Par-4 Transcriptionally Regulates Bcl-2 through a WT1-binding Site on the bcl-2 Promoter*

Sangeeta K. Cheema‡, Sandip K. Mishra§, Vivek M. Rangnekar¶, Ana M. Tari‡, Rakesh Kumar$, and Gabriel Lopez-Berestein$‡

Elevated expression levels of the bcl-3 proto-oncogene have been extensively correlated with the appearance of androgen independence in prostate cancer. Although bcl-2 was first cloned as the t(14:18) translocation breakpoint from human follicular B cell lymphoma, the mechanism of overexpression of bcl-2 is largely undefined for advanced prostate cancer because there are no gross alterations in the gene structure. We investigated the role of the product of the prostate apoptosis response gene-4 (Par-4) and the product of the Wilms’ tumor 1 gene (WT1) in the regulation of Bcl-2 expression in prostate cancer cell lines. We observed growth arrest and apoptosis, upon decreasing Bcl-2 protein and transcript in the high Bcl-2-expressing, androgen-independent prostate cancer cell line, by all-trans-retinoic acid treatment (ATRA), but this did not occur in the androgen-dependent cell line expressing low levels of Bcl-2. The decrease in the Bcl-2 protein and transcript following all-trans-retinoic acid treatment was accompanied by changes in localization of Par-4 and an induction in the expression of WT1 protein. In stable clones expressing ectopic Par-4 and in ATRA-treated cells, we observed decreased Bcl-2 protein and transcript. This was accompanied by an induction in WT1 expression. The involvement of WT1 in the Par-4-mediated down-modulation of Bcl-2 was further defined by blocking endogenous WT1 expression, which resulted in an increase in Bcl-2 expression. Finally, we detected Par-4 and WT1 proteins binding to a previously identified WT1-binding site on the bcl-2 promoter both in vitro and in vivo leading to a decrease in transcription from the bcl-2 promoter. We conclude that Par-4 regulates Bcl-2 through a WT1-binding site on the bcl-2 promoter. These data also identify Par-4 nuclear localization as a novel mechanism for ATRA-mediated bcl-2 regulation.

Overexpression of Bcl-2 in prostate cancer is a hallmark of advanced hormone-refractory disease and may account for the resistance to apoptosis that is characteristic of the late stages (1, 2). This correlation is strengthened by in vitro studies that demonstrate an increased Bcl-2 expression upon androgen withdrawal in androgen-sensitive prostate cancer cells (3–5).

Previously, we have shown that high Bcl-2 expression in androgen-independent prostate cancer cells is associated with cytoplasmic sequestration of the p53 protein, thereby impairing the nuclear transport of p53 in response to an apoptotic signal (6). Overexpression of Bcl-2 also correlates with poor prognosis for patients undergoing radical prostatectomy or hormonal ablation. In addition, high levels of Bcl-2 confer resistance to chemotherapy in prostate cancer cell lines, and current clinical efforts are aimed at modulating the expression of Bcl-2 (3). Furthermore, in vivo studies have demonstrated that increased expression of Bcl-2 accelerates the appearance of the androgen-independent phenotype in prostate cancer and that decreasing Bcl-2 expression delays progression to androgen independence along with enhancing chemosensitivity of these tumors (7–9).

The bcl-2 gene is a key determinant of neoplastic cell expansion whose oncogenic activity has been ascribed primarily to its ability to promote cell survival (10). It was first cloned as the t(14:18) translocation breakpoint from follicular lymphoma (11, 12). This translocation results in the juxtaposition of the bcl-2 gene in front of the immunoglobulin heavy-chain enhancer that leads to aberrant expression. In addition to lymphomas with t(14:18) translocations, high levels of Bcl-2 protein, aberrant patterns of Bcl-2 protein production, or both have been observed in a variety of solid tumors (13, 14). However, in contrast to lymphomas, no evidence for gross alterations in the bcl-2 gene structure has been obtained for these other types of cancer, suggesting that expression is controlled through transcription initiation at the bcl-2 promoter (15). Two promoter regions have been identified in the 5′-regulatory region of the bcl-2 gene. P1, the predominant promoter in B cells, is a TATA-less, GC-rich region containing multiple initiation sites (16–18). The second promoter, P2, is located 1.3 kb downstream of P1, contains a CCAAT box, an octamer motif, and a TATA element. This promoter is active in neuronal cells and some pre-B cells (18, 19). bcl-2 promoter usage in prostate cancer cells and the identity of transcription factors that regulate its expression in these cells remain uncharacterized. Recently, nuclear factor-κB has been identified to bind to the bcl-2 P2 promoter via κB sites and activate its transcription in prostate cancer cells in response to tumor necrosis factor-α treatment (20). However, the mechanisms of Bcl-2 overexpression in androgen-independent prostate cancer are undefined.

The product of the prostate apoptosis response gene-4 (Par-4), although not a classical transcription factor, is co-expressed along with Bcl-2 in the normal prostate basal epithelia (23). Following differentiation of these cells to luminal/secretory epithelia, Par-4 expression is decreased and mainly restricted to the nucleus, and Bcl-2 expression is lost (23). Previously, ectopic expression of Par-4 led to the down-modulation of Bcl-2...
experimental implantation of LNCaP in nude mice (41). All cell lines were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. The following antibodies were used in the immunoblotting experiments: (a) anti-Bcl-2, a mouse monoclonal antibody; (b) anti-Par-4, a rabbit polyclonal antibody; and (c) anti-WT1, a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was either anti-rabbit or anti-rat horseradish peroxidase conjugated to horseradish peroxidase (Amersham Biosciences). Enhanced chemiluminescence reagents were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Cell Growth Assay—Cell survival was determined using the cell titer MTS/phenazine methosulfate cell proliferation assay (Promega, Madison, WI). Briefly, log phase growing prostate cancer cells were cultured in flat-bottomed 96-well plates in RPMI 1640, 10% fetal bovine serum, and 1% penicillin-streptomycin. Cells were lysed at density 1,000 cells per well in MTS/phenazine methosulfate buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) to which 2 mM phenylmethylsulfonyl fluoride, 33 μg/ml leupeptin, and 5 μg/ml pepstatin were added fresh before use. Lysis was carried out at 4 °C for 30 min. Cells were then centrifuged at 14,000 rpm for 10 min, and the supernatants were collected. Fifty micrograms of protein from each cell line was loaded on precast SDS/Tris/glycine gels (Bio-Rad), and after electrophoresis, proteins were transferred to nitrocellulose membranes, which were blocked in 5% nonfat dry milk and blotted with the appropriate primary antibody. Membranes were then incubated with the appropriate secondary antibody linked to horseradish peroxidase and developed using the ECL kit according to the manufacturers’ protocols.

Fluorescence-activated Cell Sorter Analysis—LNCaP and LNIin3 cells were cultured in T75 flasks and either treated with 10 μM ATRA or left untreated. Cells were collected by trypsinization 96 h after treatment and washed in PBS. Subsequently, cells were fixed in 70% ethanol overnight at −20 °C. After removal of the ethanol by centrifugation, cells were incubated with propidium iodide containing RNase A (1 mg/ml) at 37 °C for 15 min. DNA fluorescence of propidium iodide-stained cells was measured by flow cytometric analysis (Coulter Epics Profile, Coulter Corp., Miami, FL).

Immunofluorescence Microscopy—LNCaP and LNIin3 cells were cultured in 2-chambered Falcon culture slides in the presence or absence of 10 μM all-trans-retinoic acid for 96 h. The cells were fixed for 10 min in methanol/acetone (50:50) at −20 °C. Following permeabilization with 0.1% Triton X-100 in PBS, the cells were incubated with rabbit anti-Par-4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 2% fetal calf serum, overnight at 4 °C. Subsequently, the cells were incubated with anti-rabbit rhodamine antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mounted in anti-bleaching solution, and analyzed by laser confocal microscopy Olympus Fluoview FV500.

Real Time Quantitative PCR—Duplicate samples of 1 μl of each cDNA were used as a template. Real time quantitative PCR was performed using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Wellesley, MA). We used primers and a probe generated by the program Primer Express (PerkinElmer Life Sciences; www.perkinelmer.com) designed by Dr. Michael Andreeff (University of Texas M. D. Anderson Cancer Center). Bcl-2 primers were F (forward) 5′-GAG-GAGGACTCTTCCAGGAGCGG-3′ and Bcl-2 R (reverse) 5′-GGTGCCCGGT-GACAGTACTA-3′. TaqMan probe (FAM-labeled 5′-AGGTTGGGCGC-CCTTGAGTGTCGAGG-3′) was added to the PCR mixture to a final concentration of 200 nM. GAPDH primers were F (forward) 5′-GAAGGCAGTGGGTGCTCAGG-3′ and GAPDH R (reverse) 5′-AATGTCGT-GATGGGATTCC-3′ and GAPDH TaqMan probe fluorescence labeled 5′ F 5′-CAACGCTTCCGTCTTCAAGC3′- (PerkinElmer Life Sciences) were used as internal controls. Amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 60 s. GAPDH was co-amplified with Bcl-2.

**EXPERIMENTAL PROCEDURES**

All-trans-Retinoic Acid—All-trans-retinoic acid (ATRA) has been used previously (37, 38) to down-modulate the expression of Bcl-2. Because ATRA is a hydrophobic drug and to prevent its rapid clearance due to enhanced metabolism, we used liposomes to deliver the drug (39). 1,25-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
Northern Blot Analysis—Isolation of total RNA was accomplished using the TRizol® reagent (Invitrogen). The probe for Bcl-2 was generated with the 8.0-kb cDNA fragment from the Bcl-2 expression plasmid p442 (gift from Dr. T. J. McDonnell, University of Texas M. D. Anderson Cancer Center) by cutting it with EcoRI. The probe thus generated was random primer-labeled with [32P] using the Megaprime™ DNA-labeling system (Amersham Biosciences). The GAPDH probe has been described previously (11) and was used as a loading control. Prehybridization and hybridization were performed at 62 °C for 30 min overnight. The blot was subjected to low and high stringentity washes using the buffers provided in the NorthernMax™ kit for Northern blots (Ambion, Austin, TX).

Transfection and Generation of Stable Clones Expressing Par-4—LNln3 prostate cancer cell line was stably transfected with cytomegalovirus Par-4 expression construct expressing the full-length Par-4 using the calcium phosphate method (26). Stable clones expressing Par-4 were selected using the G418 antibiotic. The clones were analyzed for Bcl-2 and Par-4 expression by immunoblotting and were analyzed for the transcript of Bcl-2 by Northern blot and quantitative real time PCR. Bcl-2 and Par-4 expression construct expressing the full-length Par-4 using the calcium phosphate method (26). Stable clones expressing Par-4 were selected using the G418 antibiotic. The clones were analyzed for Bcl-2 and Par-4 expression by immunoblotting and were analyzed for the transcript of Bcl-2 by Northern blot and quantitative real time PCR.

WT1 Antisense—For inhibition of WT1 expression, we used antisense (AS) deoxynucleotides (5′-GTCGGAGCCATTTGCTG-3′) that were complementary to the region spanning the initiation codon of the human WT1 (42). The F-ethoxy-modified oligonucleotide was incorporated into a dioleoylphosphatidylcholine liposome formulation as described previously (43). Briefly, LNln3 cells were plated in 6-well plates (1 x 10^5 well) and 10 μM antisense was added. Cells treated were harvested after 3 days of WT1 antisense incubation. Expression of Bcl-2 and WT1 proteins was determined by Western blotting.

Isolation of Nuclear Proteins Binding to Biotinylated Probes—Oligonucleotides representing the WT1-binding site on the bcl-2 promoter sequence were synthesized, and 5 μg of the sense strand was 3′-labeled using the terminal deoxynucleotidyltransferase (Promega) and biotin-14-dATP (Invitrogen) (34, 44). The biotin-labeled sense strand was annealed to its complementary antisense strand and purified over a Sephadex column. The concentration of purified oligonucleotide was measured by absorbance at 260 nm, and equal amounts of annealed oligonucleotide were incubated with streptavidin-coated magnetic beads (Promega) for 30 min at room temperature. Coupling of the oligonucleotides to the beads was measured by absorbance. For the binding reaction, 50 μg of nuclear extract alone or preincubated with anti-Par-4 antibody (R334) was used. The beads were captured by a magnet, washed three times with a high salt buffer, and resuspended in Laemmlı buffer. The samples were heated at 95 °C for 5 min to elute all the proteins, loaded onto a 12% SDS-PAGE, electrophoresed, and transferred to a nitrocellulose membrane. Par-4 was detected using the Par-4 antibody (R334) by immunoblotting as described earlier.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSAs)—The double-stranded oligonucleotides used for EMSA of the WT1 region are shown with the WT1 site underlined and the mutated bases in boldface. The B1 WT1 site in the insulin-like growth factor II (IGF-II) P3 promoter was used as the positive control (45). Bcl-2 WT1 site, CCTTCCCTCCCCGCCCGGG; and mutated WT1 site, CCTTCCCTCCCCGCCCGGG. The oligonucleotides were 5′-end-labeled with [γ-32P]dATP and T4 polynucleotide kinase. EMSA reactions (20 μl) contained 20 μg HEPES, pH 7.9, 0.5 mM EDTA, pH 8.0, 5% glycerol, 5 μg of poly(dI-dC), 3 x 10^6 cpm of end-labeled oligonucleotide probe, and 10 μg of nuclear extracts. Leupeptin (0.3 mg/ml), phenylmethylsulfonyl fluoride (5 mM), and aprotinin (2 μg/ml) were included in all nuclear extract buffers. Samples were incubated at 37 °C for 1 h. Electrophoresis was performed at 30 mA in a 0.5% Tris borate, EDTA, 5% polyacrylamide gel. For the blocking reaction, 400 ng each of the anti-Par-4 antibody (R334) and the anti-WT1 antibody specific for a region near the COOH terminus of WT1 (C-19) were used.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was done as described earlier with little modification (46). LNln3 cells and LNln3 clones stably expressing Par-4 were plated in a 100-mm
Bcl-2 expression was normalized to Bcl-2 expression. Values from densitometric analysis of Bcl-2 expression in prostate cancer cell lines were used as a positive control for androgen-independent, high Bcl-2-expressing prostate cancer cell lines.

A bcl-2 promoter are as follows:
bcl-2

5'-AAAACTGTCTGCTTGCGCGGTA;
bcl-2

3'-GTAGCTTCTCATGGTTCGC;
GenBankMT accession number AC021803 (Homo sapiens).

Due to high GC content (36,182–36,560), PCR of the immunoprecipitated chromatin sample has been performed a little upstream (about 900 bases) adjacent to the predicted WT1-binding site (37,426–37,443).

For that matter the modified sonication was done to have the fragments in the range of 500–2000 bp.

Forward and reverse primers representing a site upstream of the WT1-binding site, used as negative controls, are as follows: bcl-2 5'-AGTTTGCGCATGTTTACT; bcl-2 3' CAGCTTGGTTGACAGACT- GATAC. The negative primer was designed targeting the site further upstream and is about 3664 bases away from the known WT1-binding site on the bcl-2 promoter. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

**Plasmid Constructs**—The bcl-2 promoter constructs are a generous gift from Linda Boxer, Division of Hematology, Stanford University School of Medicine, Stanford, CA. The bcl-2 5' promoter (P1) deletions were inserted into pBluescript II KS-plasmid from Stratagene. These deletions all end −1287 bp from the bcl-2 translation start site. The Socl site at −1287 has been changed to a PsiI site, which is followed by a sequence of multiple cloning site of pBluescript KS− and then the luciferase gene sequence. The LB124 is a P1 promoter deletion containing the previously identified WT1-binding sites. The LB360 is also a P1 promoter deletion lacking the WT1-binding sites. These constructs have been described previously (34, 47). Full-length Par-4 expression plasmid is a 1.4-kb EcoRI fragment of Par-4 cDNA subcloned in the EcoRI site of pCB6 driven by cytomegalovirus promoter. The Par-4 plasmids are a generous gift from Vivek Rangnekar (26).

**Transient Transfection and Luciferase Assays**—Transfections were performed in 6-well plates using FuGENE 6 (Roche Applied Science) according to the manufacturer’s direction. The transfected DNA mixture consisted of 4 μg of the full-length construct of human bcl-2 gene 5′-promoter region cloned upstream of the firefly reporter gene and 400 ng of pRL-Tk (Promega), which served as an internal control for transfection efficiency. pRL-Tk directs expression of the Renilla luciferase (RL) (HER) using the herpes simplex virus thymidine kinase promoter. A minimal bcl-2 P1 promoter lacking the WT1 sites described previously was also used. Where appropriate the transfection mixture also contained either the full-length Par-4 expression plasmid or an empty expression vector (2–4 μg). Cell extracts were prepared 24 h post-transfection and assayed for firefly and Renilla luciferase activity using the stop and glo kit (Promega) as directed by the manufacturer.

**RESULTS**

The Vitamin A Derivative, All-trans-retinoic Acid, Causes a Down-modulation of the Bcl-2 Protein and Transcription in the Androgen-independent Prostate Cancer Cell Lines—Our initial
Fig. 4. Par-4 undergoes a change in localization following L-ATRA treatment in the androgen-independent, high Bcl-2-expressing prostate cancer cell line. Immunofluorescence analysis for Par-4 in LNCaP and LNln3 prostate cancer cells followed 96 h of treatment with 10 μM L-ATRA. A, LNCaP cells with no treatment, stained for Par-4. B, nucleus of untreated LNCaP cells, stained with TO-PRO-3 iodide nuclear dye; C is a superimposed image of Par-4 and the nuclear stain. D, LNCaP treated with 10 μM L-ATRA stained for Par-4. E, nucleus of L-ATRA-treated LNCaP cells, stained with TO-PRO-3 iodide nuclear dye; F is a superimposed image of Par-4 and the nuclear stain. G represents LNln3 cells without any treatment, stained for Par-4. H, nucleus of untreated LNln3 cells, stained with TO-PRO-3 iodide nuclear dye; I is a superimposed image of Par-4 and the nuclear stain it represents. J, LNln3 cells treated with 10 μM L-ATRA stained for Par-4. K, nucleus of L-ATRA-treated cells stained with TO-PRO-3 iodide nuclear dye; L is a superimposed image of Par-4 and the nuclear stain. The R334 rabbit polyclonal antibody against Par-4 and the anti-rabbit antibody tagged with Rhodamine were used for detecting Par-4.

investigations of the regulation of Bcl-2 expression in prostate cancer have focused on signals that are known to down-modulate Bcl-2. One known biological agent that has been shown to down-modulate Bcl-2 in various systems is ATRA (37, 38). Retinoids are known to induce and repress numerous genes that modulate cell growth and differentiation and to exert their anti-cancer action by inducing cells to undergo apoptosis (48). The relatively slow progression of prostate cancer and its association with vitamin A deficiency set precedence for use of ATRA as a therapeutic agent (49).

It has been shown previously (37) that L-ATRA, but not free ATRA, induces apoptosis in follicular lymphoma cell lines. This is accompanied by a decrease in the expression of Bcl-2 and an increase in the expression of Bax, a pro-apoptotic molecule. In prostate cancer, ATRA in conjunction with lariuzole, a cytotoxic p53 inhibitor, induces apoptosis accompanied by down-modulation of Bcl-2 (38). Prostate cancer cells are known to display an increased rate of ATRA clearance and metabolism. In order to circumvent the problem of increased clearance and metabolism of ATRA, we used L-ATRA for our studies.

In an attempt to modulate Bcl-2 expression, we treated the prostate cancer cell lines with ATRA. We first examined the effects of L-ATRA on the proliferation of prostate cancer cell lines. L-ATRA induced a dose-dependent growth inhibition in the androgen-insensitive line, LNln3, but not in the androgen-sensitive line, LNCaP (Fig. 1A).

Microscopic examination of LNln3 cells treated with 10 μM L-ATRA for 96 h revealed many flattened cells with membrane blebbing and condensed nuclei, which are changes typical of cells undergoing apoptosis. No such changes were observed for the LNCaP cell line following treatment with L-ATRA (Fig. 1B).

L-ATRA induced a 50% decrease of the Bcl-2 protein in LNln3 cells, as determined by densitometric scanning. No changes in Bcl-2 expression were observed in the LNCaP cell line (Fig. 2A).

The Bcl-2 message was also down-modulated in LNln3 cells corresponding to the observed decrease of the Bcl-2 protein, following L-ATRA treatment. No decrease in the Bcl-2 transcript was observed in the LNCaP cell line (Fig. 2B and Table I).

Decrease in Bcl-2 Expression Correlates with G1 Arrest and Apoptosis in Androgen-independent Prostate Cancer Cell Lines—Previous studies (37) in follicular lymphoma have demonstrated that decreasing Bcl-2 leads to apoptosis in these cells due to alteration of the Bcl-2:Bax ratio. In solid tumors, including prostate cancer, decreased Bcl-2 is associated with enhanced sensitivity to chemotherapeutic drugs (7, 8). Hence, we next determined whether the decrease observed in Bcl-2, at the protein and transcript level, exerted an effect on the cell cycle profile of the prostate cancer cells. The cell cycle profile of the LNCaP cells remained unaltered following 96 h of treatment with L-ATRA (Fig. 3A). A significant G1 arrest (19%) was seen for the LNln3 cell line and was accompanied by a decrease in the S phase (14.3%) and by the appearance of a sub-G0/G1 population (15.5%), as compared with none seen for the untreated cells (Fig. 3B). These data demonstrate that down-modulating Bcl-2 in the androgen-independent prostate cancer cells produces an effect in terms of growth arrest and apoptosis in these cells. These data are in agreement with previous reports (8, 9) that demonstrated in vivo that decreasing Bcl-2 in
Par-4 Regulates Bcl-2 Expression

Altered Par-4 Localization and Induction of WT1 Expression Accompany Decreased Bcl-2 Expression—Because the down-modulation of Bcl-2 in response to L-ATRA treatment was a late event (occurring 96 h after treatment) and because the bcl-2 promoter does not have any retinoic acid response element, we next examined the potential involvement of Par-4. The rationale for investigating this is based on the strong correlation observed for the expression of Bcl-2 and Par-4 in normal prostate basal epithelia. In addition, a previous study documented that ectopic expression of Par-4 results in down-modulation of Bcl-2 in the mouse fibroblast cell line NIH3T3 and in the prostate cancer cell line PC3 (24). Kinetics done for Par-4 expression following treatment with 10 μM ATRA did not reveal any change in Par-4 expression at various time points tested in the two cell lines (data not shown).

However, Par-4 has a bipartite localization that may contribute to the different interactions and functionality of this protein (26, 27, 29, 30). The localization of Par-4 following L-ATRA treatment was analyzed in the LNCaP and LNln3 cells using immunofluorescence microscopy. In LNCaP cells Par-4 was found to be cytoplasmic and nuclear in its localization. Following treatment with L-ATRA, Par-4 remains in the cytoplasm (Fig. 4, A and D). Par-4 is observed to be mainly cytoplasmic in the LNln3 cells; however, following treatment with L-ATRA for 96 h, a rise in concentration of Par-4 in the nucleus is observed, which correlated with the decreased Bcl-2 expression and transcript in these cells (Fig. 4, E and H). The concentration of Par-4 in the nucleus accompanied by a decrease in Bcl-2 expression correlates well with the nuclear localization of Par-4 in the luminal/secretory epithelial cells of the normal prostate that lack Bcl-2 expression.

Par-4 via its leucine zipper is capable of interacting with two different domains of WT1. Upon binding to the exon 5 domain of WT1, Par-4 acts as a co-activator of WT1-mediated transcriptional activation, whereas upon binding to the zinc finger domain of WT1, Par-4 augments the transcriptional repression and inhibits the transcriptional activation being mediated by WT1 (27, 36). WT1 is known to bind to two different sites on the bcl-2 promoter to activate as well as repress transcription of this promoter (34, 35). Hence, we next examined the effects of L-ATRA treatment on WT1 expression.

WT1 expression was increased in LNCaP and LNln3 cell lines following L-ATRA treatment (Fig. 5). However, LNCaP cells did not show any down-modulation in the Bcl-2 expression, which may be attributed to the unchanged Par-4 localization in this cell line following L-ATRA treatment, as compared with LNln3 cells. Data from this set of experiments suggest that altered localization of Par-4 and induction of WT1 expression may be the two events required for successful down-modulation of Bcl-2 in the prostate cancer cells.

Ectopic Expression of Par-4 Down-modulates Bcl-2 Protein and Transcript—Based on the strong correlation observed between Par-4, WT1, and Bcl-2 from our L-ATRA study, we next examined the relationship between these molecules. Previous studies (24) have shown an inverse correlation between Par-4 and Bcl-2 expression. We generated stable clones expressing Par-4 in the LNln3 cell line, and we examined the effect of this on the Bcl-2 protein and message. Bcl-2 protein expression was found to be down-modulated in LNln3 clones stably overexpressing Par-4 (Fig. 6A). The Bcl-2 transcript was assessed by Northern blotting (Fig. 6B) and real time quantitative PCR (Fig. 6C) and was found to be decreased, suggesting a transcriptional mechanism for the Par-4-mediated down-modulation of the Bcl-2 protein that was previously not defined. By using Western blotting, we also assessed the expression of WT1 in the stable clones expressing Par-4. WT1 expression was increased in the clones stably overexpressing Par-4 (Fig. 6D). The induction in WT1 expression observed following ectopic expression of Par-4 corresponds to that accompanying L-ATRA-induced decrease in Bcl-2 in the same cell line. Taken together, these data suggest that WT1 expression needs to be induced above basal levels in the LNln3 cell line for Bcl-2 to be down-modulated.

Blocking Endogenous WT1 Results in an Increase in Bcl-2 Protein—To characterize the involvement of WT1 in the regulation of Bcl-2 in the LNln3 cell line, we blocked the expression of the endogenous WT1 protein using antisense (Fig. 7). We then determined the effect of WT1 inhibition on Bcl-2 expression. Bcl-2 expression was increased following inhibition of WT1 expression (Fig. 7). These data indicate that endogenous Par-4 requires high levels of WT1 to cause a decrease in Bcl-2 expression.

Par-4 Is Part of a Protein Complex Interacting with the WT1-Binding Site on the bcl-2 Promoter—To characterize further the relationship between Par-4, WT1, and Bcl-2, we used a previously identified WT1-binding site (−1460 relative to ATG) on the bcl-2 P1 promoter as bait to pull out potential interacting molecules (34). This bcl-2 promoter sequence was labeled with biotin using terminal deoxynucleotidyltransferase and incubated with nuclear extracts from the LNln3 cell line. Following this, the samples were subjected to high salt washes to remove any nonspecific binding. The washed complex was pulled out, using streptavidin conjugated to magnetic beads, with the help of a magnet. The proteins binding to the promoter were eluted by boiling in Laemmli buffer and subjected to SDS-PAGE, and Western blotting for Par-4 was performed using anti-Par-4 antibody. Indeed, we observed that Par-4 is present in a complex interacting with the WT1-binding site on the bcl-2 promoter (Fig. 8). The Par-4-specific antibody (R334) has been shown previously (36) to disrupt the interaction between Par-4 and WT1. We could not detect Par-4 in nuclear extracts preincubated with the R334 antibody and then incubated with the biotin-labeled WT1-binding site on the bcl-2 promoter. Hence, these data would suggest that Par-4 interacts with the previously identified WT1-binding site on the Bcl-2 promoter, possibly through WT1. These results also show the presence of Par-4 on the bcl-2 promoter, reinforcing transcriptional control of the bcl-2 gene by Par-4.

FIG. 5. Selective induction of WT1 following treatment with L-ATRA. Immunoblot analysis for WT1 expression in the prostate cancer cell lines. Fifty micrograms of whole-cell lysate from untreated (−) or following treatment with 10 μM L-ATRA for 96 h (+) was immunoblotted for WT1 using the monoclonal antibody for WT1. Whole-cell lysates from MCF-7, a breast cancer cell line, served as a positive control for the expression of WT1. For loading control, the membranes were also blotted with antibody against β-actin.
Par-4 and WT1 Proteins in LNln3 Cells Bind to the bcl-2 Site In Vitro—To ascertain whether both Par-4 and WT1 interact with the bcl-2 promoter, previously defined WT1-binding sites on the bcl-2 P1 promoter (−1460 and −1807) were used as probes in EMSA experiments (34, 35). The bcl-2 (−1807) oligonucleotide site did not show any complex formation upon incubation with nuclear extracts from LNln3 cells (Fig. 9A, lane 6). Two complexes were formed with the bcl-2 (−1460) oligonucleotide (Fig. 9A, lane 8). Competition with increasing amounts of cold competitor demonstrated that both complexes were specific (Fig. 9A, lanes 9 and 10). To determine the presence of Par-4 and WT1 in the complexes formed, nuclear extracts from LNln3 cells were incubated with the −1460 probe along with antibodies directed against Par-4 and WT1. The anti-Par-4 antibody is directed against the COOH terminus of Par-4 and has been demonstrated previously (36) as a blocking antibody. This antibody caused disappearance of the faster migrating complex and reduction in intensity of the slower migrating complex, thus indicating the presence of Par-4 in both the complexes (Fig. 9A, lane 11). The anti-WT1 antibody caused a reduction in intensity of the slower migrating complex indicating the presence of WT1 (integrated density value for this complex is 76,874 in comparison to the integrated density value for the slower migrating complex in lane 8, which is 100,096, as revealed by spot densitometric analysis). The anti-WT1 antibody is also directed against the COOH terminus of WT1 and has been shown previously (34) to cause reduction in intensity of the slower migrating complex indicating the presence of WT1 (integrated density value for this complex is 76,874 in comparison to the integrated density value for the slower migrating complex in lane 8, which is 100,096, as revealed by spot densitometric analysis). The anti-WT1 antibody is also directed against the COOH terminus of WT1 and has been shown previously (34) to cause reduction in intensity of specific complex containing the WT1 protein. The mutated bcl-2 (−1460) site showed very weak complex formation (Fig. 9A, lane 4). The IGF-II promoter, P3, containing the WT1-binding site was used as a positive control and demonstrated the formation of a single complex (Fig. 9A, lane 2) (34). These results support the participation of Par-4 and WT1 in the DNA-protein complex formed at the −1460 site on the bcl-2 promoter.

The −1460 WT1-binding site was also used as a probe to perform EMSA with nuclear extracts from LNCaP and LNPro5 cell lines (Fig. 9B). The −1460 site, on the bcl-2 P1 promoter, was not bound by any proteins when LNPro5 nuclear extracts were used (Fig. 9B, 13th lane). The lack of any retarded complex formation with the nuclear extracts of LNPro5 correlates very well with the barely detectable levels of Bcl-2 and Par-4 in this cell line. However, the same nuclear extracts do show formation of DNA-protein complex when the WT1-binding site on the IGF promoter was used as a probe (Fig. 9B, 9th lane). No complex formation was observed upon using a mutated WT1-
FIG. 8. Par-4 is present in the complex of proteins binding to the previously defined WT1-binding site on the bcl-2 promoter. Denaturing SDS-PAGE of protein complexes formed with the WT1-binding site on the bcl-2 promoter. Fifty micrograms of nuclear extracts from LNln3 was either electrophoresed alone (N.E.), incubated with the WT1-binding site on the bcl-2 promoter, WT1RE (−1460 relative to the transcription initiation codon) (N.E. + WT1RE), or preincubated with anti-Par-4 antibody (R334) (N.E. + R334 + WT1RE) before being incubated with WT1RE. Lanes 4 and 5 represent supernatants removed from N.E. + WT1RE and N.E. + R334 + WT1RE, respectively, following binding.

Par-4 and WT1 Proteins in LNln3 Cells Bind to the bcl-2 (−1460) WT1 Site in Vivo—To assess directly the potential significance of physical interaction between Par-4, WT1, and the bcl-2 promoter, we investigated whether WT1 and Par-4 associate on the chromatin of endogenous bcl-2 promoter using the chromatin immunoprecipitation assay. We immunoprecipitated chromatin from the LNCAp, LNln3 cells and the clones of LNln3 stably overexpressing Par-4, using specific antibodies against WT1 and Par-4 or no antibody at all as a control. Genomic DNA fragments bound to WT1 or Par-4 were analyzed by PCR using primers upstream of the −1460 WT1 site on the bcl-2 promoter. Analysis of genomic DNA immunoprecipitated with anti-WT1 revealed bcl-2 promoter in both the parental LNln3 cells and Par-4 clones (Fig. 10A, 2nd and 3rd lanes, middle panel). Immunoprecipitates from anti-Par-4 antibody revealed the presence of the bcl-2 promoter in both the parental LNln3 cells and the clone with greater amounts of bcl-2 promoter being associated with Par-4 in the clone as compared with the parental cell line (Fig. 10, 2nd and 3rd lanes, top panel). The samples subjected to the immunoprecipitation procedure minus the antibody as expected did not pull out any genomic DNA (Fig. 10A, bottom panel). Chromatin immunoprecipitated with anti-Par-4 antibody or anti-WT1 antibody, subjected to PCR using primers representing a non-WT1-binding site on the bcl-2 promoter, did not amplify any DNA, thus demonstrating that Par-4 and WT1 specifically bind to the WT1-binding site on the bcl-2 promoter. Together, these findings strongly support the idea that Par-4 and WT1 interact with a previously defined WT1-binding site on the bcl-2 promoter in the LNln3 cells and that the ectopic expression of Par-4 causes an enhanced binding of the two molecules Par-4 and WT1 at this site. Neither Par-4 nor WT1 were found to be present on the WT1-binding site on the bcl-2 promoter in the low Bcl-2-expressing androgen-sensitive LNCAp cells (Fig. 10A, 1st lane top and middle panels, respectively).

Upon examining LNln3 cells treated with 10 μM all-trans-retinoic acid for 96 h, we observed greater amounts of the bcl-2 promoter pulled down with the chromatin immunoprecipitated with the anti-Par-4 antibody as compared with the untreated cells, indicating enhanced amounts of Par-4 at the WT1-binding site on the bcl-2 promoter that correlated with decreased Bcl-2 protein and transcript observed in these cells following all-trans-retinoic acid treatment (Fig. 10B, 1st and 2nd lanes). Chromatin isolated from the same cells (untreated and treated with ATRA) immunoprecipitated with the anti-Par-4 antibody, and PCR amplified using primers representing non-WT1-binding site on the bcl-2 promoter, failed to amplify any DNA thereby demonstrating that the WT1-binding site on the bcl-2 promoter is required by Par-4 to bind the promoter (Fig. 10B, 5th and 6th lanes).

Transrepression of Transcription by Par-4 from the bcl-2 P1 Promoter in the LNln3 Cell Line—The physical data presented above demonstrate that Par-4 can bind to the bcl-2 promoter via a WT1-binding site. However, to determine whether the WT1-binding site on the bcl-2 P1 promoter had any functional activity in presence of Par-4 in LNln3 cells, transient transfection experiments were performed. The constructs for the transient transfection experiment are illustrated in Fig. 11A. We demonstrated that co-transfection with increasing amounts of full-length Par-4 resulted in decreased activity of the bcl-2 promoter in a dose-dependent manner (Fig. 11B). Par-4 does not mediate its repression when co-transfected with a construct of bcl-2 promoter that lacks the WT1-binding site (Fig. 11B), thus indicating that the WT1-binding site is required by Par-4 to transcriptionally down-modulate Bcl-2 expression in the LNln3 cell line.

DISCUSSION

We demonstrated that Par-4 regulates the transcription of the bcl-2 gene by interacting with a previously defined WT1-binding site on the bcl-2 promoter. We provide evidence that Par-4 and WT1 are involved in regulating Bcl-2 expression in prostate cancer cell lines and that ectopically expressed Par-4 is involved in the transcriptional repression of Bcl-2 in the androgen-insensitive prostate cancer cell line LNln3. Previous studies have reported an inverse correlation between Par-4 and Bcl-2; however, here we demonstrate the presence of Par-4 at the promoter of a bcl-2 both in vitro and in vivo. We have also demonstrated the presence of Par-4 at the bcl-2 promoter following all-trans-retinoic acid treatment, which correlates with the decreased Bcl-2 protein and transcript, thus defining a mechanism for retinoic acid-mediated decrease of Bcl-2 in prostate cancer cells. So far the regulation of Bcl-2 expression in prostate cancer has not been extensively defined. A recent study (20) has demonstrated the transcriptional activation of Bcl-2 in prostate cancer cell lines by NF-kB through κB sites on the P2 promoter upon treatment with TNF-α. We have
of androgen-insensitive prostate cancer cells. The significance of this comes from the fact that previous studies (7–9) have clearly demonstrated that decreased Bcl-2 levels sensitize prostate cancer cells to chemotherapeutic agents and also delay progression to androgen independence. As such, our data implicate Par-4 and its interacting partner WT1 in the transcriptional regulation of bcl-2 via a previously defined WT1-binding site on the bcl-2 P1 promoter.

In normal prostate epithelia, Par-4 and Bcl-2 are co-expressed in the basal epithelial cells, which do not require androgens to survive. As these cells differentiate to luminal or secretory epithelia, the growth and differentiation of these cells is tightly regulated by androgens, making these cells sensitive to apoptosis upon androgen ablation. The secretary epithelium expresses no Bcl-2 and lower levels of Par-4, which is inducible upon hormonal ablation (4, 23, 24). An inverse correlation has been reported for Par-4 and Bcl-2 expression in androgen-dependent and androgen-independent prostate tissues from human prostate cancer xenografts in the rat (the CWR22 and the CWR22R models) (4, 23, 24). It has also been demonstrated that exogenous apoptotic insults, such as androgen withdrawal from prostate cancer cells, cause a decrease in Bcl-2 expression (4). Androgen withdrawal also results in induction of Par-4 in androgen-sensitive prostate tissue (25). Previously, ectopic ex-

Fig. 10. Demonstration of in vivo binding of WT1 and Par-4 to the −1460 WT1-binding site on the bcl-2 P1 promoter by chromatin immunoprecipitation assay. A, chromatin lysates from LNCaP, LNln3 cells, and clone of LNln3 stably overexpressing Par-4 were immunoprecipitated (IP) with either antibody against WT1 (middle panel) or antibody against Par-4 (top panel). Samples were processed as described under “Experimental Procedures.” Immunoprecipitated DNA was amplified using primers representing the −1460 WT1-binding site on the bcl-2 promoter. The bottom panel shows PCR analysis of the total input DNA. Results are representative of two independent experiments. B, chromatin lysates from LNCaP, LNln3 cells, and clone of LNln3 stably overexpressing Par-4 were immunoprecipitated with either antibody against WT1 (middle panel) or antibody against Par-4 (top panel). Samples were processed as described under “Experimental Procedures.” Immunoprecipitated DNA was amplified using primers representing a site upstream of the −1460 WT1-binding site (negative control) on the bcl-2 promoter. C, chromatin lysates from LNln3 cells either treated with 10 μM L-ATRA for 96 h (2nd lane) or untreated (1st lane, C, control), immunoprecipitated with antibody against Par-4 (top panel). The 3rd and 4th lanes represent PCR analysis of the total input DNA. The adjacent panel represents chromatin lysates from LNln3 cells either treated with 10 μM L-ATRA for 96 h (6th lane) or untreated (5th lane), immunoprecipitated with antibody against Par-4, and the resulting DNA is amplified using the “negative control” primers as described above. The last two lanes represent total input genomic DNA, amplified using the “negative control” primers.

Our data also indicate that an induction in the expression of WT1, over and above endogenous levels, is required for Par-4 to successfully down-modulate Bcl-2. Our study has defined a mechanism of regulating Bcl-2 expression in androgen-insen-

also demonstrated that Par-4 requires the WT1-binding site on the bcl-2 promoter to mediate repression of bcl-2 gene transcription.

Our data also indicate that an induction in the expression of WT1, over and above endogenous levels, is required for Par-4 to successfully down-modulate Bcl-2. Our study has defined a mechanism of regulating Bcl-2 expression in androgen-insen-
Par-4 Regulates Bcl-2 Expression

Fig. 11. Par-4 trans-represses the bcl-2 promoter activity via a WT1-binding site. A, schematic representation of Bcl-2 luciferase reporter constructs. B, LNln3 prostate cancer cells were co-transfected with bcl-2 reporter constructs (P1 containing both the WT1-binding sites or P1 without the WT1-binding sites) and the pRL-TK vector as an internal control for transfection efficiency. At the same time the cells were also transfected with either an empty vector or increasing amounts of full-length Par-4 (2 or 4 μg). Luciferase activity was determined 24 h after the transfection. Non-transfected cells were used as the background. All values were normalized for expression of Renilla luciferase and expressed as X-fold induction relative to the activity of the P1 promoter co-transfected with the empty expression vector control (equal to 100). Shown is a representative experiment of the two that were performed.

Pression of Par-4 in the NIH3T3 mouse fibroblast and in the PC3 prostate cancer cell line has been shown to down-modulate Bcl-2 expression (24). Furthermore, an inverse correlation between Bcl-2 and Par-4 has also been reported in acute lymphocytic leukemia. These observations suggest that Par-4 is an important component of the pathway regulating Bcl-2 expression. However, the molecular pathway(s) leading to Bcl-2 regulation by Par-4 have not been defined.

Apart from translocation and amplification, the bcl-2 gene is also subject to transcriptional, post-transcriptional, translational, and post-translational regulation, which could result in its deregulated expression. We were able to demonstrate a decrease in the bcl-2 transcript corresponding to decreased protein following ectopic expression of Par-4, suggesting that Par-4-mediated down-modulation of Bcl-2 expression occurs at the transcriptional level. Par-4 is a leucine zipper molecule possessing two nuclear localization signals; however, no DNA binding for Par-4 has been demonstrated so far. Par-4 has been identified as a co-activator and a repressor for WT1-mediated transcription. WT1, a zinc finger transcription factor, is known to bind to the bcl-2 promoter and modulate its transcription (27, 36). This led us to investigate the role of WT1 in the Par-4-mediated regulation of Bcl-2. We observed an induction in WT1 expression accompanying L-ATRA-mediated down-modulation of Bcl-2 in the LNln3 cell line. Furthermore, the ectopic expression of Par-4 in the LNln3 cell line also caused an induction in the expression of WT1, which led us to believe that Par-4 may require high levels of WT1 protein for the successful down-modulation of Bcl-2. This inference was confirmed when we blocked endogenous WT1 and observed an increase in endogenous Bcl-2 expression indicating that basal levels of WT1 are also involved in repression of Bcl-2 to some extent; however, increasing endogenous WT1 levels over and above the basal magnifies this effect.

Par-4 is known to regulate the transcriptional activity of WT1 depending on which WT1 domain to which it binds (27, 36). By binding to the zinc fingers of WT1, Par-4 can augment the transcriptional repression and inhibit the transcriptional activation being mediated by WT1. Upon binding to the exon 5-encoded domain of WT1, Par-4 has been shown to act as a co-activator of WT1-mediated transcription and is involved in rescuing cells from apoptosis. WT1 binds to two different GC-rich sites on the bcl-2 P1 promoter, −1460 and −1807, relative to the translation start site, and can both repress and activate transcription of the bcl-2 gene (33–35).

We have shown both in vitro and in vivo that both the Par-4 and the WT1 proteins are a part of the complex that binds to the −1460 site on the bcl-2 P1 promoter, thus suggesting that Par-4 mediates transcriptional control of the bcl-2 gene. The −1460 site on the bcl-2 P1 promoter was identified as a site where WT1 binds and represses transcription of the normal bcl-2 allele in the t(14:18) follicular lymphoma. The same site seems to be driving the expression of the bcl-2 gene in the prostate cancer cell lines examined because this site was bound up in the high bcl-2-expressing cell line LNln3 and was not bound in the LNCaP cell line, which has barely detectable levels of the Bcl-2 protein and also showed a weak complex formation in the LNCaP cell line, which has lower levels of the Bcl-2 protein than the LNln3 cell line. However, increased levels of WT1 protein, achieved either by L-ATRA treatment or by ectopic expression of Par-4, change the dynamics of the complex at the −1460 site leading to increased amounts of Par-4 at this site and decreased levels of Bcl-2 in the LNCaP cell line. We have demonstrated in vivo that Par-4 and WT1 are present at the −1460 WT1-binding site on the bcl-2 promoter in the LNln3 cells, whereas these proteins are barely detectable in the LNCaP cells at the same site. Ectopic expression of Par-4 resulted in an increased amount of Par-4 and WT1 at the −1460 WT1-binding site on the bcl-2 promoter in vivo. Treatment with all-trans-retinoic acid also caused an increased amount of Par-4 at the −1460 WT1-binding site on the bcl-2 promoter. By our promoter assays we have demonstrated that increased binding of Par-4 and WT1 at the −1460 site on the bcl-2 promoter translates into decreased transcription of the bcl-2 gene which requires the WT1-binding site on the bcl-2 promoter.

Hence our results suggest that in the presence of basal or endogenous levels of WT1, Par-4 activates bcl-2 transcription via the −1460 WT1-binding site of the bcl-2 promoter. However, in the presence of higher levels of WT1, Par-4 represses bcl-2 transcription via the same binding site. It can be speculated based on our data and previous studies (27, 36) that in the presence of endogenous levels of Par-4, this protein binds the exon 5 domain of WT1 and behaves as a co-activator of WT1-mediated transcription of the bcl-2 gene. However, when the levels of Par-4 in the nucleus are increased, the excess Par-4 may now bind the zinc fingers of WT1, the exon 5 domain being already bound, and inhibit transcriptional activation of bcl-2 thereby changing this from an activator complex to a repressor complex. In addition to this, WT1 is known to have numerous isoforms, the functions of which are not totally characterized (31). Hence, there is a need to explore isoforms that may be involved, and whether upon induction of WT1 by L-ATRA or ectopic Par-4 there is a change in isoforms of WT1 that changes the Par-4-WT1 complex at the bcl-2 promoter from an activator complex to a repressor complex. Currently, we are in the process of characterizing these interactions, and future experiments should yield valuable information regarding this effect.
Par-4 Regulates Bcl-2 Expression

20005

REFERENCES

1. Oh, W. K., and Kantoff, P. W. (1999) J. Clin. Oncol. 17, 3664–3675
2. Lara, P. N., Jr., and Meyers, F. J. (1999) Cancer Invest. 17, 137–144
3. Raffo, A. J., Perlman, H., Chen, M. W., Day, M. L., Streitman, J. S., and Buttyan, R. (1995) Cancer Res. 55, 4438–4445
4. McDonnell, T. J., Tronoce, P., Brisby, S. M., Logothetis, C., Chung, L. W., Hsieh, J. T., Tu, S. M., and Campbell, M. L. (1992) Cancer Res. 52, 6940–6944
5. Colombel, M., Symmans, F., Gil, S., O’Toole, K. M., Chaplin, D., Benson, M., Olsson, C. A., Korsmeyer, S., and Buttyan, R. (1993) J. Pathol. 143, 390–400
6. Beham, A., Marin, M. C., Fernandez, A., Herrmann, J., Brisby, S., Tari, A. M., Oh, W. K., and Kantoff, P. W. (1999) J. Clin. Oncol. 17, 137–144
7. Miyake, H., Tolcher, A., and Gleave, M. E. (1999) N. Engl. J. Med. 341, 1333–1341
8. Pleasure, D. (1991) Cancer Res. 51, 1440–1443
9. Buttyan, R. (1995) Am. J. Pathol. 146, 137–140
10. Hsieh, J. T., Tu, S. M., and Campbell, M. L. (1992) Cancer Res. 52, 5288–5292
11. Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., and Croce, C. M. (1984) Oncogene 3, 22687–22691
12. Lancaster, P. N., Jr., and Meyers, F. J. (1999) Cancer Invest. 17, 269–272
13. Tsakirides, Z., and Frenkel, Y. (1996) Cancer Invest. 14, 137–140
14. Moztarzadeh, A., Shokri, M., and Shokrzadeh, M. (1996) Cancer Invest. 14, 137–140
15. Kallakury, B. V., Figge, H. L., Nazeer, T., and Ross, J. S. (1996) Science 273, 5546–5556
16. Wilson, B. E., Mochon, E., and Boxer, L. M. (1996) Mol. Cell. Biol. 16, 5546–5556
17. Chen, H. M., and Boxer, L. M. (1995) Mol. Cell. Biol. 15, 3840–3847
18. Smith, M. D., Enser, H. A., Coffin, R. S., Boxer, L. M., and Latchman, D. S. (1998) J. Biol. Chem. 273, 16715–16722
19. Cott, S. D., and Johnson, J. L. (2000) Oncogene 20, 7342–7351
20. Boeher, S. C., Chow, K. U., Puccetti, E., Rutherford, M., Godzisz, T., Krapohl, A., Schneider, B., Hoezler, D., Mitrou, P. S., Rangnekar, V. M., and Weidmann, E. (2001) Hematol. J. 2, 103–107
21. Boeher, S. C., Chow, K. U., Beske, F., Kokoc-Ziwnowska, N., Puccetti, E., Rutherford, M., Baum, C., Rangnekar, V. M., Hoezler, D., Mitrou, P. S., and Weidmann, E. (2002) Cancer Res. 62, 1768–1775
22. Hsieh, J. T., Tu, S. M., and Campbell, M. L. (1992) Cancer Res. 52, 6940–6944
23. Hsieh, J. T., Tu, S. M., and Campbell, M. L. (1992) Cancer Res. 52, 6940–6944
24. Qiu, G., Ahmed, M., Sells, S. F., Mhudiuddin, M., Weinstein, M. H., and Rangnekar, V. M. (1999) Oncogene 18, 623–631
25. Sells, S. F., Wood, D. P., Jr., Jashi-Barve, S. S., Muthukumar, S., Jacob, R. J., Crist, S. A., Humphreys, S., and Rangnekar, V. M. (1994) Cell Growth Differ. 5, 457–466
26. Sells, S. F., Han, S. S., Muthukumar, S., Maddiwar, N., Johnstone, R., Boghaert, E., Gillis, D., Liu, G., Nair, P., Munsing, S., Collini, P., Mattson, M. P., Sukhatme, V. P., Zimmer, S. G., Wood, D. P., Jr., McRoberts, J. W., Shi, Y., and Rangnekar, V. M. (1997) Mol. Cell. Biol. 17, 3823–3832
27. Johnstone, R. W., See, R. H., Sells, S. F., Wang, J., Muthukumar, S., Engelert, C., Haher, D. A., Licht, J. D., Sugrue, S. P., Roberts, T., Rangnekar, V. M., and Shi, Y. (1996) Mol. Cell. Biol. 16, 6945–6956
28. Fraser, A., and Evans, G. (1996) Cell 85, 781–784
29. Diaz-Meco, M. T., Munoz, M. M., Pratas, S., Sanchez, P., Lozano, J., Sanz, L., and Moscat, J. (1996) Cell 86, 777–786
30. Page, G., Kogel, D., Rangnekar, V., and Scheidtmann, K. H. (1999) Oncogene 18, 7265–7273
31. Reddy, J. C., and Licht, J. D. (1996) Biochem. Biophys. Acta 1287, 1–28
32. Rauscher, F. J. D. (1993) PASEB J. 7, 896–903
33. Hewitt, S. M., Hamada, S., McDennell, T. J., Rauscher, F. J. III, and Saunders, G. F. (1995) Cancer Res. 55, 5386–5389
34. Heckman, C., Mochon, E., Arcinas, M., and Boxer, L. M. (1997) J. Biol. Chem. 272, 19609–19614
35. Mayo, M. W., Wang, C. Y., Drouin, S. S., Madrid, L. V., Marshall, A. F., Reed, J. C., Weissman, B. E., and Baldwin, A. S. (1999) EMBO J. 18, 3990–4003
36. Richard, D. J., Schumacher, V., Royer-Pokora, B., and Roberts, S. G. (2001) Genes Dev. 15, 328–339
37. Sundaresan, A., Clappel, K., Mehta, K., Lopez-Berestein, G., Cabanillas, F., and Ford, R. J., Jr. (1997) Cell Growth Differ. 8, 1071–1082
38. Hall, A. K. (1996) Anti-Cancer Drugs 7, 312–320
39. Trump, D. L., Smith, D. C., Stiff, D., Adedoyin, A., Day, R., Bahnson, R. R., and Stiff, D., A. A. (1980) Proc. Natl. Acad. Sci. USA 77, 781–785
40. Hall, A. K. (1996) Anti-Cancer Drugs 7, 312–320
41. Hsieh, J. T., Tu, S. M., and Campbell, M. L. (1992) Cancer Res. 52, 5288–5292
42. Yamagami, T., Sugiyama, H., Inoue, K., Ogawa, H., Tatekawa, T., Hirata, M., Kudoh, T., Akiyama, T., Murakami, A., and Masakawa, T. (1996) Blood 87, 2878–2884
43. Tari, A. M. (2000) Methods Enzymol. 331, 372–388
44. Heckman, C. A., Mehew, J. W., Ying, G. G., Introna, M., Golay, J., and Boxer, L. M. (2000) J. Biol. Chem. 275, 6499–6508
45. Werner, H., Rauscher, F. J. III, Sukhatme, V. P., Drummond, I. A., Roberts, C. T., Jr., and LeRoith, D. (1994) J. Biol. Chem. 269, 12577–12582
46. Mishra, S. K., Mandal, M., Mazumdar, A., and Kumar, R. (2001) FEBS Lett. 507, 83–84
47. Ji, L., Mochon, E., Arcinas, M., and Boxer, L. M. (1997) J. Biol. Chem. 272, 22687–22691
48. De Luca, L. M. (1991) PASEB J. 5, 2924–2933
49. Pasquali, D., Thaller, C., and Eichele, G. (1996) J. Clin. Endo. Metabol. 81, 2186–2191

Downloaded from http://www.jbc.org/ by guest on July 23, 2018
Par-4 Transcriptionally Regulates Bcl-2 through a WT1-binding Site on the bcl-2 Promoter
Sangeeta K. Cheema, Sandip K. Mishra, Vivek M. Rangnekar, Ana M. Tari, Rakesh Kumar and Gabriel Lopez-Berestein

J. Biol. Chem. 2003, 278:19995-20005.
doi: 10.1074/jbc.M205865200 originally published online March 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M205865200

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 49 references, 26 of which can be accessed free at
http://www.jbc.org/content/278/22/19995.full.html#ref-list-1