 mRNA present in DU145 and MDAPCa2a cells (Fig. 1, B and C). The data showed a significant loss of the Cdc6 steady-state transcript level in MDAPCa2a cells but a moderate reduction of the same in DU145 cells. Western blot analysis of Cdc6 using an anti-Cdc6 antibody further confirmed the loss of Cdc6 expression in the metastatic PC3 cells compared with the benign BPH-1 (Fig. 1D). The upper band in the BPH-1 whole cell extract lane (Fig. 1D) is probably the phosphorylated form of Cdc6, because human Cdc6 does not become degraded upon inactivation by phosphorylation as observed in S. cerevisiae. Analysis of the distribution of Cdc6 in subcellular fractions showed an accumulation of Cdc6 in the nuclear fraction of BPH-1 cells as expected and a substantial reduction of CDC6 in both nuclear and cytoplasmic fractions of PC3 cells (Fig. 1E).

Reduced Concentration of Cdc6 Transcripts in Human Prostatic Adenocarcinoma Tissues—To validate the expression profile of Cdc6 in human prostatic adenocarcinoma, we have monitored Cdc6 mRNA concentration by semiquantitative RT-PCR in histologically normal prostate epithelium and prostatic adenocarcinoma. To ensure homogeneous cell populations, laser-captured microdissected areas of histologically normal and carcinomatous tissues were used. Amplification of Cdc6 cDNA was normalized by co-amplified 18 S ribosomal mRNA. 18 S competitor oligonucleotides were used along with the specific 18 S primers to maintain a comparable amplification intensity of the highly abundant 18 S rRNA compared with a very low copy number of the input RNA. Southern blot analysis followed by densitometric analysis of the amplified products showed a consistently reduced expression of Cdc6 mRNA in adenocarcinoma tissues compared with that in matched normal prostatic epithelium in
six out of eight sample pairs tested (Fig. 2, A and B). Similar final digits among the sample numbers designate matched samples. In samples N4 and T4 and in P10 (prostate intraepithelial neoplasia) and T10, Cdc6 mRNA concentrations were below the detection limit, whereas Cdc6 mRNA concentration was higher in adenocarcinoma T5 than in the matched normal epithelium N5. In adenocarcinoma samples without matching normal epithelium, T9, T11, and T12, Cdc6 mRNA concentrations were lower than that in four out of seven normal epithelial samples (N3, N6, N7, and N8).

**Loss of Cdc6 Transcripts Correlated Well with the Tumor Characteristics and Pathological Report**—Histologically normal prostate epithelium and adenocarcinomas were obtained from radical prostatectomy specimens from patients with prostate cancer. The mean patient age at the time of surgery was 63.3 ± 4.3 ranging between 55 and 71 years. The Gleason scores of the adenocarcinoma ranged from 6 to 9 at the following frequencies: A Gleason score of 6 equals four tumors, a Gleason score of 7 equals six tumors, and a Gleason score of 9 equals one tumor. The cases were divided into two groups: 1) cases with organ-confined disease and no invasion into seminal vesicle or bladder and 2) cases with extension of tumors to the seminal vesicle or bladder. Group 1 tumors all had a Gleason score of 6 (two) whereas group 2 tumors had a Gleason score of 7 or higher (six) and 6 (one). Analysis showed that group 2 tumors are associated with a significant loss of Cdc6 transcripts, ranging from 86 to 99% of that of the normal epithelium, whereas group 1 tumors exhibit a moderate loss of transcripts ranging from 28 to 50% of that measured in matched histologically normal tissues (Table I). Carcinomas without matching normal epithelium showed 79–99% reduction in the transcript concentration compared with the average concentration of all uninvolved tissues.

The univariate analysis of Cdc6 expression showed an odds ratio of 3.1 and an AUC value of 0.800 (Table I). The higher the AUC, the better the predictor; therefore, in the univariate case, Cdc6 is a better predictor because the AUC is closer to 1.0. The interpretation of the odds ratio is that for every 10-fold increase in expression, the odds of being an uninvolved case relative to not being uninvolved increases by the amount given above, e.g. for every 10-fold increase in Cdc6, the odds of being uninvolved as compared with uninvolved increased 3.1 to 1.

**Differential Expression of Cdc6 in Intact Histologic Sections of Prostate Tissue**—To correlate Cdc6 expression with the histologic features of normal prostatic epithelium and prostatic adenocarcinoma, Cdc6 proteins were detected in prostate tissue samples by immunostaining using an anti-CDC6 antibody (Fig. 3). Tissue sections containing histologically normal epithelium, adenocarcinoma, and prostatic intraepithelial neoplasia (PIN) were chosen for comparative analysis. Areas with positive signals were compared with the H&E-stained, parallel sections for accurate identification of normal and carcinomatous areas. As shown in Fig. 3, normal epithelium showed a positive homogeneous staining for Cdc6, with granular cytoplasmic and nuclear staining observed in the prostatic luminal cells (Fig. 3, A and B). PIN also showed distinct staining similar to that observed in the histologically normal glands (Fig. 3, C and D). In carcinoma, a significant reduction in overall staining for Cdc6, representing loss of expression can be seen (Fig. 3, E and F). A similar expression pattern of CDC6 was observed in histologic sections from eight different prostate tissues. This observation is also in agreement with our semiquantitative RT-PCR analysis showing the reduced Cdc6 transcripts in prostate adenocarcinoma.

**Sub-optimal Transcription May Lead to Low Cdc6 Expression in PC3 Cells**—To understand the regulation of CDC6 expression in PC3 cells, we conducted a luciferase gene assay using a 1.654-kb fragment of Cdc6 promoter. Sequence analysis of Cdc6 promoters amplified from PC3 and BPH-1 or from a normal prostate cancer cell line (PrEC, Clonetics) did not show any mutations (data not shown). Computer analysis of the promoter sequence revealed a variety of putative transcription factor binding sites, including E2F, Sp1, NF1, NFκB, Oct1, and C/EBP (Fig. 4A), which may or may not be utilized in a particular cell line. Interestingly, the pGL3-Cdc6 construct C containing the entire 1.654-kb promoter fragment showed a considerable difference in luciferase expression upon transfection of BPH-1 versus PC3 cells (Fig. 4B). Approximately 50- to 100-fold higher transcription efficiency (1040 ± 128 versus 10 ± 6) of construct C was noted in transfected BPH-1 cells compared with that in PC3 cells. The construct B, containing a truncated promoter of the 794-bp fragment, showed an overall decline in the transcription efficiency compared with that obtained by the entire promoter, construct C, as expected. Although construct B was less efficient because of the lack of three C/EBP, two NFκB, and one Oct1 binding sites, a 16-fold difference in relative luminescence units (RLU) (373 ± 93 versus 22 ± 16) was detected in BPH-1 cells compared with PC3 cells transfected with the same construct. Construct A, composed of a 391-bp promoter fragment containing three E2F, three Sp1, and one NF1 sites, however, was inefficient in driving the luciferase transcription in either cell line. The RLU for construct A measured in both cell lines was close to the basal RLU of the basic pGL3 without any promoter. Although construct B did not have any putative transcription factor binding sites, the additional 403-bp sequence in construct B was able to stimulate luciferase transcription, suggesting interaction/binding of unknown proteins with this region. The control vector containing an SV40 promoter was equally effective in luciferase transcription in both cell lines. Further mapping of the upstream sequence showed a significant increase in the luciferase expression by the transfected BPH-1 cells following inclusion of the NFκB binding site in construct B (construct D) (Fig. 4B). Inclusion of the region containing a C/EBP (construct E) binding site suppressed luciferase transcription substantially. Addition of the NF1 (construct F) and Oct1 (construct G) binding sites increased transcription back to the level obtained with construct C. Addition of two other C/EBP sites (construct C) did not make any difference in the level of luciferase transcription. None of the constructs were able to improve transcription efficiency of the Cdc6 promoter when transfected into PC3 cells, implying that the reduced transcription of Cdc6 was not due to interaction of any negative regulators with the Cdc6 promoter in this cell line.

**Differential Expression of Transcription Factors May Be Responsible for the Altered Transcription of Cdc6**—It is likely that the sub-optimal interaction of the putative transcription factors with the Cdc6 promoter resulted in the reduced Cdc6 transcription in PC3 cells. Promoter mapping showed a dramatic reduction in the transcription efficiency not only between BPH-1 and PC3 cells but also between constructs C and B. The difference between these two constructs was that, in construct B three C/EBP sites (two in tandem), two NF1 sites in tandem, one NFκB, and one Oct1 transcription factor binding sites were
deleted. To rule out the possibility of reduced transcription of Cdc6 in PC3 cells linked to reduced expression of specific transcription factors, the concentration of Oct1, NF1, NFxB, and C/EBP transcription factors in BPH-1 and PC3 cells was determined next by Western blot using specific antibodies and nuclear extracts from both cell lines (Fig. 5). Our result showed a substantially reduced expression of Oct1 (Fig. 5 A) in the nuclear extracts from PC3 cells compared with BPH-1 cells, whereas NFxB was up-regulated in PC3 cells (Fig. 5B). The expression of NF1 did not differ between these two cell lines (Fig. 5C). C/EBP expression was undetectable in both cell lines. We have also monitored the concentration of the E2F transcription factor in BPH-1 and PC3 cells by Western blot, because E2F plays an important role in Cdc6 transcription. Interestingly, a significant reduction in the expression of E2F in PC3 cells compared with BPH-1 cells was noted (Fig. 5D).

**FIG. 3.** Immunohistochemical analysis of CDC6 in prostate tissues. A and B, normal; C and D, PIN; E and F, tumor. A2, A3, B2, and B3, immunohistochemical staining for CDC6 showing strong reactivity in non-neoplastic glands in comparison with weak staining in an adenocarcinoma (E2, E3, F2, and F3). C2, C3, D1, and D2, immunohistochemical staining for CDC6 in areas of PIN showing distinct reactivity. (Magnification: A2, A3, C2, C3, E2, and E3, ×100; A1, B1, B2, B3, C1, D1, D2, E1, F1, F2, and F3, ×400).

**FIG. 4.** Luciferase expression in transiently transfected BPH and PC3 cells. A, Cdc6 promoter-luciferase constructs and putative transcription factor binding sites. B, relative levels of luciferase transcription. Data represent mean ± S.D. of five different experiments.

**FIG. 5.** Western blot analysis of the transcription factors in nuclear extracts (50 µg) of BPH and PC3 cells.
elimination of repression by a possible Rb-E2F complex as proposed by other investigators (Fig. 6) (37, 38). The activity of the mutant Cdc6 promoter construct transfected into PC3 cells was also increased significantly (86%, p < 0.003) but not to the same level as that obtained by the wild type or mutated construct C following transfection into BPH-1 cells. These results suggest that other transcription regulatory proteins may be involved in regulating Cdc6 expression in PC3 cells.

**DISCUSSION**

In this study, the expression of CDC6, a key molecular component that regulates the onset of S phase, in prostate cancer has been assessed. Using a variety of methods a 25–70% loss of Cdc6 transcripts in prostate adenocarcinoma cells was detected. It was unlikely to detect a reduced transcription of Cdc6 in prostate cancer, because it is one of the evolutionarily conserved key proteins that maintain DNA replication in tight control. Our study showed that the down-regulation of CDC6 in prostate cancer was associated with phenotypic characteristics of aggressive prostate cancer (Figs. 2 and 3).

Although prostate cell lines provide a homogeneous cell population that is convenient for data interpretation, especially for gene expression profiling, they do not represent the true phenotype of prostate cancer. Analysis of the transcript profile of Cdc6 in human prostate provided a similar pattern of Cdc6 expression in adenocarcinoma to the prostate cancer cell lines (Fig. 2). To avoid the heterogeneity inherent in prostate tissue samples of adenocarcinoma, which often exhibit normal uninvolved prostatic epithelium, PIN, and adenocarcinoma in close proximity to one another, laser-captured microdissected areas of histologically normal and carcinomatous tissues were used, which provided nearly homogeneous cell populations. A consistent loss of Cdc6 transcripts in adenocarcinoma compared with matched normal tissues authenticates its down-regulation. In addition, the extent of loss of expression correlates with the phenotypic characteristics of the carcinoma showing an inverse relationship with the Gleason score and invasiveness (Table I). Organ-confined disease with a Gleason score of 6 showed a relatively higher level of Cdc6 expression compared with the carcinoma with a Gleason score greater than or equal to 7 and with extension to the margin of seminal vesicle or the bladder. Univariate analysis of the Cdc6 expression profile in 24 human prostate samples containing eight matched pairs of adenocarcinoma and adjacent normal tissue indicates the strong potential of Cdc6 as a marker for advanced prostate cancer (Table II). Our RT-PCR result also correlates with the immunohistochemical detection of the protein product in histologic section of human prostate with a loss of staining in adenocarcinoma and uniform staining of both nucleus and cytoplasm of histologically normal prostate glands. Staining of CDC6 in some carcinoma cell nuclei may indicate partial expression and nuclear targeting of the expressed CDC6 or nonspecific staining by the antibody, although there is a substantial loss of overall staining (Fig. 3).

A number of studies (23, 39–41) on the precise role of CDC6 in the regulation of initiation of DNA replication have reported a periodic transcription, subcellular localization, and degradation of CDC6 in proliferating cells that restricts DNA synthesis to only once per cell cycle and that maintains proper timing of mitosis. The potential uses of CDC6 and MCM proteins as markers of proliferation of and for the detection of abnormal cells in cervical smears and tumors in brain tissues have been demonstrated also (32, 33). Contrary to these reports, our study showed that a decreased expression of CDC6 is associated with aggressive prostate cancer. Prostate cancer is a heterogeneous and complex disease that may shift from an androgen-dependent growth phase to androgen independence, and this may result in gene expression profiles that are dissimilar to other cancers.

The mechanism by which CDC6 expression is differentially regulated in BPH-1 and PC3 cells is unclear. Our Cdc6 reporter assay indicated a 50- to 100-fold reduced promoter activity in PC3 cells (Fig. 4B, construct C). Because no mutation could be detected in the promoter sequence obtained from PC3 cells and luciferase transcription driven by SV40 promoter (control plasmid) remained the same in both cell lines, it is possible that a variation in the pool of specific transcription factors between PC3 and BPH cell lines contributes to the reduced efficiency of Cdc6 transcription. Although a truncated Cdc6 promoter without putative C/EBP, NFkxB, Oct1, and NF1 binding sites (construct B) exhibited an overall decrease in the luciferase activity in both cell lines, it maintained a 16-fold higher promoter activity in BPH-1 cells compared with PC3 cells. Interestingly, deletion of 403 bases from the 5′-end of construct B (construct A) in which no consensus transcription factor binding site was detected completely abolished the promoter activity in both cell lines. Despite the fact that the truncated promoter in construct A contained three E2F, three Sp1, and one NF1 site it was unable to transcribe luciferase at a detectable level, indicating possible interaction of unknown transacting factors with the element upstream of this region.

Fine mapping of the upstream sequence of the Cdc6 promoter showed that the addition of the NFkxB binding site (construct D) restored the transcription level similar to that of construct C in both cell lines. However, inclusion of 11 addi-

![Fig. 6. Luciferase expression driven by the doubly mutated Cdc6 promoter. Data represent mean ± S.D. of four separate experiments (*, p < 0.02; **, p < 0.002).](image-url)
tional bases upstream of the NFκB binding site containing a putative C/EBP binding site (construct E) inhibited luciferase expression significantly in BPH-1 cells, suggesting a possible nearby negative regulatory region. Addition of the NF1 and Oct1 binding sites (constructs F and G) increased the transcription efficiency back to the same level as obtained with construct C. It is noteworthy that none of the constructs containing various lengths of the promoter were able to restore luciferase expression in PC3 cells. It is possible that either a lack of specific transcription factors or the inability of specific factors to assemble to trans-activate Cdc6 transcription in PC3 cells is responsible for the difference in transcriptional efficiency.

Our data also provide evidence that there is a disproportional expression of transcription factors, such as Oct1, NFκB, and E2F, between these two cell lines. Expression of NFκB was noticeably higher but Oct1 and E2F concentrations were significantly lower in PC3 cells compared with that in BPH-1 cells. It can be speculated that the differential expression of these transcription factors may influence Cdc6 expression in these prostate cell lines, although, other than E2F, it is not known whether these transcription factors are true regulators of Cdc6 transcription. E2F is one of the key regulators of Cdc6 transcription, which acts as a positive and negative regulator of Cdc6 depending on the cell cycle. In early phases of the cell cycle, E2F sites are occupied by pRB, p107, and/or p130 bound to E2F-DP complexes, but Cdc6 transcription does not occur until cells enter mid-to-late G1 phase (42). Transcription activation by E2F probably requires displacement of the inhibitory complex through interaction with other transcription factors. It is interesting that none of the constructs were able to improve luciferase expression in PC3 cells. It is possible that the interaction of E2F with the E2F binding sites is essential for orchestrating interaction of other transcription factors even though the promoter containing only the E2F sites was unable to drive luciferase transcription. Inefficient transcription by the Cdc6 promoter following point mutations in the two E2F binding sites in serum-stimulated cells suggests a pivotal role of E2F in Cdc6 transcription through positive regulation (37). At the same time, point mutation in two E2F binding sites also eliminates the negative regulation of Cdc6 transcription by E2F that is mediated through occupying the E2F binding sites as an inhibitory complex (37). In support of this finding, our experiments using the Cdc6 promoter containing two mutated E2F sites significantly improved Cdc6 promoter activity of both transfected cell lines (Fig. 5). Nevertheless, luciferase expression in PC3 cells was far from being close to that in BPH-1 cells. It is possible that the reduced expression of E2F in PC3 cells may be responsible for the lack of interaction with the E2F sites despite the intact binding sites. Inefficient interaction of the E2F may be the determining factor for the loss of Cdc6 transcription in PC3 cells. However, it is unclear whether this is the primary reason for the loss of Cdc6 transcription, and further research is needed to understand the roles of E2F and other interactors on altered transcription of Cdc6 in prostate cancer.

Studies are underway to understand the molecular events of Cdc6 regulation and whether these transcription factors are directly involved in Cdc6 transcription in prostate cells. However, this report demonstrates a significant difference in the efficiency of Cdc6 transcription and a corresponding alteration in the concentration of transcription factors that may be involved in regulating Cdc6 transcription in PC3 cells.

Analysis of expression of other negative regulators of the cell cycle, such as p16\(^{(INK4a)}\), p15\(^{(INK4b)}\), and p18\(^{(INK4c)}\) in prostate cancer, did not show any apparent correlation to tumor grade, stage, and Gleason score, with the exception of p16 (43). Despite the fact that p16 is a negative regulator of cell cycle, it is up-regulated in prostate cancer (43). p16 is inactivated in many primary tumors and encodes a protein that inhibits cyclin-dependent kinases (44). This counterintuitive expression profile also demonstrates the complexity of prostate carcinoma and may represent a feedback mechanism to counteract increased cell proliferation. Similarly, overexpression of mutant p53 is associated with advanced prostate carcinoma (45) and is responsible for the loss of function of the wild type p53 by forming heterodimers. In contrast, a consistent allelic loss at the Rb locus and mutations in the Rb gene, a negative regulator of cell cycle, have been identified in clinical samples of prostate cancer and prostate cancer cell lines. Loss of function of Rb is associated with increased availability of active E2F, which eventually stimulates transcription of S phase proteins.

It is unusual that the lack of expression of Cdc6 is associated with aggressive prostate cancer. A significant understanding of the role of Cdc6 in regulating DNA replication has been achieved in the past few years. Cdc6 is the key regulator of initiation of DNA synthesis, and a lack of functional Cdc6 can delay S phase entry. It is also proposed that Cdc6 may be responsible for the timing of mitosis, because human CDC6 is present during S, G2, and M phases unlike its ortholog in Schizosaccharomyces pombe (Cdc18), and degradation of Cdc6 is required for exiting mitosis (38). In support of this assumption, inactivation of Cdc18 and Cdc6 in S. cerevisiae and S. pombe resulted in mitosis in the absence of DNA replication (29, 30). It can be speculated that Cdc6 may play an equally important role in regulating the G2 and M phases, and this function could be linked to aberrant cell proliferation in cancer as a result of the loss of functional Cdc6. Further research in this area is needed to understand the role of Cdc6 in prostate cancer.

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