

# Antisense TR3 Orphan Receptor Can Increase Prostate Cancer Cell Viability with Etoposide Treatment\*

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## ABSTRACT

In androgen-responsive LNCaP human prostatic cancer cells, human TR3 orphan receptor, a member of the steroid receptor superfamily, can be rapidly induced by androgen. In contrast, ablation of androgen by castration can induce the expression of the TR3 orphan receptor gene in rat ventral prostate that has undergone apoptosis. This phenomenon prompted us to further analyze the potential role of human TR3 orphan receptor in prostate cancer cells in which apoptosis had been induced. Northern blot analysis shows that hu-

man TR3 orphan receptor expression can be induced rapidly after treatment of LNCaP and PC-3 prostate cancer cells with calcium ionophore or etoposide. Our data further demonstrate that a much higher concentration of etoposide was needed to kill the same number of cells in LNCaP and PC-3 cells transfected stably with antisense TR3 orphan receptor compared with that in control vector transfectants. Together, our data suggest that the human TR3 orphan receptor may play an important role in modulating drug-induced prostate apoptosis. (*Endocrinology* 139: 2329–2334, 1998)

**A**POPTOSIS is morphologically characterized by several features, such as cell shrinkage, separation from neighboring cells, nuclear condensation, nuclear membrane breakdown, cytosol membrane blebbing, and cytolysis. In terms of biochemical and molecular aspects, DNAs degraded by apoptosis-inducing stimuli are shown on electrophoresis as a ladder in 180-bp units (1, 2).

Apoptosis in the prostate has been investigated in earlier studies. Androgen ablation by castration can induce regression of the rat ventral prostate gland, mainly in glandular epithelial cells, but not in basal epithelial cells or stromal cells (1, 2). Intranucleosomal DNA fragmentation can start to appear on day 1 after castration (3). Furthermore, several specific genes associated with prostate apoptosis have been studied (4). Testosterone-repressed prostatic message-2 levels can increase after castration or after the administration of apoptosis-inducing agents (5–9). Another gene, *bcl-2*, which can function as an inhibitor for apoptosis, was found to be expressed at a much higher level in androgen-independent prostate cancer cells than in androgen-dependent prostate cancer cells (10). Recent data have suggested that the expression of tumor growth factor may be linked to prostate apoptosis (11, 12). Androgen removal may increase intracellular free  $Ca^{2+}$ , which, in turn, may activate a  $Ca^{2+}/Mg^{2+}$ -dependent endonuclease to degrade DNA in prostate apoptosis (1, 13). Rat androgen-independent prostatic cancer cell apoptosis can also be induced with a modest elevation of intracellular  $Ca^{2+}$  (14).

Several genes, such as *c-myc*, *c-fos*, and *c-jun*, are reported to play important roles in apoptosis events in different cell types, particularly in lymphoid cells (15, 16). More interest-

ingly, Liu *et al.* demonstrated that mouse *nur77*, one of the orphan receptors in the steroid/thyroid receptor superfamily, can be induced in T cell hybridoma apoptosis (17, 18). Human TR3 orphan receptor, isolated in our laboratory (19), is the human homolog of mouse *nur77* (20), N10 (21), and rat NGFI-B (22, 23) genes.

Here we report that human TR3 orphan receptor can be induced rapidly after stimulation of apoptosis-inducing agents in human prostate cancer cells. Furthermore, the stable transfectants with antisense TR3 orphan receptor showed resistance to etoposide-induced cytotoxicity in a dose-dependent manner compared with control vector transfectants. In summary, our data clearly demonstrate that the human TR3 orphan receptor may have a regulatory role in human prostate apoptosis.

## Materials and Methods

### Vector constructs

The vector p-2149TR3, which includes the 2.3-kilobase (kb) promoter region of human TR3 orphan receptor, was created in our laboratory (24, 25). From the results of human TR3 orphan receptor promoter analysis using chloramphenicol acetyltransferase assays, this 2.3-kb fragment was demonstrated to include a strong enhancer element (–200 to –181 bp in front of the transcriptional start site) for transcriptional activity in HeLa cells. This 2.3-kb promoter was then ligated with 1.9-kb antisense TR3 complementary DNA (cDNA; +1927 to +56 nucleotides) and inserted into pBluescript II SK<sup>–</sup> (Stratagene, La Jolla, CA). The 1.9-kb cDNA fragment of human TR3 orphan receptor includes amino-terminal domains (A and B domains), a DNA-binding domain (C domain), and partial ligand-binding domains (D and E domains). In brief, this fragment was ligated to the TR3 promoter fragment in reverse orientation. The pGKneo DNA fragment (1.7 kb) encoding neomycin resistance, which enables selection of stably transfected cells using the antibiotic G418 (Geneticin, Life Technologies, Grand Island, NY), was also inserted into the antisense TR3 vector (pASTR3neo). pGKneo was inserted into p-2149TR3 (pGKneo) as a control vector.

### Cell culture and transfections

LNCaP and PC-3 cell lines, two human prostate cancer cell lines, were used in all experiments. Cells were maintained in DMEM with 5% FBS.

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Synthetic androgen R1881 and 4-bromo-calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO) were dissolved in ethanol (100 mM). Etoposide (VP-16, Sigma) was dissolved in dimethylsulfoxide (Sigma) at 100 mM and used at varying final concentrations of 25–300  $\mu$ M. LNCaP and PC-3 cells were transfected with pASTR3neo or pGKneo vectors using a calcium phosphate method described previously (26). Forty-eight hours after transfection, the cells were cultivated in DMEM with 5% FBS containing G418 at 700  $\mu$ g/ml for LNCaP cells and 600  $\mu$ g/ml for PC-3 cells. G418-resistant clones of LNCaP cells were maintained until confluent in 100-mm dishes and were used for all experiments. Furthermore, several single clones of PC-3 cells transfected with pASTR3neo or pGKneo were independently selected and maintained in DMEM with 5% FBS containing G418. Of the stable transfectants in PC-3 cells, the antisense TR3 clone, AS-4, and the control clone, CON-1, were used for the experiments.

### Cell viability

Stably transfected cells ( $2 \times 10^5$ ) were plated on six-well dishes. Medium was changed after 24 h and was supplemented with etoposide at varying concentrations. After treatments of etoposide, the cells were washed with PBS, trypsinized, collected, and resuspended in fresh medium, and viable cells were counted using 0.4% trypan blue staining.

### DNA electrophoresis

Cells ( $2 \times 10^6$ ) treated under various conditions were centrifuged, washed with PBS, and resuspended with a solution of 100 mM Tris-HCl, 5 mM EDTA, and 0.2% SDS containing 100  $\mu$ g/ml proteinase K. These samples were incubated at 37 C for 18 h, extracted with phenol/chloroform, and treated at 37 C for several hours with 200  $\mu$ g/ml ribonuclease A. Samples were precipitated in ethanol and loaded onto 1.5% agarose gel. Gels were stained with 2.5  $\mu$ g/ml ethidium bromide and photographed under UV light.

### Northern blot analysis

Total RNA was isolated by guanidium thiocyanate followed by centrifugation in CsCl solutions as described previously (24). Twenty micrograms of total RNA were analyzed by electrophoresis through 1% agarose-formaldehyde gel, followed by Northern blot, and transferred to a nylon filter (Hybond-N, Amersham, Arlington Heights, IL). The filters were prehybridized at 42 C for 2 h and then hybridized with [ $\alpha$ - $^{32}$ P]deoxy-CTP-labeled 400-bp cDNA of human TR3 orphan receptor at 42 C overnight. The filters were also hybridized with an [ $\alpha$ - $^{32}$ P]deoxy-CTP-labeled cDNA of  $\beta$ -actin (Oncor) as a control. After hybridization, filters were washed twice with  $2 \times$  SSC and 0.1% SDS at 60 C and finally washed twice with  $0.1 \times$  SSC and 0.1% SDS at 60 C. After washing, the filters were exposed to x-ray films at  $-80$  C overnight.

### RT-PCR analysis of TR3 transcript

Male rats at 42–45 days of age (Sprague-Dawley, Madison, WI) were castrated and then killed 1, 2, 3, 4, and 5 days after castration. Total RNAs of ventral prostate were isolated by the method described above. One microgram of total RNA was converted into complementary DNA (cDNA) by Moloney murine leukemia virus reverse transcriptase (Life Technologies). Then each sample was amplified by *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in an automated thermal cycler (Perkin-Elmer/Cetus Instruments, Norwalk, CT) at 95 C for 1 min, 37 C for 2 min, and 72 C for 3 min. Primers were synthesized as follows: NGFI-B forward and backward, 5'-ACAACGCTTCGTGCCAGCAT-3' and 5'-CCGGACAACCTTCCTTAC-3', respectively. Each primer was end labeled with [ $\gamma$ - $^{32}$ P]ATP. Amplification was stopped at 20 cycles, and then PCR products were run on a 5% polyacrylamide gel. Dried gel was exposed to x-ray film at  $-80$  C overnight.

### Southern blot analysis

Stably transfected cells of antisense TR3 vector (pASTR3neo) or control vector (pGKneo) were collected and resuspended with PBS. DNA extractions were performed using the method described above. DNAs were digested with *Hind*III (Promega, Madison, WI) and electropho-

resed on 0.8% agarose gel, followed by transfer to nylon filters. The techniques of prehybridization, hybridization, and washing were same as those used for Northern blot analysis.

## Results

### Androgen effects on the expression of TR3 orphan receptor in human prostate cancer cells and rat ventral prostate

The growth of prostate LNCaP cells is known to be sensitive to androgen. Accordingly, we used Northern blot analysis to examine androgen effects on the expression of human TR3 orphan receptor in these cells. The synthetic androgen R1881 (20 nM) dramatically induced the human TR3 orphan receptor messenger RNA (mRNA) as early as 30 min after stimulation. The induced TR3 orphan receptor mRNA levels reached a peak at 90 min and then declined to a basal level 3 h after stimulation (Fig. 1A). To further extend this *in vitro* cell line study to the *in vivo* condition, we treated rats with androgen ablation by castration and detected the mRNA expression in the ventral prostate. As shown in Fig. 1B, the induction of TR3 orphan receptor mRNA appeared 3 days after castration, reaching a peak at 4 days. Together, our data demonstrated that both the addition of androgen and the removal of androgen by castration induce expression of the TR3 orphan receptor in the prostate.

### Calcium ionophore and etoposide induction of TR3 orphan receptor expression

As shown in Fig. 2A-a, 10  $\mu$ M calcium ionophore, an apoptosis-inducing agent, dramatically induced TR3 orphan receptor expression at 1 h, peaking at 4 h, and gradually disappearing 8 h after treatment. In the same experiment, a stereotypic DNA fragmentation ladder was observed 24 h after treatment (Fig. 2A-b). Another apoptosis-inducing reagent, etoposide (VP-16), which has been used as a chemotherapeutic drug in the later stages of prostate cancer, was tested to further confirm the potential roles of TR3 orphan receptor in prostate apoptosis. As shown in Fig. 2B-a, Northern blot analysis clearly demonstrated that 300  $\mu$ M etoposide can transiently induce TR3 orphan receptor expression 2 h after treatment. Under the same conditions, we easily observed the DNA fragmentation ladders at 8 and 24 h after treatment (Fig. 2B-b). We also observed a similar TR3 orphan receptor induction pattern and DNA degradation fragments when etoposide was applied to PC-3 cells, an androgen-independent prostatic cancer cell line (data not shown). Taken together, these data indicated that human TR3 orphan receptor can be induced in the early phases after treatment by reagents that instigate apoptosis in human prostate cancer LNCaP and PC-3 cells.

### Antisense TR3 orphan receptor may increase cell viability in prostate cancer LNCaP and PC-3 cells

To further confirm the role of TR3 orphan receptor in prostate apoptosis, we established a stably transfected LNCaP cell line with an antisense TR3 orphan receptor expression vector based on the hypothesis that antisense TR3 orphan receptor may block chemotherapeutic drug-induced cell death in prostate cancer cells. LNCaP cells were transfected with an antisense TR3 orphan receptor expression vector,

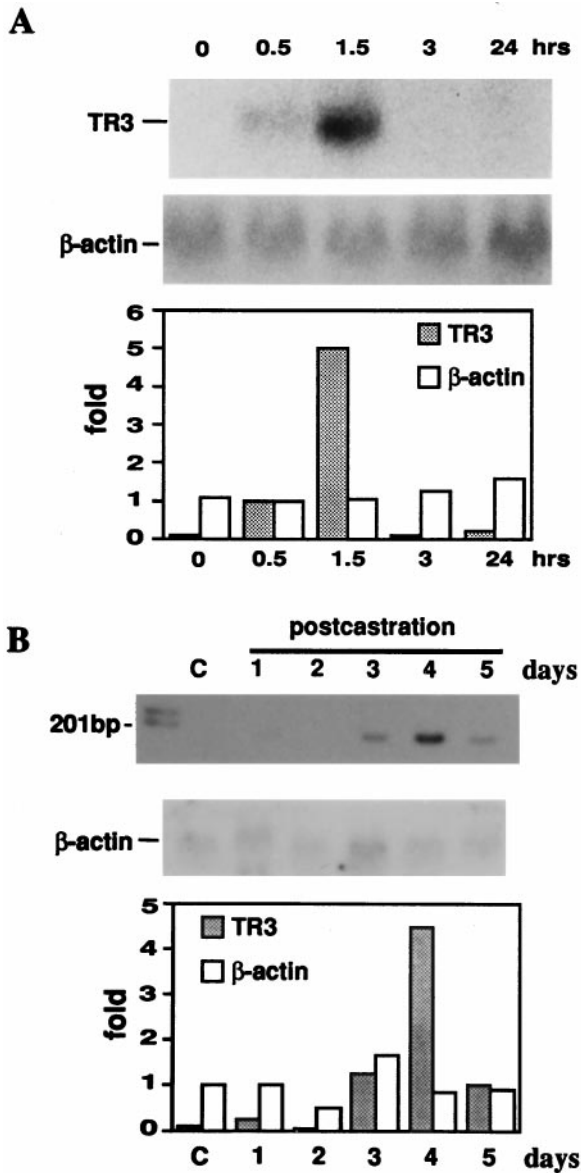


FIG. 1. Androgen effects on the expression of TR3 orphan receptor in prostate. A, LNCaP cells were cultivated in DMEM containing charcoal-treated serum and treated with 20 nM R1881. Total RNA was extracted at the indicated times and hybridized with TR3 orphan receptor and  $\beta$ -actin cDNA. B, Induction of TR3 orphan receptor mRNA in castrated rat ventral prostate. Samples were taken 1, 2, 3, 4, and 5 days postcastration and compared with intact controls. Total RNAs were isolated and reverse transcribed to cDNAs. The same cDNA synthesis reaction was then used in PCR with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  end-labeled NGFI-B primers. After 20 cycles, the amplification reaction was electrophoresed through a 5% polyacrylamide gel. For A and B controls, the same amount of RNA was loaded per well and hybridized with  $\beta$ -actin cDNA.

pASTR3neo, or the parent vector, pGKneo, as a control. After selection in antibiotic G418 medium, the surviving cell colonies were grown, and DNAs were isolated for Southern blot analysis. As shown in Fig. 3, a 1.9-kb band of antisense TR3 orphan receptor cDNA could be detected only in cells transfected with pASTR3neo, but not in those transfected with the parent vector, pGKneo. Furthermore, our Northern blot anal-

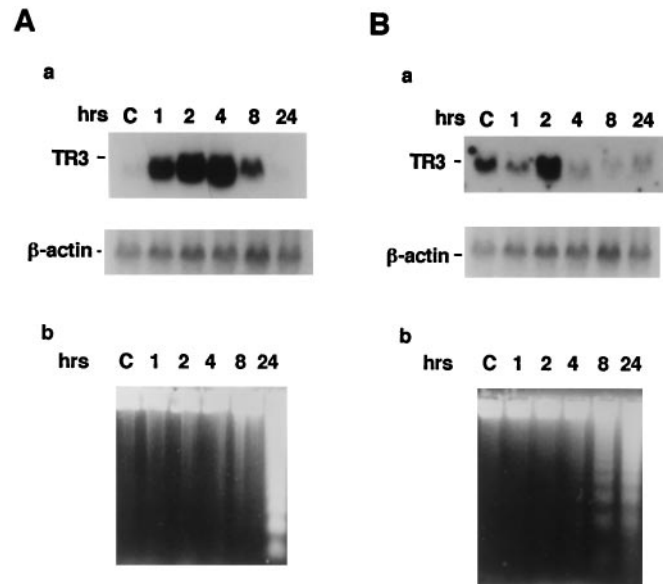


FIG. 2. The expression of TR3 orphan receptor in etoposide- or calcium ionophore-treated prostate LNCaP cells. A-a, Calcium ionophore effects. LNCaP cells were cultivated in DMEM containing 10  $\mu\text{M}$  calcium ionophore. Total RNAs were extracted at various times after cultivation and examined by Northern blot analysis for TR3 orphan receptor gene and  $\beta$ -actin expression. A-b, DNAs were isolated from the cells at various times after exposure of 10  $\mu\text{M}$  calcium ionophore. B-a, Etoposide effects. LNCaP cells were cultivated in DMEM containing 300  $\mu\text{M}$  etoposide (conditions were the same as in A, except 300  $\mu\text{M}$  etoposide was used). B-b, DNAs were isolated from the cells at various times after exposure to 300  $\mu\text{M}$  etoposide. For A and B controls, the same amount of RNA was loaded per well and hybridized with  $\beta$ -actin cDNA.

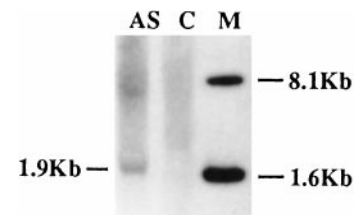


FIG. 3. Southern blot analysis of DNAs extracted from antisense TR3 transfectant and control transfectant. DNAs of both transfectants were digested with *Hind*III and examined by Southern blot analysis. AS, Antisense TR3 transfectants; C, control transfectants; M, 1-kb DNA ladder marker (Life Technologies) end labeled with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . (Control transfectant means transfected with parent vector without antisense TR3 insertion.)

ysis (Fig. 4, lane 1 *vs.* 2) clearly demonstrated that after treatment with etoposide, antisense TR3 orphan receptor could reduce or block the expression of TR3 orphan receptor to 15% compared with that in stably transfected control cells (cells transfected with parent vector only). This result was similar to the blockage of lymphoid cell apoptosis by the addition of antisense *c-fos* and *c-jun* (16).

In a second series of experiments using stable transfectants, we further examined whether the antisense TR3 stable transfectants can increase cell viability after treatment of etoposide at different concentrations. Both stable transfectants of LNCaP cells (antisense *vs.* control) were exposed to etoposide at concentrations of 50–300  $\mu\text{M}$  for 24 h; a trypan blue exclusion assay (in triplicate) was then applied to quan-

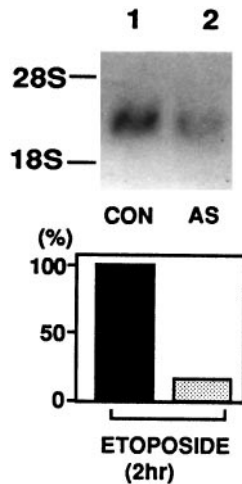


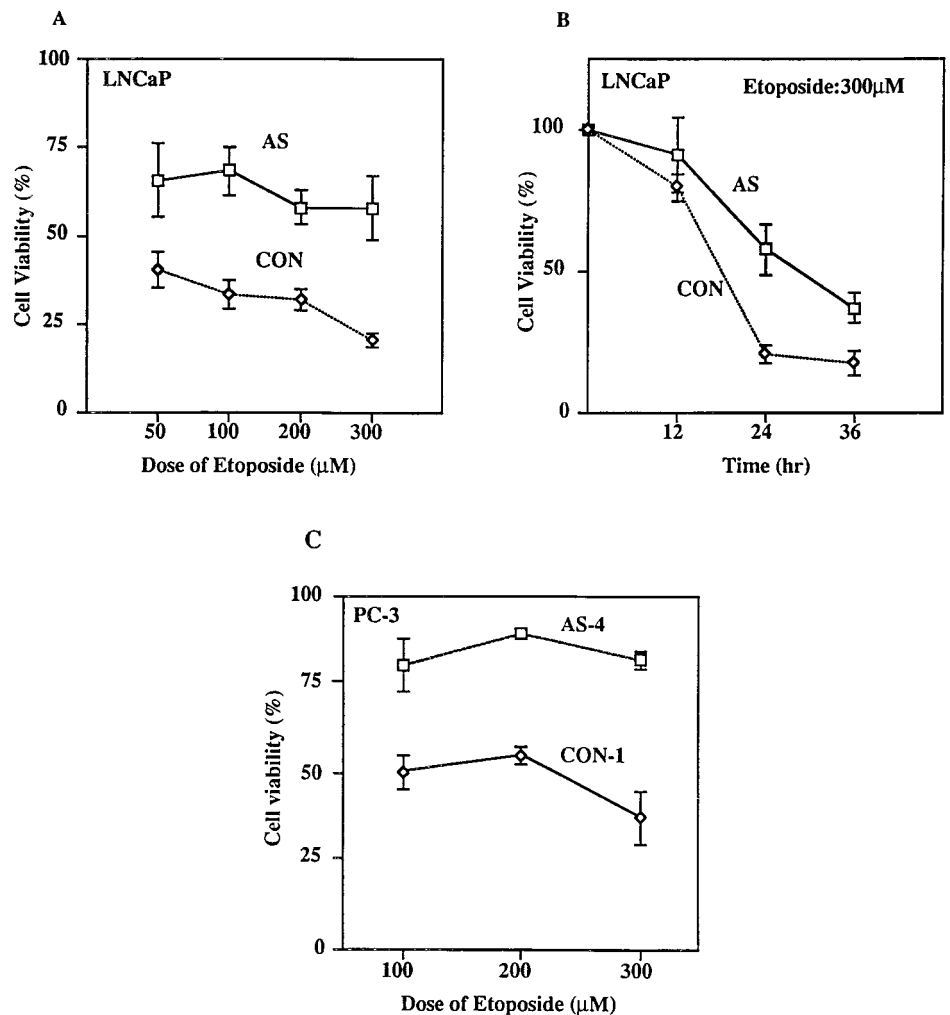
FIG. 4. Expression of TR3 orphan receptor in antisense TR3 transfectants and control transfectants treated with etoposide. Both transfectants were cultivated in DMEM containing  $300 \mu\text{M}$  etoposide. After 2 h, total RNAs were extracted and examined by Northern blot analysis for TR3 orphan receptor gene expression. Equal 28S and 18S ribosomal RNAs were verified by staining with methylene blue for the guarantee of equal loading in each well. Lane 1, Control transfectants treated with etoposide; lane 2, antisense TR3 transfectants treated with etoposide.

tify cell death. As shown in Fig. 5A, etoposide at  $300 \mu\text{M}$  effected significantly different levels of viability in antisense TR3 stable transfectants *vs.* control transfectants ( $57.9 \pm 9.1\%$  *vs.*  $20.5 \pm 1.8\%$ ). Our data also showed the effects of etoposide to be dose dependent; the 50% lethal doses for antisense and control transfectants were approximately 400 and  $40 \mu\text{M}$ , respectively.

In subsequent studies, both stably transfected LNCaP cells with antisense TR3 orphan receptor and control vectors were exposed to high dose etoposide ( $300 \mu\text{M}$ ) for various time periods (12, 24, and 36 h). Cell viability at different time points (Fig. 5B) clearly demonstrated a significant difference induced by etoposide. Within 36 h after treatment with etoposide, cell viability in the control vector transfectants decreased to  $17.4 \pm 4.4\%$ , whereas viability in the antisense TR3 transfectants decreased to  $37.1 \pm 5.3\%$ , suggesting that antisense-transfected cells have a 20% higher survival rate than control vector transfectants. Like Fig. 5A, Fig. 5B reproducibly shows that 24 h after treatment with  $300 \mu\text{M}$  etoposide, antisense TR3 transfectants could increase cell viability by nearly 37% ( $57\%$  *vs.*  $20\%$ ) compared with control vector transfectants.

A similar approach was applied to another prostate cell line, PC-3, by isolation of a single clone transfected with

FIG. 5. Cell viabilities in stably transfected LNCaP and PC-3 cells treated with etoposide. A, Cell viability in stably transfected LNCaP cells treated with etoposide. Stably transfected cells with antisense TR3neo (AS) and control vector pGKneo (CON) were treated with etoposide at different concentrations for 24 h (as described in Fig. 2A), and viable cells were evaluated by trypan blue exclusion. Values are the mean  $\pm$  SD of three different experiments; in each experiment, all counts were performed in triplicate. B, Cell viability of stable transfectants. Antisense TR3neo (AS) and control vector pGKneo (CON) were assessed at various times by trypan blue dye exclusion. Values were the mean  $\pm$  SD of three different experiments. In each experiment, all counts were performed in triplicate. C, Stably transfected clones of PC-3 cells, antisense TR3 stable transfectant AS-4, and control vector stable transfectant CON-1 were treated with etoposide at different concentrations for 24 h. Viable cells were evaluated by trypan blue exclusion. Values are the mean  $\pm$  SD of three different experiments; in each experiment, all counts were performed in triplicate.



antisense TR3 (AS-4) or vector control (CON-1). Southern blot analysis again confirmed the existence of antisense TR3 construct (pASTR3neo) or vector control (pGKneo; data not shown). AS-4 and CON-1 were then exposed to various concentrations of etoposide (100, 200, and 300  $\mu\text{M}$ ). After 24 h of exposure, cell viabilities were measured in triplicate by trypan blue exclusion assay. As shown in Fig. 5C, a significant difference in cell viability between AS-4 and CON-1 stable transfectants was detected. For example, at 300  $\mu\text{M}$  etoposide, high cell viability ( $82.1 \pm 2.6\%$ ) of AS-4 clone *vs.* low cell viability ( $37.6 \pm 7.6\%$ ) of CON-1 was detected. Together, our antisense TR3 orphan receptor data demonstrate clearly that the expression of TR3 orphan receptor may play an important role in etoposide-induced prostate LNCaP and PC-3 cell death.

### Discussion

Apoptosis in the prostate or in prostate cancer cells (especially androgen-dependent cells) can be induced by androgen ablation, antiandrogens, irradiation, and chemotherapeutic agents (27). Other steroid hormones, such as glucocorticoids, can also repress apoptosis in the rat ventral prostate (28). The fact that apoptosis can be prevented by RNA or protein inhibitors (29–31) implies that gene transcription and translation may be required in the apoptotic process. From the results presented here, human TR3 orphan receptor gene could be one such gene induced in prostate apoptosis. The mouse homolog of TR3 orphan receptors, *nur77*, has been demonstrated to be required for apoptosis in T cell hybridomas (17), and the expression of *nur77* in apoptosis can be regulated by its restricted promoter region. Recently, we were able to identify a novel *cis*-acting element, NCAE-TR3, in the 5'-promoter region of the TR3 orphan receptor gene that is required for the expression of this gene (25). Interestingly, a DNA fragment very similar to this NCAE-TR3 *cis*-acting element was also found in the promoter area (–322 to –151 nucleotides) of *nur77* that proved to play a role in apoptosis of mouse T cell hybridomas (17). Determining whether NCAE-TR3 may also play a role in human prostate apoptosis may be an intriguing study.

Genes such as *c-myc*, *c-fos*, and *c-jun* have been linked to cell proliferation, differentiation, and apoptosis (32, 33). The human TR3 orphan receptor can also be rapidly induced by several mitogenesis inducers, such as androgen and the growth factors epidermal growth factor and fibroblast growth factor (24), that play important roles in the cell cycle. The data presented here demonstrated that the human TR3 orphan receptor can be induced by androgen or apoptosis-inducing agents (calcium ionophore and etoposide), and the fact that the antisense TR3 orphan receptor can reduce the cell death caused by these apoptotic reagents further suggests that the TR3 orphan receptor could be an important regulator for the induction of both mitogenesis and apoptosis in the prostate.

Quarmany *et al.* (34) demonstrated that *c-myc* mRNA in rat ventral prostate increased 4- to 7-fold within 2 days after castration. They also demonstrated that *in situ* hybridization of *c-myc* mRNA was expressed in epithelial cells of ventral

acinar glands. These data indicated that androgen may regulate the expression of *c-myc* in rat ventral prostate. Another group demonstrated a number of genes, including *c-myc*, participating in the mechanism by which androgens induce mitogenesis of prostate cells (35). Their data showed that within 6–12 h after androgen replacement to castrated rat, mRNA transcripts for *c-myc* in ventral prostate were induced 5- to 9-fold compared with those in the untreated rat.

Recently, Jenkins *et al.* (36) showed clear data in which *c-myc* amplification in prostate cancer tissues and metastatic lymph nodes was detected using fluorescence *in situ* hybridization. Our *in situ* hybridization data in human prostate cancer, benign prostate hypertrophy, and normal prostate tissue revealed that the expression patterns of TR3 orphan receptor mRNA are very similar to those of *c-myc*. More interestingly, TR3 orphan receptor mRNAs are more highly expressed in prostate cancer areas than in adjacent normal or benign prostate hypertrophic tissue (data not shown). These data suggest that the TR3 orphan receptor may play some important roles in development or progression of prostate cancer in the same manner as the *c-myc* oncogene.

At this time, we still do not know the molecular mechanism operating during the induction of TR3 orphan receptor in prostate cells with either the addition of androgen or the removal of androgen by castration. Like *c-myc*, which requires dimerization with Max for its role in mitogenesis or apoptosis (37), TR3 orphan receptor may also need other proteins as partners for its potential function. Using the yeast two-hybrid system, we were able to isolate several potential candidates. Further characterization of these TR3 orphan receptor-associated proteins may allow us to answer the above hypothesis.

Hormone refractory prostate cancer is incurable and accounts for the death of more than 40,000 American men each year. The median survival of patients with hormone refractory prostate cancer is 6–9 months (38). Multiple cytotoxic agents used in combination or as single agents have failed to significantly prolong survival in this disease. Recently, Pienta *et al.* have shown an overall median survival of 11 months in 42 patients treated with a combination of oral etoposide (VP-16) and estramustine, despite limited activity for either agent alone (39–41). Etoposide is a podophylotoxin derivative that is known to inhibit topoisomerase II, to interact with the nuclear matrix, and to induce apoptosis. The data presented in this paper indicate a strong relationship between the level of expression of the TR3 orphan receptor and the dose of etoposide required to kill prostate cancer cells *in vitro*. Ongoing studies *in vivo* will determine whether increased TR3 orphan receptor expression can enhance the sensitivity of prostate cancer cells to etoposide. If true, strategies to increase TR3 orphan receptor expression could be used in combination with agents such as etoposide to improve the efficacy of antiprostate cancer therapy.

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