

## Characterization of a Zinc-Finger Protein and Its Association With Apoptosis in Prostate Cancer Cells

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**Background:** The transition from androgen-dependent to androgen-independent prostate cancer is not fully understood but appears to involve multiple genetic changes. We have identified a gene, GC79, that is more highly expressed in androgen-dependent LNCaP-FGC human prostate cancer cells than in androgen-independent LNCaP-LNO human prostate cancer cells. Physiologic levels (0.1 nM) of androgens repress expression of GC79 messenger RNA (mRNA) in LNCaP-FGC cells. To determine the role of GC79, we cloned its complementary DNA (cDNA) and functionally characterized its product. **Methods:** The differentially expressed GC79 gene was cloned from human prostate cDNA libraries, sequenced, and transfected into mammalian cells to study its function. Expression of GC79 was analyzed in various adult and fetal human tissues and in prostate glands of castrated rats. The association of GC79 expression and apoptosis was investigated in COS-1 and LNCaP cells transfected with GC79 cDNA. All statistical tests are two-sided. **Results:** Sequence analysis indicates that GC79 encodes a large, complex, multitype zinc-finger protein, containing nine C<sub>2</sub>H<sub>2</sub>-type zinc-finger domains, a cysteine-rich region, and a GATA C<sub>4</sub>-type zinc-finger domain. Castration-induced androgen withdrawal increased the expression of GC79 mRNA in the regressing rat ventral prostate, suggesting that the expression of GC79 mRNA is associated with the process of apoptotic cell death in the rat ventral prostate. Transfection and induction of GC79 cDNA in both COS-1 and LNCaP prostate cancer

cells led to an apoptotic index that was eightfold higher ( $P < .001$ , two-sided Student's *t* test) than that observed in uninduced transfected cells. **Conclusions:** We have cloned an androgen-repressible gene, GC79, that is potentially involved in apoptosis. This finding may have implications for the development of androgen-independent prostate cancer and, ultimately, for the treatment of prostate cancer. [J Natl Cancer Inst 2000;92:1414-21]

Prostate cancer is the second leading cause of cancer-related deaths and the most commonly diagnosed cancer in elderly men in the Western world (1). Androgens play a pivotal role in normal prostate development and differentiation (2). Initially, prostate cancer is androgen dependent and can be treated by blocking endogenous androgen production by castration or androgen action with antiandrogen therapy (3). Castration can trigger apoptotic cell death in the rat ventral prostate (4) and in a human prostate cancer xenograft (5). Apoptosis is a physiologic process of programmed cell death that may be actively induced by chemicals or genetic factors (6) or passively induced by withdrawal of growth factors or hormones (4,5). Androgen withdrawal induces apoptosis in most androgen-dependent prostate cancer cells. However, androgen-independent prostate cancer cells are refractory to apoptosis and can be considered to be apoptosis resistant. It has been reported (7) that the emergence of androgen-independent cells and subsequently the generation of apoptosis-resistant cells are associated with the expression of the apoptosis-resistant gene *bcl-2*. Androgen-independent cells can still be induced to undergo apoptosis by certain compounds or by transfection of complementary DNAs (cDNAs) encoding proteins, such as p53 or p21 (8), illustrating that most factors of the apoptotic cascade are still functional in these resistant cells. However, apoptosis in androgen-independent cells is not induced by androgen withdrawal because proteins (such as *bcl-2*) that suppress apoptosis may inhibit apoptosis in these cells. Genes expressed during castration-induced apoptosis of the prostate include testosterone-repressed prostate message-2 [TRPM-2 (9,10)],

transforming growth factor- $\beta$  (11), prostate apoptosis response-4 (12), and *c-fos*, *c-myc*, and the 70-kd heat-shock protein (13).

The mechanism of prostate cancer progression and subsequent development of hormone-refractory prostate cancer is not fully understood (14,15). Cancer progression from a hormone-dependent state to a hormone-independent state seems to be caused by a cascade of genetic changes, reflected by activation of oncogenes and/or inactivation of tumor suppressor genes (16,17). Comparison of androgen-dependent and androgen-independent prostate cancer tissues showed very similar losses and gains of chromosomes. This finding suggests that the majority of chromosomal changes occurred during the growth of androgen-dependent prostate cancer (18). It seems that subtle but essential genetic differences are more likely involved in the transition to growth of androgen-independent prostate cancer (18-20). As a first step toward determining the molecular mechanism(s) underlying the progression toward hormone-refractory disease, we identified these genetic differences. By use of well-defined model systems, we cloned and subsequently characterized genes involved in this transition to determine their role in androgen-independent prostate cancer development.

We have used differential-display, reverse transcription-polymerase chain reaction (RT-PCR) analysis of human prostate cancer LNCaP sublines that are androgen dependent and androgen independent (21,22) to clone several differentially expressed cDNAs, and thus we have

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identified several genes (23–25). One of these cDNAs was novel and derived from a gene designated GC79, which is expressed more highly in androgen-dependent prostate cancer cells than in androgen-independent prostate cancer cells. Physiologic levels (0.1 nM) of androgens repress expression of GC79 messenger RNA (mRNA) in LNCaP-FGC cells (22). To determine the role of GC79 in prostate cancer cells, we have cloned its cDNA and functionally characterized its product.

## MATERIALS AND METHODS

### Cell Culture

The LNCaP-FGC and LNCaP-LNO cell lines were used to identify GC79 (21–23). The LNCaP-FGC cell line is identical to the LNCaP cell line provided by the American Type Culture Collection (Manassas, VA). LNCaP-FGC cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>–95% air and cultured in RPMI-1640 medium supplemented with penicillin (200 IU/mL), streptomycin (200 µg/mL), and 7.5% fetal calf serum. LNCaP-LNO cells originate from cultures of an early passage of the parental LNCaP cells and grow under the same conditions as the LNCaP-FGC cells, except that the medium contained 5% fetal calf serum depleted of steroids by treatment with dextran-coated charcoal (0.1% dextran and 1% charcoal), as described previously (22,23).

To study the effects of androgen and antiandrogen, we used the nonmetabolizable synthetic androgen R1881, 17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one (New England Nuclear, Boston, MA), and the nonsteroidal antiandrogen bicalutamide, (*RS*)-*N*-(4-cyan-3-[trifluoromethyl]phenyl)-3-(4-fluorophenylsulfonyl)-2-hydroxy-2-methylpropanamid (Zeneca Pharmaceuticals, Macclesfield, U.K.). LNCaP-FGC cells were grown in 80-cm<sup>2</sup> culture flasks until 80% confluent. Before hormones were added, medium containing fetal calf serum was exchanged for medium containing fetal calf serum treated with dextran-coated charcoal for 18–24 hours. R1881 (0.1 nM) alone or R1881 (0.1 nM) with 1 µM bicalutamide was then added to the cells. At various times, medium was discarded, and cells were snap-frozen at –80°C until isolation of total RNA.

For transfection studies, COS-1 or LNCaP prostate cancer cells were grown in six-well plates until 50% confluent. An ecdysone-inducible mammalian expression system (Invitrogen Corp., San Diego, CA) was used to study GC79 expression. The full-length GC79 cDNA was isolated from pBS79FL by digestion with *Bam*HI and *Eco*RI and cloned into pIND, digested with *Bam*HI and *Eco*RI. We sequenced the resulting plasmid pINDGC79 to verify that it carried the GC79 sequence and then used this plasmid for transfection studies. Transfections were performed with the Fugene 6 reagent (Boehringer Mannheim GmbH, Mannheim, Germany). Briefly, a total of 2 µg containing pINDGC79, pVgRXR, and pEGFP-C1 (1 : 1 : 1) was incubated for 15 minutes at room temperature with 100 µL of diluted Fugene 6

reagent (3 µL of Fugene 6 reagent preincubated with 97 µL of RPMI-1640 medium for 5 minutes at room temperature). The mixture was added to cells for 18–24 hours. Expression of GC79 was induced by addition of muristerone A (final concentration = 1 µM; Invitrogen Corp.) for 18–24 hours. To detect apoptotic cells, we stained nuclei *in situ* with a mixture of Hoechst 33342 (final concentration = 1 µg/mL) and propidium iodide (final concentration = 3.5 µg/mL) (Molecular Probes Inc., Eugene, OR) for 15–30 minutes and then viewed the nuclei with a fluorescent microscope (Zeiss, Jena, Germany).

### Morphologic Analysis of Apoptosis

To determine the fraction of apoptotic cells, we counted 300 transfected cells by fluorescent microscopy at a magnification of x40. Only the transfected cells are green because of constitutively expressed green fluorescent protein. Briefly, COS-1 or LNCaP cells were cotransfected with pINDGC79 (a plasmid that carries multiple ecdysone response elements in its promoter), pVgRXR (a plasmid that carries genes for constitutively expressed ecdysone and the retinoid X receptor), and pEGFP-C1 (a plasmid that carries a gene for constitutively expressed green fluorescent protein, to identify transfected cells). When muristerone A (an ecdysone analogue) is added to the transfected cells, formation of ecdysone–retinoid X receptor heterodimers occurs, and the dimers subsequently bind to the ecdysone response elements of pINDGC79, leading to the expression of GC79. Apoptotic cells are identified by the presence of blue, condensed, fragmented nuclei. The presence of apoptotic cells was also established independently by use of annexin V coupled to fluorescein isothiocyanate, which specifically binds to membranes of apoptotic cells. In the absence of muristerone A, cultures of transfected cells have substantially fewer apoptotic cells. In addition, when cultures of cells transfected with pIND (the empty plasmid), pVgRXR, and pEGFP-C1 were treated with muristerone A, no apoptotic cells were detected. The apoptotic index was determined by dividing the number of apoptotic transfected cells by the total number of normal transfected cells, and the result was expressed as a percentage [(number of apoptotic green cells/number of normal green cells) × 100].

### Animal Experiments

Young adult male RP Wistar rats (250–300 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), and were maintained under normal laboratory conditions. Animal protocols were in accord with the guidelines of the experimental animal health care center (EDC or Experimenteel Dier Centrum) of the Erasmus University Rotterdam, The Netherlands. Castration was performed by the abdominal route in rats under ether anesthesia; testes, fat pads, and epididymides were removed. The rats were killed by decapitation while under CO<sub>2</sub> anesthesia 0, 4, and 7 days after castration. The ventral prostate glands were removed rapidly, snap-frozen in liquid nitrogen, and stored at –80°C until isolation of total RNA.

### Molecular Cloning

The 255-base-pair (bp) GC79 fragment (nucleotides 3237–3492) obtained from the differential display RT-PCR analysis (22) was <sup>32</sup>P labeled and

used as probe. Screening a *Xba*I-(dT)<sub>15</sub> [an oligo(dT) primer including an *Xba*I restriction site]-primed human prostate 5′-STRETCH cDNA library λDR2 (Clontech Laboratories, Inc., Palo Alto, CA) yielded three positive clones. The largest clone (pDR2-79) had an insert of 1570 bp (nucleotides 2106–3676) that contained zinc-finger domains 6 and 7, a GATA-type zinc-finger domain, and the 255-bp sequence and ended with a *Xba*I restriction endonuclease site. Because of this *Xba*I site, another round of screening was performed with the 1570-bp fragment as probe on an oligo(dT)<sup>+</sup> random-primed human prostate 5′-STRETCH cDNA library λgt10 (Clontech Laboratories, Inc.). This second-round screening yielded six positive clones, and the largest overlapping clones (pCR2-79.5.3, nucleotides 1038–2173; pCR2-79.3′-4.3, nucleotides 3192–4541; and pCR2-79.3′, nucleotides 3389–5507) contained the sequence from zinc-finger domain 2 to a putative poly(A) tail (nucleotides 1038–5507). The most 5′ clone (pCR2-79.5.3, nucleotides 1038–2173) was used as probe for a third-round screening on λgt10, and this screening yielded 14 positive clones. One of these clones (pCR2B, nucleotides 1–2800) contains a putative ATG start codon, harboring a Kozak consensus sequence (26).

To facilitate cloning of full-length GC79 for further studies, we introduced a *Bam*HI site upstream of the start codon (nucleotides 174–179) by using the primer 5′-CGATGGATCCACAGATATGGTC-CGG-3′ (where the *Bam*HI site is underlined). PCR was performed with this primer, a GC79 primer (79t8r, nucleotides 806–829, 5′-GTCTGGGTTGT-CATTACCAG-3′), and pCR2B as template. The PCR product was subcloned by use of the TA (i.e., thymidine adenosine) cloning procedure (Invitrogen Corp.) and sequenced as follows: Briefly, the GC79 fragment was isolated by digestion with *Bam*HI and *Bsu*36I (nucleotides 174–615) and ligated into pCR2B, which had been digested with *Bam*HI (at a site located upstream of the introduced *Bam*HI site) and *Bsu*36I. The resulting plasmid was sequenced and designated pCR2E. When *Bam*HI and *Apa*I were used, a larger part of GC79 was isolated (nucleotides 174–2779) from pCR2E. The sequence that included the stop codon was isolated from the most 3′ clone (pCR2-79.3′, nucleotides 3389–5507). The GC79 fragment was isolated after digestion with *Apa*I (nucleotides 2776–2782) and *Eco*RI (nucleotides 4079–4085), whose restriction sites are located downstream of the stop codon (nucleotides 4029–4031). Both fragments were ligated to pBlue-script KS(+) (Stratagene, La Jolla, CA) that had been digested with *Bam*HI and *Eco*RI. The resulting plasmid now harbored the full-length open reading frame of GC79 (nucleotides 174–4085), as verified by sequencing, and was named pBS79FL.

### Northern Blot Analysis

For cell lines, total RNA was isolated by the urea/lithium chloride method as described previously (22). For rat ventral prostate glands, total RNA was isolated with a guanidine isothiocyanate-based micro-RNA isolation kit (Stratagene). Total RNA was subjected to electrophoresis in a 1.5% denatured agarose gel, blotted onto a nylon membrane (Hybond N; Amersham, Buckinghamshire, U.K.), and fixed to the membrane by use of a Stratalinker UV crosslinker (Stratagene). The 255-bp GC79 cDNA probe was purified from agarose gels with the

QIAEXII gel extraction kit (Qiagen, Chatsworth, CA) and labeled with deoxyadenosine 5'-[ $\alpha$ - $^{32}$ P]triphosphate (Amersham) by random primed labeling. The rat TRPM-2 probe was used to confirm apoptosis (8). Hybridization was performed overnight at 42°C ( $2\text{--}5 \times 10^6$  cpm/mL), and the blots were washed with 2 $\times$  standard saline citrate for 2 minutes, followed by a washing in 2 $\times$  standard saline citrate-0.25% sodium dodecyl sulfate for at least 5 minutes. Radioactive material was monitored with a Geiger-Müller counter. The blots were exposed to x-ray film (Hyperfilm MP; Amersham) at -80°C with intensifying screens for at least one night.

### Dot Blot Analysis

A human dot blot (Master blot; Clontech Laboratories, Inc.) was used that contained poly(A)<sup>+</sup> RNA (range = 89-514 ng/dot) isolated from 50 different normal human tissues obtained from individuals who died of trauma. The amounts of RNA on the blot were normalized against eight different housekeeping genes. Hybridization with a  $^{32}$ P-labeled 255-bp GC79 probe was performed according to the manufacturer's instructions. The blot was exposed to an x-ray film at -80°C with intensifying screens for at least one night or analyzed alternatively for several hours at room temperature with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

### Chromosomal Localization

The 255-bp GC79 fragment (22) was  $^{32}$ P labeled and used as a probe to screen a genomic human PAC (P1 phage artificial chromosome) library on gridded filters (Genome Systems, St. Louis, MO). Hybridization was performed according to the manufacturer's instructions. Genomic DNA was isolated from positive PAC clones according to the manufacturer's protocol and used for fluorescence *in situ* hybridization analysis (24). Briefly, genomic DNA was labeled with digoxigenin by use of a Dig-Nick kit (Boehringer Mannheim GmbH). Chromosome preparations were made from phytohemagglutinin-stimulated lymphocytes. The hybridization signal was visualized by incubation with anti-Dig-FITC (i.e., digoxigenin coupled to fluorescein isothiocyanate; Boehringer Mannheim GmbH). The slides were mounted in a Vecta Shield (Vector Laboratories, Inc., Burlingame, CA) containing 4',6'-diamino-2-phenylindole (Sigma Chemical Co., St. Louis, MO). Analysis was performed with a Leica DM-RXA microscope equipped with a PowerGene image analysis system (Perceptive Scientific Instruments, Inc., Chester, U.K.).

### Statistical Analysis

Data on apoptotic cell death are presented as the means and 95% confidence intervals. All statistical analyses were performed with two-tailed Student's *t* tests in the SPSS version 8.0 computer software package (SPSS, Inc., Chicago, IL). Data were considered to be statistically significantly different at  $P < .001$ .

## RESULTS

The overlapping GC79 cDNA clones yield a cDNA of 5507 bp. A homology search in the EMBL/GenBank databases was performed with the use of the

BLAST server (27). GC79 was found to be novel and encoded a protein of 1281 amino acids with a calculated molecular mass of approximately 141 kd. GC79 has nine C<sub>2</sub>H<sub>2</sub>-type zinc-finger motifs, a cysteine-rich region, and a C<sub>4</sub> GATA-type zinc-finger motif (Fig. 1).

Previously, we have shown that GC79 mRNA expression is repressed by androgen in a time-dependent and concentration-dependent fashion (22). Fig. 2, A, shows that androgen (0.1 nM R1881) repressed GC79 mRNA levels after 4 hours, whereas simultaneous addition of 1  $\mu$ M bicalutamide, an antiandrogen, reversed the repression (Fig. 2, B). Bicalutamide alone did not alter GC79 mRNA expression under androgen-deprived culture conditions (data not shown). These results strongly implicate the androgen receptor in the androgen regulation of GC79.

GC79 mRNA was ubiquitously expressed in adult tissues (Fig. 2, C). In fetal tissues, expression was lower than that in the corresponding adult tissues.

Castration-induced androgen withdrawal shows that, in rat ventral prostate, the levels of GC79 mRNA were increased at 4 days and decreased at 7 days after castration (Fig. 2, D). During this period, apoptotic cell death is assumed to be induced, as illustrated by a similar pattern of mRNA from the apoptotic-associated gene TRPM-2 (Fig. 2, E). These results show that increased GC79 mRNA is directly associated with induction of apoptosis of the rat ventral prostate.

GC79 was cloned in an inducible vector and transfected in both COS-1 cells (Fig. 3, A-E) and LNCaP cells (Fig. 3, F-J). In Fig. 3, A, blue intact nuclei and blue condensed fragmented nuclei are shown, representing apoptotic cells. In Fig. 3, B, expression of green fluorescent protein, cotransfected with GC79 in the same cells, is shown. These results demonstrate that expression of GC79 is associated with apoptosis. Cultures of noninduced transfected cells have fewer apoptotic cells (Fig. 3, C and D). Fig. 3, E, illustrates that cells induced to express GC79 had an eightfold greater apoptotic index (two-sided,  $P < .001$ ) than uninduced cells. Fig. 3, F-J, shows similar results for LNCaP cells.

The chromosomal location of the GC79 gene was 8q23-24.1 (Fig. 4).

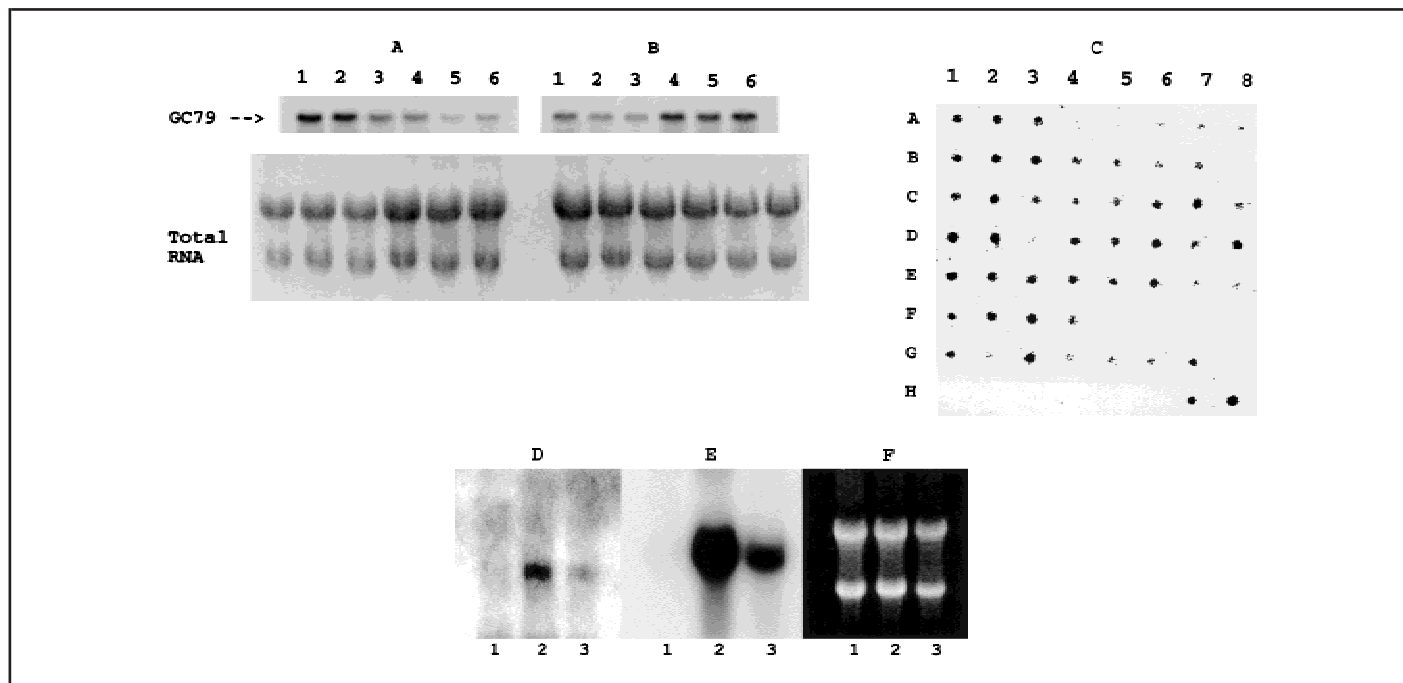
## DISCUSSION

Molecular cloning analysis showed that GC79 is a complex and multitype

zinc-finger protein. Many proteins containing zinc-finger structures have been reported to be involved in oncogenesis and growth regulation (28-31). Because GC79 has two or three different types of zinc-finger structures, we speculate that GC79 is a unique novel transcription factor. With the use of different probes located throughout the full-length GC79 cDNA, a single hybridization signal of approximately 6 kilobases was observed on northern blots. This finding demonstrates that GC79 is not a fusion product. To our knowledge, a protein that contains both C<sub>2</sub>H<sub>2</sub> and GATA types of zinc-finger domains has not been described before. Thus, GC79 might be the first member of a novel class of zinc-finger transcription factors. The GATA domain of GC79 has 77% homology with the C-terminal GATA domain of the superfamily of GATA transcription factors, GATA-1 to GATA-6 (29,30). These transcription factors bind DNA sequences with the (A/T)GATA(A/G) motif. All six vertebrate members of the GATA family contain two C<sub>4</sub> zinc-finger domains, whereas GC79 only has a single C<sub>4</sub> zinc-finger domain. The two C<sub>4</sub> zinc-finger domains of the GATA family are 50% homologous with each other (29,30). The GATA transcription factors are involved in cell proliferation, differentiation, and regulation of genes (29,30), which indicates that GC79 may also be involved in these activities. For example, the GATA domain of GC79 could bind to the DNA consensus sequence (A/T)GATA(A/G) and thus regulate gene expression.

We have shown previously (22) that the expression of GC79 mRNA in LNCaP-FGC cells is repressed by androgen (0.1 nM R1881) in a time-dependent and concentration-dependent manner. Repression was relatively fast (after 4 hours) and was observed at a physiologic concentration (0.1 nM R1881). Fig. 2 shows that an antiandrogen (bicalutamide) is able to reverse the effect of androgen (R1881) and indicates that the androgen repression activity of GC79 is mediated by the androgen receptor. It is not known whether GC79 binds to the androgen receptor. It is interesting to note that, when a steroid (estrogen) binds to its receptor (estrogen receptor), the activity of a GATA-binding protein (GATA-1) is repressed (32). This estrogen-mediated repression could be reversed by an anti-steroid (4-hydroxytamoxifen), which suggests involvement of the estrogen recep-





**Fig. 2.** Analysis of GC79 messenger RNA (mRNA) expression. **Panels A and B:** regulation in androgen-dependent human LNCaP-FGC prostate cancer cells by R1881. LNCaP-FGC prostate cancer cells were cultured with 0.1 nM R1881 in the absence or presence of 1  $\mu$ M bicalutamide. Total RNA was isolated at various times, and 20  $\mu$ g was loaded per lane of a 1.5% denatured agarose gel, subjected to electrophoresis, blotted onto nylon filters, and hybridized with the  $^{32}$ P-labeled 255-base-pair GC79 probe. **Each lane** contains an equal amount of total RNA, as measured by absorbance at 260 nm and ethidium bromide staining. In **panel A**, 0.1 nM R1881 was used. In **panel B**, 0.1 nM R1881 with 1  $\mu$ M bicalutamide was used. **Lanes 1** = 0 hour, **lanes 2** = 1 hour, **lanes 3** = 2 hours, **lanes 4** = 4 hours, **lanes 5** = 6 hours, and **lanes 6** = 8 hours. **Panel C:** expression of GC79 in different human tissues. A dot blot containing poly(A)<sup>+</sup> RNA (range = 89–514 ng/dot) from 50 different normalized human tissues was hybridized with the  $^{32}$ P-labeled 255-base-pair GC79 probe. **Row A** is as follows: **1** = whole brain, **2** = amygdala, **3** = caudate nucleus, **4** = cerebellum, **5** = cerebral cortex, **6** = frontal lobe, **7** = hippocampus, and **8** = medulla oblongata. **Row B** is as follows: **1** = occipital lobe, **2** = putamen, **3** = substantia nigra, **4** = temporal lobe, **5** = thalamus, **6** = subthalamic nucleus, and **7** = spinal cord. **Row C** is as follows: **1** = heart, **2** = aorta, **3** = skeletal muscle, **4** = colon, **5** = bladder, **6** = uterus, **7** = prostate, and **8** = stomach. **Row D** is as follows: **1** = testis, **2** = ovary, **3** = pancreas, **4** = pituitary gland, **5** = adrenal gland, **6** = thyroid gland, **7** = salivary gland, and **8** = mammary gland. **Row E** is as follows: **1** = kidney, **2** = liver, **3** = small intestine, **4** = spleen,

**5** = thymus, **6** = peripheral leukocyte, **7** = lymph node, and **8** = bone marrow. **Row F** is as follows: **1** = appendix, **2** = lung, **3** = trachea, and **4** = placenta. **Row G** is as follows: **1** = fetal brain, **2** = fetal heart, **3** = fetal kidney, **4** = fetal liver, **5** = fetal spleen, **6** = fetal thymus, and **7** = fetal lung. **Row H** is as follows: **1** = yeast total RNA (100 ng), **2** = yeast transfer RNA (100 ng), **3** = *Escherichia coli* ribosomal RNA (100 ng), **4** = *E. coli* DNA (100 ng), **5** = poly[r(A)] (100 ng), **6** = human C<sub>ot</sub> 1 DNA (genomic DNA enriched for repetitive sequences) (100 ng), **7** = human genomic DNA (100 ng), and **8** = human genomic DNA (500 ng). **Panels D–F:** expression of GC79 in the rat ventral prostate gland undergoing castration-induced apoptosis. Adult male RP Wistar rats were castrated, and total RNA was isolated from the regressing ventral prostate glands at various times as indicated. With the use of a 1.5% denatured agarose gel, 20  $\mu$ g of total RNA was loaded per lane, subjected to electrophoresis, blotted onto nylon filters, and hybridized with the  $^{32}$ P-labeled 255-base-pair GC79 probe or a probe for the apoptosis-associated rat TRPM-2 gene (9). It should be noted that rat GC79 mRNA has the same size as human GC79 mRNA (data not shown). Each lane contains an equal amount of total RNA as measured by absorbance at 260 nm and ethidium bromide staining. **Panel D** shows GC79. **Panel E** shows TRPM-2. **Panel F** shows ethidium bromide staining of total RNA. **Lanes 1** = normal intact ventral prostate gland, **lanes 2** = 4-day castration ventral prostate gland, and **lanes 3** = 7-day castration ventral prostate gland.

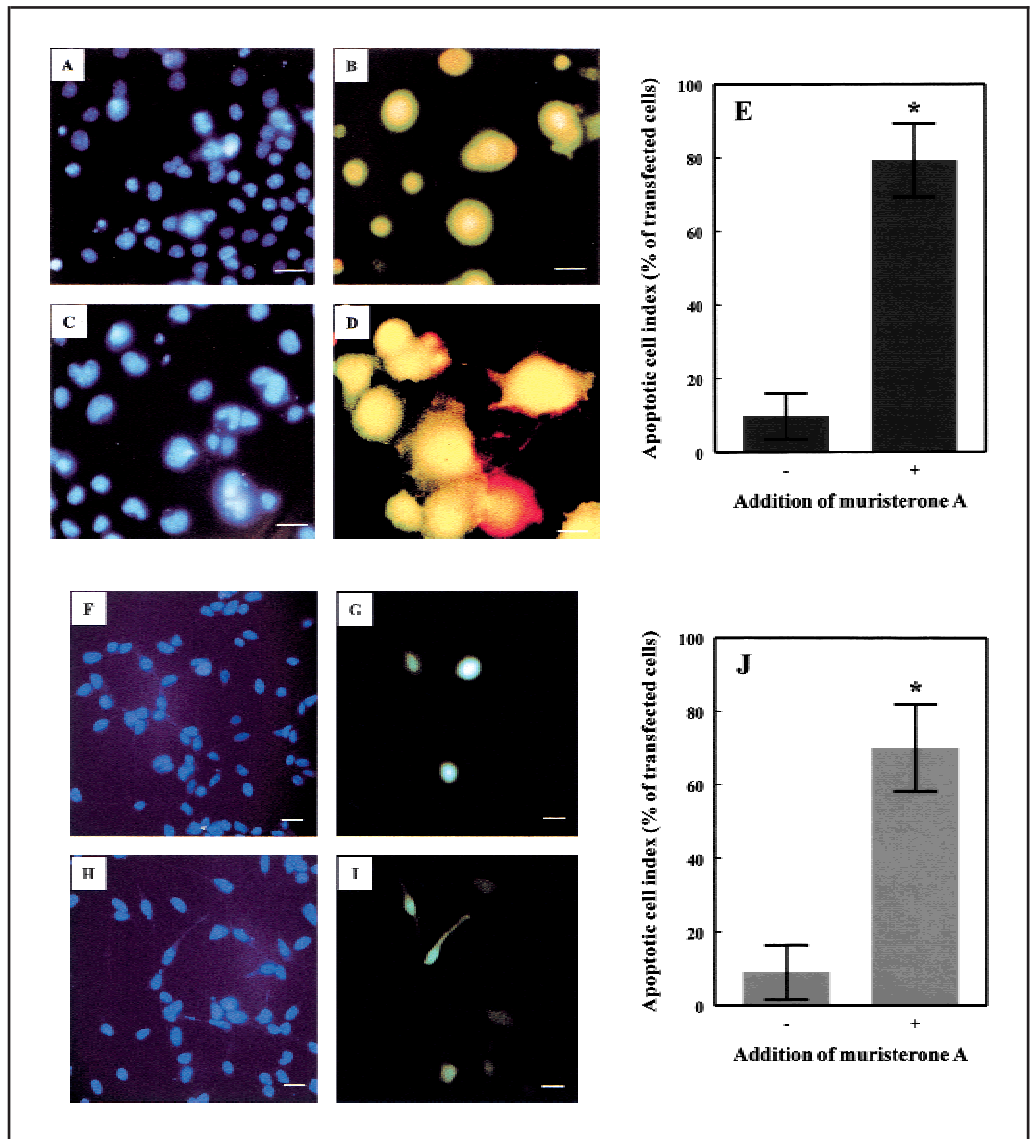
tor. Furthermore, protein–protein binding between the estrogen receptor and GATA-1 *in vitro* was reported (32). Whether these observations are applicable to GC79 and the androgen receptor is speculative as yet. Alternatively, androgen repression activity of GC79 involving the androgen receptor is indicative of the presence of a negative hormonal responsive element in the GC79 promoter. To our knowledge, the only negative hormonal responsive element recognized by the androgen receptor is in maspin, a tumor-suppressing serine protease inhibitor (33). Molecular cloning, sequencing, and functional analysis of the promoter region of GC79 could identify regulatory

elements involved in GC79 gene expression.

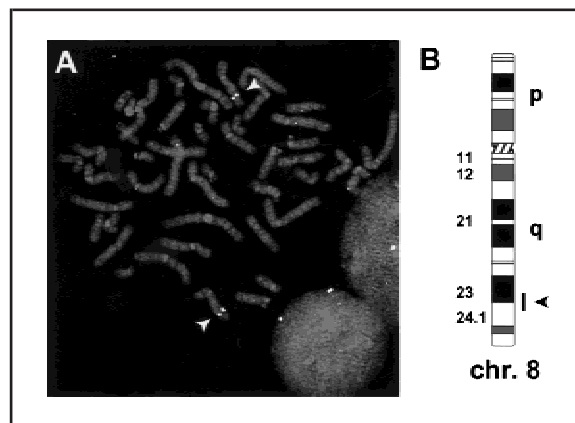
The tissue distribution of GC79 mRNA expression was studied with an RNA dot blot containing RNAs from multiple human tissues. Expression of GC79 was found to be high in the prostate, testis, ovary, kidney, lung, and mammary gland. Lower levels of expression were found in the liver, colon, heart, uterus, and brain. It is interesting that in fetal tissues (kidney, lung, liver, heart, and brain) GC79 expression was lower than that in the corresponding adult tissues. This result could suggest that during adulthood GC79 might be involved in growth suppression of these

tissues. GATA-4, GATA-5, and GATA-6 have been described in developing heart and gut (34), and GATA-2 has been reported to have a vital role during urogenital development (35). Recently, it has been reported that GATA transcription factors are involved in gonadal development. For example, GATA-4 and GATA-6 are expressed and hormonally regulated in mouse ovary (36) and in mouse testis (37). Furthermore, GATA-4 is expressed during early gonadal development and sexual differentiation of mouse gonads (38). GATA-4 is a potent activator of the promoter for Müllerian inhibiting substance (38). Potential GATA-binding sites have also been iden-

**Fig. 3.** GC79 expression is associated with apoptosis in COS-1 (panels A–E) and LNCaP prostate cancer (panels F–J) cells. GC79 complementary DNA was ligated in the ecdysone-inducible expression vector and transiently cotransfected with ecdysone receptor, retinoic acid X receptor, and green fluorescent protein. GC79 expression was induced by addition of muristerone A, an ecdysone analogue. Panels A, C, F, and H: Hoechst 33342–propidium iodide staining of nuclei. Panels B, D, G, and I: GC79 expression, colocalized with green fluorescent protein. After transfection, cells were treated with (A, B, F, and G) or without (C, D, H, and I) 1  $\mu$ M muristerone A for 18–24 hours to induce GC79 expression. Panels E and J: apoptotic index ([number of apoptotic transfected cells/number of normal transfected cells]  $\times$  100). The percentage of apoptotic cells was determined by scoring 300 transfected cells. Values are expressed as the means and 95% confidence intervals of three independent experiments carried out in triplicate. \* $P$ <.001 as assessed by two-tailed Student's  $t$  test. Scale bars = 20  $\mu$ m.



**Fig. 4.** Chromosomal localization of GC79. Southern blot analysis using the  $^{32}$ P-labeled 255-base-pair GC79 probe on LNCaP-FGC genomic DNA, digested with *Hind*III, *Eco*RI, or *Bam*HI, demonstrated that GC79 is encoded by a single copy gene (data not shown). Screening of a human genomic PAC (P1 phage artificial chromosome) library yielded three positive clones (62P9, 136H8, and 212M23). Genomic DNA was isolated from PAC clone 212M23, verified by polymerase chain reaction analysis, and used as probe. Panel A: *in situ* hybridization of digoxigenin-labeled GC79 PAC clone 212M23 on



a metaphase spread of human chromosomes derived from phytohemagglutinin-stimulated lymphocytes. Specific labeling was observed at 8q23–24.1 (arrowheads). Panel B: a representative idiogram showing the 8q23–24.1 region. Images were obtained with a Leica DM-RXA microscope equipped with a PowerGene analysis system.

identified in the promoter of Müllerian inhibiting substance type II receptor (39). GATA-1 and GATA-4 can activate the promoter of the gonadal gene inhibin  $\alpha$

(40). Because GC79 seems to be differentially expressed in normal and fetal tissues, GC79 might be involved in urogenital or gonadal development.

During androgen ablation therapy (e.g., antiandrogen treatment or castration), when androgens are repressed, it is hypothesized that apoptosis occurs in both the normal and the malignant human prostates as it does in the normal rat ventral prostate (4,5). Because we observed that expression of GC79 is high when androgen levels are low in LNCaP-FGC cells, we hypothesize that GC79 might be involved in the arrest of cell growth and/or apoptotic cell death. So that we could test this hypothesis *in vivo*, rats were castrated and GC79 mRNA expression in the regressing rat ventral prostate gland was measured. GC79 mRNA increased 4 days after castration and remained high until 7 days after castration. The rat apoptotic marker TRPM-2 (9) has a similar expression pattern, indicating that apoptotic cell death of the rat ventral prostate gland has been induced. These results demonstrate that GC79 mRNA expression coincides

with apoptotic cell death in the rat ventral prostate gland and suggests that GC79 is involved in apoptosis of normal androgen-dependent prostate epithelial cells, present in the rat ventral prostate gland. Whether the GC79 protein is present in the secretory epithelium of the involuting rat ventral prostate is under investigation. Because the function of endogenous GC79 is still unknown, we are currently studying whether inhibition of GC79 prevents castration-induced apoptosis in the rat ventral prostate. The involvement of GC79 in the apoptotic process of the mammalian cell lines COS-1 and LNCaP was shown in transient transfection studies with the use of the ecdysone-inducible mammalian expression system. Induction of GC79 expression with muristerone A in GC79-transfected cells resulted in the appearance of many apoptotic cells with condensed fragmented nuclei. This result indicates that GC79 belongs to a class of androgen-repressed genes that are associated with or involved in apoptosis (9–13). Transforming growth factor- $\beta$  (11) and prostate apoptosis response-4 (12) have been reported to be expressed during castration-induced regression of the prostate. The c-myc gene, which can be repressed by androgen (13), has also been reported to be involved in apoptosis (41,42). It is interesting that c-myc is localized to human chromosome 8q24, whereas we mapped GC79 to human chromosome 8q23–24.1. Whether there is a link between expression of GC79 and c-myc is an intriguing question. It would be interesting to determine whether GC79 and c-myc share similar or different apoptotic pathways. It has been reported that elevated levels of c-myc were observed in prostate carcinomas (43,44) and that amplification of c-myc was present only in a subset of tumors with an 8q gain (44). Recently, it was reported (45) that an 8q gain (as measured by c-myc) is associated with progression of prostate carcinomas. Identification and functional characterization of genes on the 8q arm are important and will extend our current knowledge of the involvement of chromosome 8q in prostate cancer progression.

In conclusion, we have cloned GC79, a gene with potential tumor growth-suppressing activity that is associated with apoptosis. GC79 is a zinc-finger protein and can be repressed by physiologic concentrations of androgen in optimally growing androgen-dependent prostate cancer cells. Androgen withdrawal leads

to an increase in GC79 and subsequent cell growth arrest, followed by apoptosis. GC79 is a gene that is potentially involved in prostate cancer apoptosis and may be important in the treatment of prostate cancer.

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## NOTES

*Note added in proof:* While this report was in review, a paper was published (51) that describes a novel gene, TRPS1, that is identical to GC79.

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