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Differentially Expressed Genes in Androgen-dependent and -independent Prostate Carcinomas[1]

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ABSTRACT

Differential gene expression between androgen-dependent (LNCaP-FGC) and androgen-independent (LNCaP-LNO) prostate cancer cells has been investigated using RNA arbitrarily primed and differential display PCR of mRNA. Four differentially expressed cDNA transcripts were identified, of which differential expression was confirmed by Northern blot analysis. Sequence analysis revealed two unknown (*JC19* and *GC79*) and two known genes [*B-cell translocation gene 1* and *UDP-glucuronosyltransferase 2B15* (*UGT2B15*)]. *JC19, GC79,* and *B-cell translocation gene 1* were more highly expressed in LNCaP-FGC cells compared with LNCaP-LNO cells, whereas *UGT2B15* was only expressed in LNCaP-LNO cells. Androgens and 1,25-dihydroxyvitamin D_3 were able to down-regulate *UGT2B15* mRNA in LNCaP-LNO cells. For *GC79* mRNA, down-regulation was only observed with androgens in LNCaP-FGC cells. Expression of *JC19* mRNA was studied using a panel of human prostate cancer xenografts. In androgen-dependent xenografts, expression of *JC19* mRNA was much higher compared with androgen-independent xenografts, in which significant expression was hardly detectable. The mRNA expression pattern in the xenografts is in good agreement with that observed in the cell culture system. In conclusion, the differential display technique used in the present study allows analysis of gene expression *in vitro* and *in vivo* and can be used for the identification of important genes involved in androgen-independent prostate cancer development.

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths and the most commonly diagnosed cancer in men in the Western world (1, 2). Treatment of advanced prostate cancer aims at inhibiting cancer growth by suppression of endogenous androgen action or production (3). However, almost all tumors will eventually progress, reflected by the growth of androgen-independent cells and the development of hormone-refractory disease. The background of this clinical phenomenon is poorly understood (4, 5). The transition from hormone-dependent to hormone-independent tumorigenesis is supposed to be a cascade of genetic alterations caused by activation of oncogenes and/or inactivation of tumor suppressor genes (6, 7). For example, high levels of the proto-oncogene *bcl-2* have been associated with androgen-independent prostate cancer (8). Recently, two novel candidate oncogenes (*PTI-1* and *HPC1*) have been reported to be involved in prostate cancer. The *PTI-1* gene, *prostate tumor inducing gene 1,* was shown to be expressed in prostate cancer and not in normal prostate tissue (9). The *HPC1* gene, *hereditary prostate cancer gene 1,* is probably involved in the familial form of prostate cancer and is localized on the long arm of chromosome 1 (10). The function of both genes and their encoded proteins is not known.

The involvement of genetic changes in tumor suppressor genes (*e.g.*, the *retinoblastoma* gene and the *p53* gene) in prostate cancer has been documented (11–16). However, the role of *p53* mutations in prostate cancer is still uncertain (17). Recently, three novel candidate tumor suppressor genes (*Mxi1, maspin,* and *KAI1*) have been reported to be involved in prostate cancer. *Mxi1,* a suspected repressor of the c-*myc* oncogene, has been reported to be mutated in 40% of primary prostate tumors (18, 19). *Maspin* was shown to inhibit prostatic cancer motility and invasion (20). *KAI1* was shown to have metastasis-suppressing activity in prostate cancer and is localized on chromosome 11p11.2 (21).

Novel molecular techniques (loss of heterozygosity and comparative genomic hybridization) have been developed to study chromosomal alterations. Allelotyping analysis or loss of heterozygosity has shown that the majority of prostate cancer patients had a deletion in one or both alleles of either chromosome 8p, 10q, or 16q (22–25). Comparative genomic hybridization detects loss or gain of individual chromosomes. It was shown that in prostate cancer, patient losses of either chromosome 8p or 13q are the most common chromosomal changes (26). Comparison between androgen-dependent and androgen-independent prostate cancer tissues showed very similar losses or gains of chromosomes, suggesting that the bulk of chromosomal alterations occurred during androgen-dependent prostate cancer growth (26, 27). Minor genetic differences are more likely to be involved in the transition to androgen-independent growth. Finding these minor genetic differences between androgen-dependent and androgen-independent prostate cancer is a challenge. Genes involved in these differences can be cloned and characterized to study their role in androgen-independent prostate cancer development. Horoszewics and coworkers (28, 29) have isolated an androgen-dependent cell line, the LNCaP[4] cell line, which was derived from a metastatic lesion of a prostate carcinoma. Several LNCaP sublines have been isolated and characterized (28–30), and two closely related cell lines, an androgen-dependent (LNCaP-FGC; Refs. 28 and 29) and an androgen-independent (LNCaP-LNO; Ref. 30) subline, offer unique possibilities to study gene expression and regulation. The cells can be grown under strictly controlled culture conditions and allow studying the effects of androgens.

An elegant method of identifying novel genes important for prostate cancer development is to compare mRNA populations from the LNCaP sublines grown under different hormonal conditions. This comparison can be made by RNA fingerprinting (31, 32). The basic principle of this method is to display a cDNA representation of a subset of mRNAs with the aid of reverse transcriptase and PCR. Different subpopulations can be detected and displayed on a gel by using various sets of arbitrary primers. By comparing cDNA popula-

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[4] The abbreviations used are: LNCaP, lymph node carcinoma of the prostate; FGC, fast-growing colony; LNO, lymph node original; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DCC, dextran-coated charcoal; EST, expressed sequence tag; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; *BTG1*, B-cell translocation gene 1; *UGT2B15*, UDP-glucuronosyltransferase 2B15; PSA, prostate-specific antigen.

tions from different prostate cancer cells, it should be possible to identify cDNAs related to an androgen-dependent and androgen-independent phenotype at the same time.

Applying this method to the LNCaP sublines, we have cloned several cDNA transcripts and identified two unknown and two known genes. Three of these genes were not reported previously in relation to advanced prostate cancer development.

MATERIALS AND METHODS

Materials. The nonmetabolizable synthetic androgen R1881 (17α-methyltrienolone) was purchased from DuPont NEN (Boston, MA). The biological active vitamin D metabolite 1,25-$(OH)_2D_3$ was a gift from Dr. L. Binderup of LEO Pharmaceutical Products (Ballerup, Denmark) and was donated by the department of Internal Medicine, Erasmus University (Rotterdam, the Netherlands). Cell culture flasks and disposables were obtained from Costar (Cambridge, MA). RPMI 1640 and culture chemicals were from Life Technologies, Inc. (Breda, the Netherlands). Lovastatin was a gift from Dr. Y. Chao of Merck Research Laboratories (Rahway, NJ). Mevalonic acid was from Sigma Chemical Co. (St. Louis, MO). Restriction and modifying enzymes were from Boehringer Mannheim GmbH (Mannheim, Germany). Nucleotides, one-base anchored oligo-dT primer ET12G (5′-GCGAATTCGGTTTTTTTTTTTTG-3′), and arbitrary primers SLtk3 (5′-CGATGTCGACCTTGATTGCC-3′), HB15 (5′-GCAAGCTTGCGATCCAGTAC-3′), and HB21 (5′-GCAAGCTT-GCGATCTAACCG-3′) were from Pharmacia Biotech (Roosendaal, the Netherlands). Arbitrary primer HB13 (5′-GCAAGCTTGCGTTTTCGCAG-3′), Moloney murine leukemia reverse transcriptase, DTT, and 5× first-strand buffer were from Life Technologies, Inc. Super*Taq* DNA polymerase and 10× PCR buffer were from HT Biotechnology, Ltd. (Cambridge, United Kingdom). [α-^{32}P]dATP and [α-^{33}P]dATP were from Amersham (Buckinghamshire, United Kingdom). Human GAPDH cDNA plasmid was from Clontech (Palo Alto, CA). All other reagents were of the highest grade available.

Cell Culture. The LNCaP-FGC and LNCaP-LNO cell lines were gifts from Dr. J. S. Horoszewics (Buffalo, NY). The LNCaP-FGC cell line is identical to the LNCaP cell line provided by the American Type Culture Collection (Rockville, MD). The LNCaP-FGC cells were maintained at 37°C in a humidified incubator containing 5% CO_2/95% air in RPMI 1640 supplemented with 200 IU/ml penicillin, 200 μg/ml streptomycin, and 7.5% heat-inactivated FCS. The LNCaP-LNO cells originate from cultures of an early passage (6th) of the parental LNCaP cells and grow under the same conditions as the LNCaP-FGC cells, except that the medium contained 5% FCS depleted of steroids by DCC treatment using 0.1% dextran and 1% charcoal as described (30).

For hormone deprivation experiments, the cells were seeded in 24-well culture plates at a density of 2×10^4 cells/well. After 1 day, the medium was replaced by medium containing 5% DCC-treated FCS. Variable concentrations of R1881 or 1,25-$(OH)_2D_3$ were added, as was also a mixture of 10^{-8} M R1881 and 10^{-7} M 1,25-$(OH)_2D_3$. Cells were cultured for 6 days to near confluency, with a change of medium after 3 days. Growth of the cells was determined as the amount of DNA per well, according to the fluorometric method reported by Hinegardner (33). Briefly, cells were washed with PBS, lysed with 1 M NaOH, and subsequently neutralized with 1 M HCl. Diaminobenzoic acid dihydrochloride (1.5 M) was added, followed by 1 M HCl. The amount of fluorescence was measured in a spectrofluorometer (Kontron, Tokyo, Japan) using excitation of 415 nm and emission of 500 nm. As standard, herring sperm DNA was used. In parallel culture flasks (80 cm^2), total RNA was isolated for differential display (31, 32).

For the R1881 and 1,25-$(OH)_2D_3$ time course experiments, cells were seeded in culture flasks (80 cm^2) and cultured to 30–40% confluency. The medium was replaced by RPMI 1640 DCC, and after 1 day, hormones were added and cells were cultured for appropriate times. Total RNA was isolated and used in the Northern blot analysis.

The cell cycle experiments were performed by synchronization of FGC cells. Cells were synchronized with lovastatin, a drug that inhibits the activity of 3-hydroxy-3-methylglutaryl CoA reductase, a key enzyme in the cholesterol biosynthetic pathway (34, 35). To release cells from this inhibition, mevalonic acid was added to the cells. Synchronization conditions were performed as described previously (36). Briefly, the cells were cultured in RPMI 1640 containing 10 μM lovastatin for 36 h to reach growth arrest in the G_0-G_1 stage of the cell cycle. The cells were allowed to enter into the cell cycle by the addition of 2 mM mevalonic acid. The cells were cultured for different time periods, and total RNA was isolated.

Fibroblasts (genital skin fibroblasts) were isolated from human genital skin and cultured in Eagle's MEM supplemented with nonessential amino acids. The commercially available cell lines HEK293 (human embryonic kidney), T47D (breast carcinoma), HeLa (cervix carcinoma), SnuC5 and SnuC1 (colon carcinoma), IMR32 (neuroblastoma), HEPG2 (hepatoma), T24 (vesicle carcinoma), and COS1 (SV40-transformed CV1 monkey kidney) were cultured in their appropriate media as recommended by the American Type Culture Collection.

RNA Arbitrarily Primed and Differential Display PCR of mRNA. Total RNA from 6 days of FGC and LNO cell cultures was isolated as described (37). Differential display was performed with modifications as described (31, 32). cDNA was synthesized using one-base anchored oligo-dT primer (38) or a single arbitrary primer (31, 39). Total RNA (2 μg) was reverse transcribed using 2 μM oligo-dT or arbitrary primer, 20 μM deoxynucleotide triphosphate, 10 mM DTT, and 200 units Moloney-murine leukemia virus reverse transcriptase (5 min at 65°C; 60 min at 37°C; 5 min at 95°C). PCR was performed by amplifying 10% of the cDNA mixture with 0.4 μM oligo-dT or arbitrary primer, 0.4 μM arbitrary primer, 20 μM deoxynucleotide triphosphate, 1 μCi [α-^{33}P]dATP, and 0.1 unit Super*Taq* DNA polymerase. Thermocycle reactions were performed in a Biometra Trio-Thermoblock (Tampa, FL) according to Zhao *et al.* (40). One cycle of 1 min at 94°C, 4 min at 40°C, and 1 min at 72°C and 35 cycles of 45 s at 94°C, 2 min at 60°C, and 1 min at 72°C were performed, followed by an elongation step of 5 min at 72°C. The PCR products were separated on a 6% denaturing polyacrylamide gel in the presence of 8 M urea and electrophoresed until the dye xylene cyanol reached the bottom of the gel. The gel was dried without fixation and exposed to an X-ray film (Fuji RX, Tokyo, Japan) at room temperature for 18 h. Differentially expressed bands were excised from the gel and eluted with 100 μl of distilled water for 10 min at room temperature and for 20 min at 100°C. The eluted DNA was precipitated with ethanol and dissolved in 10 μl of distilled water. Reamplification was performed using 40% of the eluate and 35 cycles of 1 min at 94°C, 2 min at 60°C, and 1 min at 72°C were performed, followed by a 5-min, 72°C elongation. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide (0.5 μg/ml). The PCR products were ligated into a pCRII vector using the TA cloning system (Invitrogen Corp., San Diego, CA) and transformed in *Escherichia coli* DH5α competent cells using standard molecular biological techniques (41).

Northern Blot Analysis. Total RNA was extracted by lysing the cells or homogenizing the tissues with 3 M lithium chloride/6 M urea according to Auffray and Rougeon (37). Electrophoresis of total RNA was performed using a 1.5% denatured agarose gel. RNA was blotted onto a nylon membrane (Hybond N; Amersham) and fixed to the membrane using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). The cDNA probes were purified from agarose gels using the QIAEXII gel extraction kit (Qiagen, Chatsworth, CA) and labeled with [α-^{32}P]dATP by random-primed labeling (42). Hybridization was performed overnight at 42°C (2–5 × 10^6 cpm/ml), and the blots were washed with 2× SSC for 2 min, followed by 2× SSC/0.25% SDS for at least 5 min, while monitoring using a Geiger-Müller counter (41). The blots were exposed to an X-ray film (Kodak X-Omat, New Haven, CT) at −80°C with intensifying screens for at least 1 night or alternatively for 3–6 h at room temperature using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The cDNA probes on the blots were stripped by incubating the blots for 10 min in distilled water of 95°C. The blots were washed with distilled water, and the GAPDH probe was hybridized on the blots to verify equal loading of total RNA and the efficiency of the blotting procedure.

Human Prostate Cancer Xenografts. The transplantation of prostate tumor tissues is performed routinely by implanting small freshly isolated tumor tissue fragments s.c. into both shoulders of an intact or testosterone-supplemented male nude mouse of the NMRI background as described (43, 44). The tumor specimens were obtained from primary carcinomas (radical prostatectomies), metastatic lesions (pelvic lymph nodes and scrotal skin), and transurethral resection material of the prostate. The prostate cancer xenograft panel consists of four androgen-dependent and five androgen-independent tumors. The tumors retained their resemblance to the original patient material, and their main characteristics have been described recently (43, 44). Having reached the

exponential growth phase, tumors were dissected from the surrounding tissues, snap frozen in liquid nitrogen, and stored at −80°C until isolation of total RNA.

RESULTS

Differential Gene Expression between LNCaP-FGC and LNCaP-LNO Cells. Two closely related LNCaP cell lines were used to isolate genes involved in androgen-independent prostate cancer development. To rule out the possibility of isolating genes only involved in the growth process itself, conditions were chosen where both cell lines grew either at equal rates or were both arrested in their growth. The FGC cell line is androgen dependent and had an optimal growth rate at 10^{-10} M R1881, whereas higher concentrations did not stimulate growth at all (Fig. 1*A*). In DCC (steroid-free) medium, the FGC cells stopped growing but were still viable, because the addition of 10^{-10} M R1881 afterwards could still stimulate growth (data not shown). None of the 1,25-$(OH)_2D_3$ concentrations tested stimulated growth. Stimulation of growth was also not observed by the combined addition of 10^{-8} M R1881 and 10^{-7} M 1,25-$(OH)_2D_3$ (data not shown). The LNO cell line is androgen independent, and growth was slightly inhibited with concentrations higher than 10^{-8} M R1881 (Fig. 1*B*). A similar growth pattern was observed for 1,25-$(OH)_2D_3$. A combination of 10^{-7} M 1,25-$(OH)_2D_3$ and 10^{-8} M R1881 inhibits the growth almost completely (results not shown). 1,25-$(OH)_2D_3$ served as a tool to create a condition where both cells lines were arrested in their growth. Differential display was performed using cell culture conditions where both cell lines grew maximally or were arrested in their growth: 0, 10^{-10} M R1881, 10^{-8} M R1881, 10^{-7} M 1,25-$(OH)_2D_3$, and a combination of 10^{-7} M 1,25-$(OH)_2D_3$ and 10^{-8} M R1881, respectively. Fig. 2 gives an example of a typical experiment and shows a band exclusively expressed in LNO cells. The relevant differentially expressed fragments were isolated and cloned. Northern blot analysis confirmed four clones to be differentially expressed (Table 1 and Fig. 3). *JC19*, *GC79*, and *BTG1* mRNA were highly expressed in FGC cells, whereas *UGT2B15* mRNA was only expressed in LNO cells. Next, the four clones were used to determine the mRNA expression levels in several established cell lines (Table 2). For *JC19*, the highest expression level was detected in the FGC cell line, whereas the other cell lines showed lower or no expression. *GC79* was more highly expressed in the FGC and T47D cell lines compared with the other cell lines. Expression of *UGT2B15* was limited to the LNO cell line. *BTG1* was ubiquitously expressed.

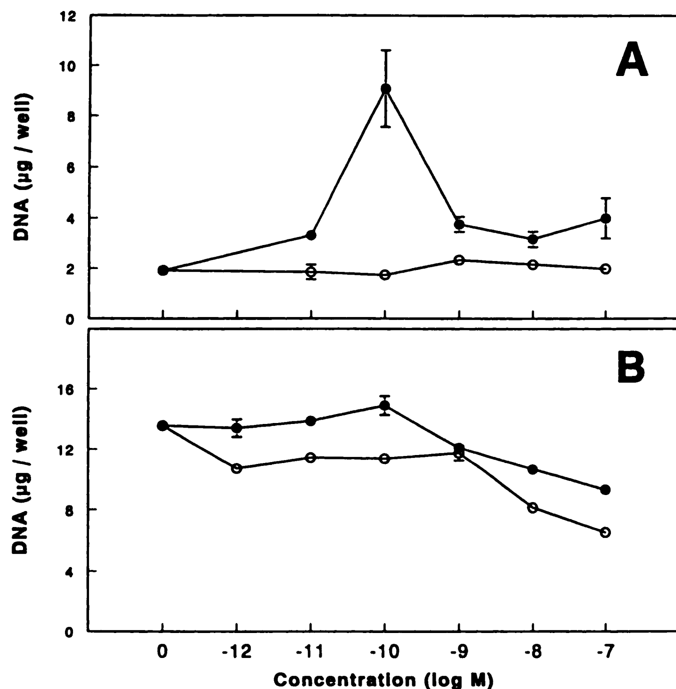

Fig. 1. The effects of R1881 (●) and 1,25-$(OH)_2D_3$ (○) on the growth of LNCaP-FGC (*A*) and LNCaP-LNO (*B*) prostate cancer cells. Various concentrations of hormones were added as indicated. The cells were cultured for 6 days in 24-well culture plates, and the DNA content was determined. The means of four measurements are shown; *bars*, SD.

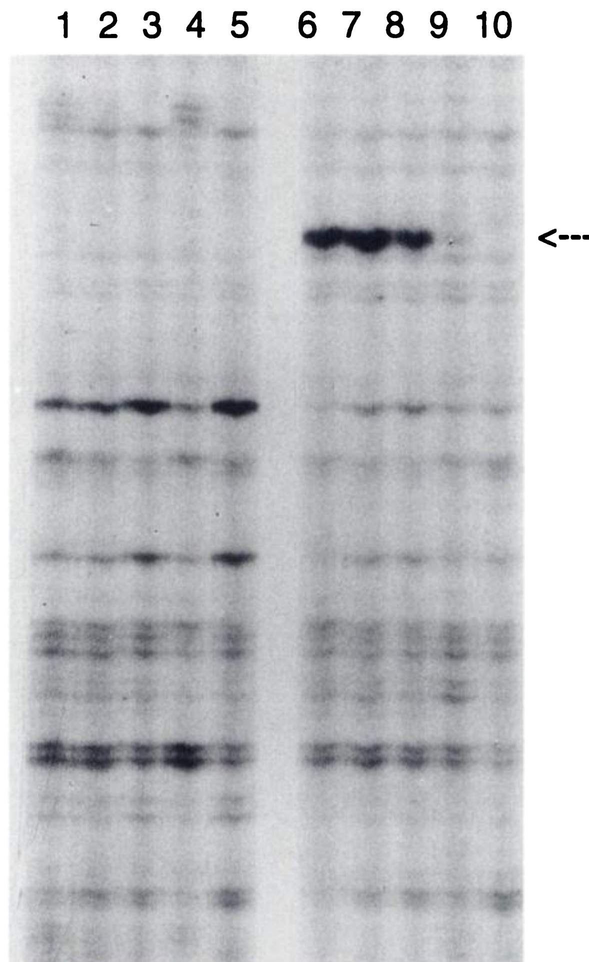

Fig. 2. Identification of differentially expressed genes in FGC and LNO cells. Cells were cultured under different hormonal conditions as described in "Materials and Methods." This figure gives an example of a representative experiment. *Lanes 1–5*, FGC cells; *Lanes 6–10*, LNO cells; *Lanes 1* and *6*, no additions; *Lanes 2* and *7*, 10^{-10} M R1881; *Lanes 3* and *8*, 10^{-8} M R1881; *Lanes 4* and *9*, 10^{-7} M 1,25-$(OH)_2D_3$; *Lanes 5* and *10*, 10^{-8} M R1881 and 10^{-7} M 1,25-$(OH)_2D_3$. mRNA was reverse transcribed and amplified by PCR in the presence of [α-^{33}P]dATP. The PCR products were separated using a 6% denaturing polyacrylamide gel, followed by autoradiography. *Arrow*, a differentially expressed band in LNO cells. The primers used are described in "Materials and Methods."

Cloning and Sequencing of cDNA Clones. The differentially expressed cDNA clones were sequenced, and a homology search in the EMBL/GenBank databases was performed, using the BLAST Server (45).

JC19, isolated by the differential display-PCR method, showed >95% homology to ESTs derived from normal liver (T72336), fetal liver spleen (R88729), and breast tissues (H44028). *JC19* is 428 bp long and contains a poly(A) sequence as well as the SLtk3 arbitrary primer sequence.

GC79 had no significant homology to known DNA sequences in the database. *GC79* is 255 bp long, and the nucleotide sequence is shown in Fig. 4.

Table 1 *Characteristics of differentially expressed genes in androgen-dependent LNCaP-FGC and androgen-independent LNCaP-LNO prostate cancer cells*

After cloning the differentially expressed bands, the cDNA clones were sequenced for identification and confirmation of primer sequences and to determine the size of the fragments. The fragments were used as probes in Northern blot analysis to determine the mRNA size and the expression levels. A homology database search was performed using the BLAST Server (45).

Clone	Homology	Primer	Fragment length (bp)	mRNA size (kb)	Expression[a]	
					FGC	LNO
JC19	ESTs	SLtk3 × ET12G	428	6	++	+−
GC79	Unknown	HB13	255	6	++	+−
GC81	*UGT2B15*	HB15	427	2	−	++
GC85	*BTG1*	HB21	270	2	++	+

[a] −, no expression; +−, low expression; +, moderate expression; ++, high expression.

The two remaining clones were homologous to known genes, *BTG1* and *UGT2B15*. Clone BTG1 is 270 bp long and is located between nucleotides 1008–1278 of 1783 bp, according to the numbering reported by Rouault *et al.* (46). Clone UGT2B15 is 427 bp long and is located between nucleotides 1478–1905 of 2090 bp, according to the numbering reported by Chen *et al.* (47).

Expression of cDNA Clones in Prostate Cancer Xenografts. Recently, we have established a unique panel of four androgen-dependent and five androgen-independent human prostate cancer xenografts (43, 44). The characteristics of the xenografts showed resemblance with the corresponding patient tumor tissue with respect to morphology and expression of PSA, prostatic acid phosphatase, and androgen receptor. The mRNA expression levels of *JC19* were analyzed in the prostate cancer xenografts as shown in Fig. 5. The androgen-dependent xenografts displayed higher expression levels, whereas in the androgen-independent xenografts, no significant expression was detected. This pattern is in good correlation with the expression pattern from the cell culture system, where the androgen-dependent FGC cell line also had a considerably higher expression level compared with the androgen-independent LNO cell line (Fig. 3). Because *GC79* and *UGT2B15* are androgen-regulated genes, expression in the xenografts was less pronounced compared with expression in the cell culture system (results not shown).

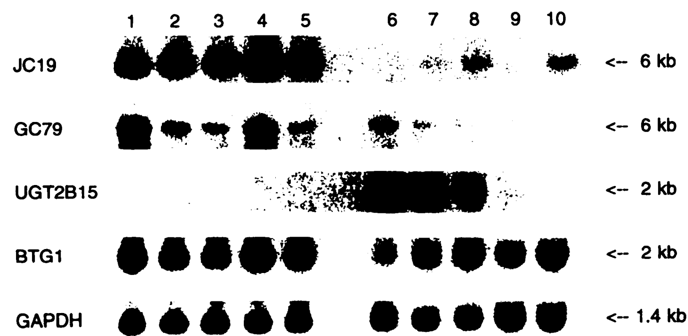

Fig. 3. Northern blot analysis of *JC19, GC79, BTG1,* and *UGT2B15* mRNA expression in FGC and LNO cells. Total RNA was loaded on each lane of a 1.5% denaturing agarose gel, electrophoresed, blotted onto nylon filters, and hybridized with the specific probes as listed in Table 1. Each lane contains 20 μg of total RNA as measured by the absorbance at 260 nm, ethidium bromide staining, and hybridization with a control GAPDH cDNA probe (1.2-kb *PstI* fragment). *Lanes 1–5,* FGC cells; *Lanes 6–10,* LNO cells; *Lanes 1* and *6,* no additions; *Lanes 2* and *7,* 10^{-10} M R1881; *Lanes 3* and *8,* 10^{-8} M R1881; *Lanes 4* and *9,* 10^{-7} M 1,25-$(OH)_2D_3$; *Lanes 5* and *10,* 10^{-8} M R1881 and 10^{-7} M 1,25-$(OH)_2D_3$. *Arrows,* positions of the mRNAs and the estimated sizes.

Androgen Regulation of Differentially Expressed Genes. *GC79* and *UGT2B15* were examined for their androgen regulation in the cell culture system. The effects of 10^{-10} M R1881 on the mRNA expression levels of *GC79* in FGC cells were studied, and results are shown in Fig. 6*A*. R1881 inhibits mRNA expression levels. Down-regulation starts to occur after 4–5 h and levels off after 8–9 h. Androgen concentration dependency on mRNA down-regulation was studied and is shown in Fig. 6*B*. The concentration at which down-regulation starts is at physiological concentrations of androgens (10^{-10} M). Down-regulation leveled off at higher R1881 concentrations.

The mRNA expression levels of *UGT2B15* in LNO cells are shown in Fig. 7. R1881 and 1,25-$(OH)_2D_3$ both down-regulate mRNA levels. Down-regulation induced by 10^{-10} M R1881 was observed already after 9 h and leveled off after 24 h (Fig. 7*A*). The kinetics of 10^{-7} M 1,25-$(OH)_2D_3$ induced down-regulation was different from that induced by R1881. Down-regulation was slower and was observed not before 48–72 h, as shown in Fig. 7*B*.

***BTG1* Expression in the Cell Cycle.** Because it has been reported that *BTG1* is involved in cell cycle regulation (46), we analyzed whether this is also the case in FGC cells. The mRNA expression of *BTG1* during the cell cycle was studied using cells synchronized by lovastatin. Expression was reduced between 3 and 10 h (Fig. 8). Between these time periods, FGC cells were at the late G_1 phase, as reported previously using flow cytometry analysis (36).

Table 2 *mRNA expression of the differentially expressed genes from FGC and LNO cells in several established cell lines*

The cells were cultured in their appropriate medium, and at near confluency, total RNA was isolated and Northern blot analysis was performed as described in "Materials and Methods." The blots were hybridized with the specific probes as listed in Table 1. Total RNA in each lane on the gel was equally loaded as measured by the absorbance at 260 nm and ethidium bromide staining (data not shown).

Cell line	Origin (species)	*JC19*[a]	*GC79*[a]	*UGT2B15*[a]	*BTG1*[a]
FGC	Prostate carcinoma (human)	++	++	−	++
LNO	Prostate carcinoma (human)	+−	+−	++	+
HEK293	Embryonic kidney (human)	+	+	−	++
GSF	Fibroblasts (human)	−	+	−	+
T47D	Breast carcinoma (human)	+	+++	−	++
HeLa	Cervix carcinoma (human)	+	+	−	++
SnuC5	Colon carcinoma (human)	+	−	−	+
SnuC1	Colon carcinoma (human)	+	−	−	++
IMR32	Neuroblastoma (human)	−	+	−	+
HEPG2	Hepatoma (human)	−	−	−	+
T24	Vesicle carcinoma (human)	−	+	−	+
COS1	Kidney (monkey)	−	+	−	+

[a] −, no expression; +−, low expression; +, moderate expression; ++, high expression.

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1    GAATTCAGCCCCAGCCTAAGTAAGTACGAAGCCCAGGGTTCATTGACTAAAAGCCATTCT

61   GCTCAGCAGCCAGTCCTGGTCAGCCAAACTCTGGATATTCACAAAAGGATGCAACCTTTG

121  CACATTCAGATAAAAAGTCCTCAGGAAAGTACTGGAGATCCAGGAAATAGTTCATCCGTA

181  TCTGAAGGGAAAGGAAGTTCTGAGAGAGGCAGTCCTATAGAAAAGTACATGAGACCTGCG

241  AAAACGCAAGCTTGC
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Fig. 4. Nucleotide sequence of clone GC79. Clone GC79 was sequenced according to Sanger *et al.* (63). The sequence of the HB13 primer used in the RNA arbitrarily primed-PCR is *underlined*. *Left*, numbering of the nucleotide sequence.

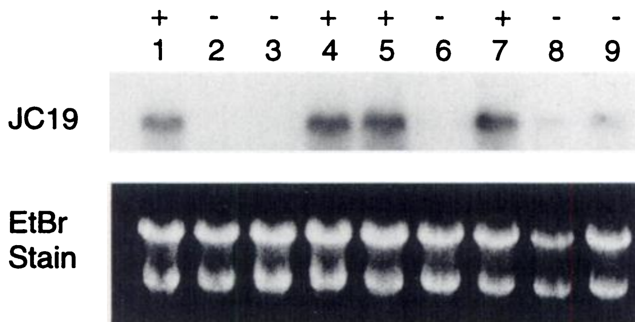

Fig. 5. Northern blot analysis of *JC19* mRNA expression in human prostate cancer xenografts. Human androgen-dependent (PC-82, PC-295, PC-310, and PC-329) and androgen-independent (PC-133, PC-135, PC-324, PC-339, and PC-374) prostate cancer xenografts were grown in athymic nude mice as described previously (43, 44). Total RNA was isolated from the xenografts, electrophoresed, blotted, and hybridized with the *JC19* probe as listed in Table 1. Each lane contains an equal amount of total RNA as measured by the absorbance at 260 nm and ethidium bromide staining. *Lane 1,* PC-82; *Lane 2,* PC-133; *Lane 3,* PC-135; *Lane 4,* PC-295; *Lane 5,* PC-310; *Lane 6,* PC-324; *Lane 7,* PC-329; *Lane 8,* PC-339; *Lane 9,* PC-374; +, androgen dependent; −, androgen independent. The numbering of the xenografts corresponds to previous reports (43, 44).

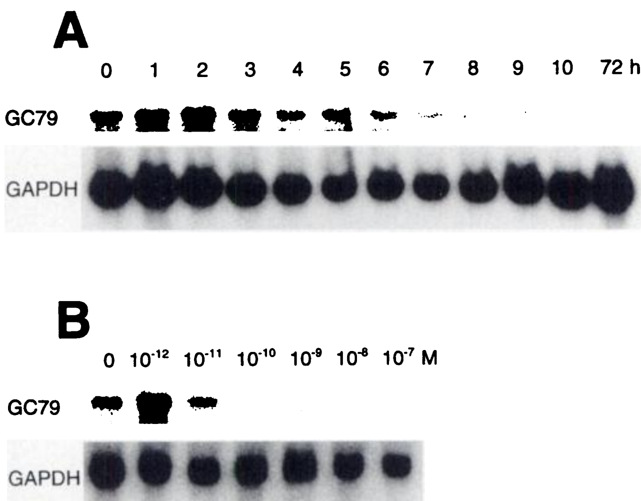

Fig. 6. Northern blot analysis of androgen down-regulation of GC79 mRNA expression in FGC cells. *A,* time course. FGC cells were cultured with 10^{-10} M R1881 as described in "Materials and Methods." At different time intervals as indicated, total RNA was isolated from the cells, electrophoresed, blotted, and hybridized with the *GC79* probe as listed in Table 1. *B,* concentration dependency of expression pattern. FGC cells were cultured for 2 days with various amounts of R1881 as indicated. Total RNA was isolated, electrophoresed, blotted, and probed. Each lane contains an equal amount of total RNA as measured by the absorbance at 260 nm, ethidium bromide staining, and hybridization with a control GAPDH cDNA probe (1.2-kb *Pst*I fragment).

DISCUSSION

To characterize genes involved in the development of androgen-independent prostate cancer, we used the differential display technique to compare gene expression between an androgen-dependent and an androgen-independent prostate cancer cell line. Genes related to androgen-independent prostate cancer have been reported recently, including PSA (48–50), early growth response gene α (36), vimentin (48), S100P (49), and several ESTs (50). However, these genes were identified by comparison of androgen-dependent to androgen-independent prostate cancer cells derived from different patients. Large chromosomal differences may be present between these cell lines. To avoid these chromosomal differences, we analyzed gene expression using genetically related cell lines: the parental androgen-dependent LNCaP cell line (LNCaP-FGC); and an androgen-independent subline, designated LNCaP-LNO (28–30). Both cell lines contain androgen receptors and express PSA and prostatic acid phosphatase (51–53). To avoid the isolation of genes only involved in the growth process itself, we used hormonally controlled conditions, where both cell lines grew either at equal rates or were arrested in their growth.

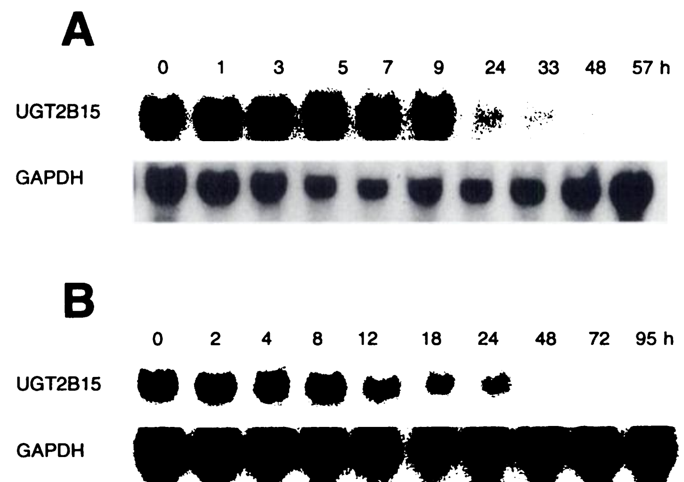

Fig. 7. Northern blot analysis of down-regulation of *UGT2B15* mRNA expression in LNO cells by R1881 and $1,25\text{-(OH)}_2D_3$. *A,* R1881. LNO cells were cultured with 10^{-10} M R1881 as described in "Materials and Methods." At different time intervals as indicated, total RNA was isolated from the cells, electrophoresed, blotted, and hybridized with the *UGT2B15* probe as listed in Table 1. *B,* $1,25\text{-(OH)}_2D_3$. LNO cells were cultured with 10^{-7} M $1,25\text{-(OH)}_2D_3$. At different time intervals as indicated, total RNA was isolated from the cells, electrophoresed, blotted, and probed. Each lane contains an equal amount of total RNA as measured by the absorbance at 260 nm, ethidium bromide staining, and hybridization with a control GAPDH cDNA probe (1.2-kb *Pst*I fragment).

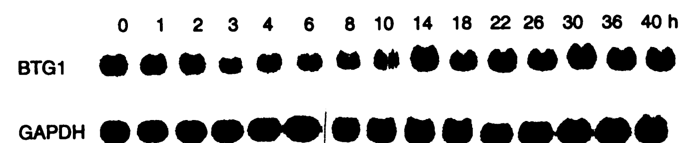

Fig. 8. *BTG1* mRNA expression during the cell cycle in FGC cells. FGC cells were arrested in their growth with 10 μM lovastatin for 36 h as described in "Materials and Methods." Mevalonic acid (2 mM) was added to release the cells from G_0-G_1 arrest. At different time intervals as indicated, total RNA was isolated from the cells, electrophoresed, blotted, and hybridized with the *BTG1* probe as listed in Table 1. Each lane contains an equal amount of total RNA as measured by the absorbance at 260 nm, ethidium bromide staining, and hybridization with a control GAPDH cDNA probe (1.2-kb *Pst*I fragment).

For the FGC cells, optimal growth was observed at 10^{-10} M R1881, whereas at higher concentrations, growth was no longer stimulated. This phenomenon was recently described to be associated with expression of transforming growth factor $\beta 1$ (54). The growth of LNO cells was independent of R1881 but was almost completely inhibited in the presence of 10^{-8} M R1881 and 10^{-7} M 1,25-$(OH)_2D_3$. Using these controlled conditions, sequence tags of two known and two unknown novel genes were isolated.

The first novel gene, *JC19,* is highly expressed in FGC cells (Fig. 3) and has a mRNA size of approximately 6 kb. Database search analysis showed that *JC19* is homologous to ESTs derived from liver and breast tissues. Using a panel of established cell lines of various origins (Table 2), we found that *JC19* is expressed in the human breast carcinoma cell line T47D but not in a liver-derived cell line HEPG2. This suggests that *JC19* mRNA expression in the liver probably does not occur in hepatocytes, or alternatively, that expression is undetectably low in HEPG2 cells. Furthermore, expression of *JC19* was observed in the human embryonic kidney cell line HEK293, the human cervix carcinoma cell line HeLa, and the colon carcinoma cell lines SnuC1 and SnuC5. Using the material of a series of human prostate cancer xenograft models, *JC19* was exclusively expressed in androgen-dependent, and not in androgen-independent, xenografts (Fig. 5). The studies of these human *in vivo* models correlate very well with the observations made in the cell culture system, indicating that the prostate cancer xenograft panel is a valuable tool to study gene expression *in vivo*. Currently, we are cloning the full-length cDNA of *JC19* and are characterizing the role of the gene in androgen-independent prostate cancer development.

The second novel gene, *GC79,* is also highly expressed in FGC cells and has a mRNA size of approximately 6 kb. *GC79* is different from *JC19,* because it is down-regulated by androgens. Down-regulation already starts after 4–5 h, which is relatively fast (Fig. 6*A*) and inversely related with androgen up-regulated expression of PSA (52). This suggests that *GC79* could be important in androgen-related functions. Down-regulation of *GC79* was observed at physiological levels of androgens (10^{-9} M). During androgen ablation therapy, where androgens are low and *GC79* mRNA is shown to be high, it is reported that apoptosis can occur (55–57). Whether *GC79* is involved in this process remains to be investigated.

The third differentially expressed gene found is *UGT2B15*. Expression was androgen regulated and only observed in LNO cells. *UGT2B15* is a member of the family of UDP-glucuronosyltransferases, enzymes localized in the endoplasmic reticulum. The enzymes are able to conjugate glucuronic acid to many different steroids and xenobiotic compounds to make them more water soluble to facilitate secretion (47, 58). Among different isozymes, UDPTh-3 or UGT2B15 has been reported to glucuronidate specifically androgens (47). It was suggested that the absence of *UGT2B15* mRNA in the liver of a patient with benign prostate hyperplasia could be of critical importance, because these patients have elevated levels of free androgens (47). We found that androgens down-regulate *UGT2B15* mRNA in androgen-independent cells, suggesting that glucuronidation of androgens will be decreased and free androgen levels will be increased. Because LNO cells are androgen independent and *UGT2B15* mRNA is down-regulated by androgens, it is probably unlikely that growth-related factors are involved in *UGT2B15* expression. *UGT2B15* mRNA was down-regulated after 9 h at 10^{-10} M R1881 (Fig. 7*A*). 1,25-$(OH)_2D_3$ could also down-regulate the expression, but only after 48–72 h, suggesting a different mechanism compared with down-regulation by R1881. Bélanger *et al.* (59) reported that *UGT2B15* mRNA is expressed in LNCaP cells, whereas in the present investigation, we could not find expression in LNCaP-FGC cells. The origin of these differences is not known. Of all of the cell lines we analyzed, LNCaP-LNO cells exclusively expressed *UGT2B15* mRNA (Table 2).

BTG1 is another differentially expressed gene, the expression of which, to our knowledge, has not been reported before in prostate cancer development. *BTG1* is a member of a family of antiproliferative genes and was originally isolated during cloning of the breakpoint of a t(8,12)(q24;q22) chromosomal translocation in a human B-cell chronic lymphocytic leukemia patient (46). *BTG1* is conserved during evolution in mammals and has been suggested to be a candidate tumor suppressor gene (46, 60). It is involved in cell cycle regulation and is localized in the nucleus (46). *BTG1* was less expressed in androgen-independent LNO cells, and it is tempting to speculate that this might be associated with the more antiproliferative character of this cell line, assuming androgen-independent cells have more uncontrolled growth and are more invasive (28, 29, 61, 62). Furthermore, mRNA levels were reduced in FGC cells just before the G_1-S phase of the cell cycle (Fig. 8). This indicates that *BTG1* is involved in cell cycle regulation. Possibly, *BTG1* is a tumor suppressor gene involved in the prevention of androgen-independent prostate cancer development because of its relative low expression in LNO cells.

In summary, by using RNA arbitrarily primed-PCR and differential display-PCR in a controlled cell culture system, we have identified three genes (*JC19, GC79,* and *BTG1*) not identified previously in the prostate and one gene (*UGT2B15*) not reported previously to be down-regulated by androgens and 1,25-$(OH)_2D_3$. Differential expression of *JC19* mRNA in androgen-dependent and androgen-independent prostate cancer xenografts demonstrates that the xenograft system, which represents various stages of prostate cancer, is a valuable *in vivo* tool to study the progression of clinical prostate cancer toward hormone-refractory disease.

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