Androgen Receptor Gene Expression in Human Prostate Carcinoma Cell Lines

W. D. Tilley, C. M. Wilson, M. Marcelli, and M. J. McPhaul

ABSTRACT

Responses to androgen vary widely among prostate cancers and prostatic carcinoma cell lines. We have explored the basis for this heterogeneity by examining the levels of androgen receptor expression in a prostate carcinoma cell line (LNCaP) that expresses the androgen receptor and two prostate carcinoma cell lines that do not contain detectable androgen receptor. We find that while the LNCaP cell line contains high levels of both the androgen receptor protein and mRNA, the receptor-negative cell lines DU-145 and PC-3 do not express androgen receptor protein as detected by immunoblotting or mRNA as detected by Northern analysis. These results indicate that the absence of androgen receptor expression in the androgen receptor-negative cell lines is caused by diminished androgen receptor mRNA levels. Genomic Southern analysis indicates that the differences in androgen receptor expression in each of these cell lines is not associated with detectable alterations in the structure of the androgen receptor gene.

INTRODUCTION

Many forms of prostate cancer are initially androgen dependent, but the response to androgen ablation is often transient (1). The mechanism of the transition from androgen-dependent to androgen-independent growth has been the subject of much interest. Studies of animal models of prostate carcinoma, particularly the Dunning rat prostatic adenocarcinoma have suggested that relapse following androgen ablation is due to the outgrowth of a population of androgen-independent carcinoma cells (2, 3). The origin of these androgen-independent cells remains to be clarified. In the current studies, we sought to examine the nature of androgen responsiveness and androgen resistance by determining the nature and level of androgen receptor ligand binding, protein expression, and mRNA expression in the prostate carcinoma cell lines, LNCaP, PC-3, and DU-145.

MATERIALS AND METHODS

Cell Culture. The LNCaP (4), PC-3 (5), and DU-145 (6) cell lines were obtained from the American Type Culture Collection. The LNCaP cell line was maintained in RPMI containing 10% fetal bovine serum. The PC-3 cell line was cultured in HAM's nutrient mixture F-12 containing 7% fetal bovine serum. The DU-145 cell line was maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. In one experiment, LNCaP cells were exposed to the effects of exogenous steroids. In this experiment, the medium was removed 5 days after plating and replaced with medium containing 5% charcoal-stripped fetal bovine serum and either no added steroid, 10^-4 M estradiol, or 10^-4 M 5a-dihydrotestosterone. The incubations were continued for an additional 48 h, the cells were harvested, and RNA was isolated as described below.

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2 The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

Radioligand Binding Assays. A whole cell monolayer binding assay similar to that described previously (10) was used to assay for specific 5a-dihydrotestosterone binding. Cultures of cells were grown to near confluence in 3-cm plates. Twenty-four h prior to performing the ligand binding assays, the medium was replaced with minimal essential medium containing 10% bovine serum albumin. To assay the level of specific 5a-dihydrotestosterone binding, the medium was washed twice with 5 ml of Tris-saline. The cells were dissociated by trypsinization and washed twice with 5 ml of ice-cold Tris-saline. The cell pellets were resuspended in 1 ml of deionized water and disrupted by sonication. The quantity of cell-associated radioactivity was determined by scintillation counting. Cells grown in parallel and treated in an identical manner were solubilized and assayed for protein (11). The ligand binding data were analyzed according to the method of Scatchard (12).

Northern Analysis. RNA samples were denatured in buffer containing 2.2 M formaldehyde and 50% formamide, loaded onto 1% agarose gels, cast, and run in buffer containing 2.2 M formaldehyde (13). After running, the gels were washed briefly in 50 mM NaOH and 10 mM NaCl and transferred to Zetaprobe nylon membrane (Bio-Rad, Richmond, CA) by capillary blotting. Following transfer, the RNA was covalently cross-linked to the membrane with UV light (Stratallinker; Stratagene, San Diego, CA). The membranes were prehybridized for 2 h in 1 ml EDTA-0.5 M NaH2PO4 (pH 7.2) and 7% SDS at 68°C. Hybridization was performed in the same buffer containing probe labeled with alpha-35PdCTP using the random hexamer method of Feinberg and Vogelstein (14). After hybridization, the membranes were washed at 60°C in 40 mM NaH2PO4 (pH 7.2), 1 mM EDTA, and SDS (5% SDS in the first wash and 1% SDS in the final wash).

Southern Analysis. Samples of genomic DNA were digested with the restriction endonuclease EcoRI, electrophoresed on 0.8% agarose gels, and transferred and cross-linked to a nylon Zetaprobe membrane. Following prehybridization at 68°C for 2 h in 7% SDS, 1 mM EDTA, and 0.5 M NaH2PO4 (pH 7.2), the filters were hybridized in another aliquot of the same buffer containing probes specific for each exon of the androgen receptor gene (8) labeled with alpha-32PdCTP using the random priming method (14). Probe concentration in all cases was approximately 1 x 10^6 cpm/ml and the probe specific activity was >5 x 10^8 cpm/ug DNA. Following hybridization, the filters were washed in 0.1% standard saline-citrate-0.1% SDS at 68°C and autoradiographed at -80°C.

RNA Probe Synthesis. Two segments of the androgen receptor gene (see below) were subcloned into the plasmid vector, BSKM13+ (Stratagene; San Diego, CA) and used as templates for RiboMAX RNA transcript kit (Promega, Madison, WI). cDNA probe synthesis was performed using 10-15 μg total RNA in a 10-μl volume using 80-100 ng DNA probe and 10 ml BSKM13+ vector DNA. The probes were labeled with alpha-32PdCTP using the random hexamer method (14). Probe concentration in all cases was approximately 1 x 10^6 cpm/ml and the probe specific activity was >5 x 10^8 cpm/ug DNA. Following hybridization, the filters were washed in 0.1% standard saline-citrate-0.1% SDS at 68°C and autoradiographed at -80°C.
tage Cloning Systems, La Jolla, CA) and used to synthesize continuously labeled antisense RNA probes for S1 nuclear protein protection assays. Region I was a 530-base pair PstI-Smal restriction endonuclease fragment derived from the 5'-untranslated region of the androgen receptor gene.2 Region II consisted of a 713-base pair HindIII-EcoRI restriction endonuclease fragment derived from the coding region (nucleotides 1850 to 2563) of the human androgen receptor complementary DNA (15). Following linearization with an appropriate restriction endonuclease (HindIII for probe I, BamHI for probe II), in vitro transcription was performed using either the T7 polymerase (probe II) or the T3 polymerase (probe I), as described by the manufacturer, in a reaction containing 0.4 mM concentrations each of unlabeled CTP, ATP, and GTP and 12 μM [α-32P]UTP (final specific activity, 400 Ci/mmol; Amersham Corp.). Following synthesis, the DNA template was digested with RNase-free DNase (RIQ DNASE; Promega Biotech) and the unincorporated nucleotides were removed by ammonium acetate precipitation.

Preparation of a Synthetic "Sense" Androgen Receptor mRNA and the Standard Curve. To allow the precise quantitation of the androgen receptor mRNA present in RNA samples, synthetic sense RNA transcripts were prepared using as template the plasmid containing region II from the coding region of the androgen receptor (see above). In these transcription reactions, the plasmid was linearized instead with the restriction enzyme EcoRI and the T3 RNA polymerase was used, leading to the synthesis of RNA transcripts with the same polarity as the native androgen receptor RNA. In vitro transcription with the T3 RNA polymerase was carried out as described for the antisense RNA probe synthesis, except that the specific activity of the sense standard was 2 orders of magnitude less than that used for the synthesis of the antisense RNA probe. After removal of the unincorporated nucleotides, the amount of purified sense RNA used in the standard curve was inferred from the nucleotide sequence of the RNA probe and the specific activity of the radiolabeled UTP (2.5 Ci/mmol).

S1 Nuclease RNA Protection Assay. S1 nuclelease protection assays were performed using a protocol similar to that of Burke (16). Approximately 8 x 10^6 cpm of the purified uniformly labeled antisense RNA probe was coprecipitated with 25 μg (receptor-positive) or 40 μg (receptor-negative) RNA samples, rinsed with ethanol, and dried. The samples were resuspended in 25 μl of a solution containing 0.4 M NaCl, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6.5), 1 mM EDTA, and 64% deionized formamide, denatured at 80°C for 5 min, and incubated for 16 h at 52°C. After hybridization, 300 μl of ice cold 5% S1 nuclease (25 μg/ml), 30 mM sodium acetate (pH 4.5), 0.6 mM bromophenol blue-80 mM Tris, pH 6.9, and 5% (v/v) glycerol were added. Nuclease digestion was performed with 4000 units of S1 nuclease (Boehringer Mannheim, Indianapolis, IN) at 42°C for 1 h. Digestion was terminated by the addition of 15 μl of buffer containing 0.5 M Tris (pH 8.0), 25 mM EDTA, and 5 μg of tRNA. The samples were extracted with phenol:chloroform (1:1, v/v), precipitated at -20°C following addition of 1 ml of chilled ethanol, and then resuspended and precipitated twice with ammonium acetate. After the final precipitation, the samples were washed in ethanol, dried, resuspended in 90% (v/v) formamide, electrophoresed on a denaturing 5% polyacrylamide gel containing 8 M urea, and visualized by autoradiography.

In order to quantify the absolute levels of androgen receptor mRNA present in these samples, in each experiment we included a standard curve that used known amounts of synthetic androgen receptor RNA transcripts of the same polarity as native androgen receptor mRNA. This synthetic mRNA was identical in structure but opposite in polarity to the labeled probe (probe II) employed in the quantitation assays (see above). Standard samples that ranged from 0.005 to 0.1 fmol of synthetic sense androgen receptor mRNA were included in the quantitation experiments. Standard samples were hybridized with labeled probe, digested with S1 nuclease, and analyzed as above. The autoradiographs of both the standard and the experimental samples were scanned using a Molecular Dynamics Computing Densitometer (Model 300A; Sunnyvale, CA), and the values obtained (arbitrary units) were plotted as a function of the amount of synthetic sense androgen receptor mRNA. Absolute levels of androgen receptor mRNA were inferred by comparison of the experimental values to the values obtained for the standard curve.

Western Analysis. Confluent monolayers of cells were scraped into ice cold PBS and pelleted by centrifugation. Pellets were washed twice with 10 volumes of PBS, resuspended in 10 volumes of electrophoresis loading buffer [70 mM SDS-100 mM dithiothreitol-10% (v/v) glycerol-0.6 mM bromophenol blue-80 mM Tris, pH 6.9] and stored at -20°C. Prior to electrophoresis samples were thawed, resuspended by sonication, heated in a boiling water bath for 3 min, diluted in loading buffer to the desired protein concentration, sonicated again to produce a homogeneous solution, and boiled again for 3 min. The final protein concentration was determined by a modification of the method of Lowry et al. (11) which included the initial precipitation of protein with 10% (w/v) trichloroacetic acid in the presence of 0.8 mM sodium deoxycholate. Bovine serum albumin was used as the protein standard.

Electrophoresis was carried out in 7.5% polyacrylamide gels containing 3.5 mM SDS according to the method of Laemmli (17) using 100 μl of sample in each lane. Ten μl of Rainbow 14C-methylated protein molecular weight markers (CFA.756; Amersham, Arlington Heights, IL) were mixed with 90 μl of loading buffer and applied to one lane of each gel. Following electrophoresis, protein bands were transferred to nitrocellulose and analyzed by immunoblotting as described by Harris et al. (18) using affinity-purified rabbit antibodies that recognize the amino (NH2)-terminal of the androgen receptor. Blots were incubated with 125I-labeled goat anti-rabbit IgG (Fab'), (New England Nuclear Research Products, Boston, MA) at 5 x 10^6 cpm/ml to detect anti-NH2-terminal antibody binding. Autoradiograms were produced by exposing Kodak XAR2 film to immunoblots for 65 h at ~70°C with one intensifying screen.

Anti-NH2-terminal antibodies were produced by immunizing rabbit U402 with the carrier protein keyhole limpet hemocyanin coupled to a synthetic 21-amino acid peptide corresponding to the NH2-terminal amino acid sequence of human and rat androgen receptor protein, purified on a peptide affinity chromatography column, and characterized as described previously (19). Affinity-purified antibodies were diluted 1:30 in BLOTTO and used at a final concentration of 33 μg protein/ml as determined by the method of Lowry et al. (11).

RESULTS

PC-3 and DU-145 Cell Lines Express No Detectable Dihydrotestosterone Binding. To establish the level of androgen receptor in the prostate carcinoma cell lines, we examined the levels of specific 5α-dihydrotestosterone binding sites present in the LNCaP, PC-3, and DU-145 cell lines (Table 1). The LNCaP cell line expresses high levels of androgen receptor, while the DU-145 and PC-3 cell lines express no detectable specific 5α-dihydrotestosterone binding. Scatchard analysis of these ligand binding data indicate that androgen receptor in the LNCaP cell line binds 5α-dihydrotestosterone with an apparent Kd of 0.7 nM.

LNCaP Cell Line Expresses High Levels of the Androgen Receptor Protein. We next examined the levels of immunore-

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Specific binding of [3H]5α-dihydrotestosterone (fmol/mg protein)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>286</td>
<td>0.7</td>
</tr>
<tr>
<td>PC-3</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>DU-145</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 1 5α-Dihydrotestosterone binding in monolayer cultures of three prostate carcinoma cell lines

The ligand binding assays were performed in monolayer cultures of the prostate carcinoma cell lines. The number of specific 5α-dihydrotestosterone binding sites and the apparent Kd of the androgen receptor were determined by Scatchard analysis of the binding data as described in "Materials and Methods." Specific 5α-dihydrotestosterone binding represented over 90% of the total binding detected at saturation in the LNCaP cell monolayers. By contrast, no specific binding was detected above background in the DU-145 and PC-3 cell lines. NA, not applicable.
We therefore measured androgen receptor mRNA levels by both Northern analysis and the more sensitive 
5a-protection assays. The results of the Northern analysis of 
rNA prepared from human prostate carcinoma 
cell lines. Samples were run on agarose gels and transferred to a nylon membrane. 
a. comparison of the androgen receptor mRNA species detected in either total 
(25 μg) or polyadenylated (Poly(A)) (10 μg) RNA derived from the prostate 
carcinoma cell line, LNCaP. In b, the lanes containing the PC-3 and DU-145 
samples contain 20 μg of total RNA per lane. The LNCaP lanes contain 25 μg 
each of total RNA prepared from cells cultured in charcoal-stripped serum 
containing no added steroid, 10^-4 M 5a-dihydrotestosterone (+DHT), or 10^-4 M 
estradiol (+E2). The probe used for these experiments was derived from the 
carboxy-terminal region of the human androgen receptor complementary DNA 
(nucleotides 1850 to 2563 in Ref. 15) and labeled by the random hexamer method 
(14). This same probe is used in the S1 protection assays shown in Fig. 3 (probe II). c, same blot in b reprobed with 32P-labeled 28S (upper portion) and 18S 
(lower portion) ribosomal RNA probes. The positions of labelled molecular weight 
markers are shown to the left.

**Fig. 2. Northern analysis of RNA prepared from human prostate carcinoma**
cell lines. Samples were run on agarose gels and transferred to a nylon membrane. 
a. comparison of the androgen receptor mRNA species detected in either total 
(25 μg) or polyadenylated (Poly(A)) (10 μg) RNA derived from the prostate 
carcinoma cell line, LNCaP. In b, the lanes containing the PC-3 and DU-145 
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(lower portion) ribosomal RNA probes. The positions of labelled molecular weight 
markers are shown to the left.

**Fig. 1. Immunoblot analysis of proteins prepared from human prostate carcinoma**
cell lines. Proteins from three prostatic carcinoma cell lines were separated by electrophoresis on a 7.5% polyacrylamide gel containing 3.5 mM sodium 
dodecyl sulfate and transferred to a nitrocellulose filter. Androgen receptor 
protein was identified by incubating the blot with affinity-purified polyclonal 
antisera that recognize the NH2-terminal segment of the receptor followed by 
incubation with 125I-goat anti-rabbit IgG F(ab')2 to label antigen-antibody 
complexes and autoradiography to visualize the labeled complex. The source and 
total μg of protein applied to each lane are indicated on the top abscissa. Horizontal 
bars and number in the center indicate the position and size in thousands of °C 
molecular weight markers run on the same gel.
represents the limit of detection of the assay. This value is at least 200-fold lower than the level of androgen receptor mRNA expression in the LNCaP cell line and less than one-tenth that found in the control (704) genital skin fibroblast cultures which express low levels of androgen receptor mRNA and protein. Similar values were obtained in experiments using probe I.

Androgen Receptor Gene in the LNCaP, DU-145, and PC-3 Cell Lines Is without Large Scale Rearrangements. The virtual absence of androgen receptor mRNA expression in the DU-145 and PC-3 cell lines raised the question as to whether the androgen receptor gene in these cell lines had undergone an alteration that had rendered it nonfunctional. This possibility was addressed by analyzing the androgen receptor gene structure in the three prostate carcinoma cell lines and in normal diploid fibroblasts by genomic Southern analysis (Fig. 5). All eight coding exons of the androgen receptor gene (8, 20) are present in each of the cell lines, and no large deletions or rearrangements of the androgen receptor gene have occurred.

DISCUSSION

While it is believed that the majority of prostate cancer is derived from the androgen-dependent prostatic epithelium, these tumors vary widely both in histological appearance and biological behavior from patient to patient. Furthermore, the responses of patients to various forms of endocrine therapy are not uniform (1).

Studies of the heterogeneity of prostate carcinoma and the hormonal responsiveness of this tumor have been most extensively performed in the rat Dunning model of prostate carcinoma (2, 21). These studies have suggested that the progression from an androgen-sensitive to an androgen-independent state is associated with the outgrowth of androgen-insensitive cells from an initially heterogeneous mixture of tumor cells. Interestingly, studies recently reported by Quarmby et al. (22) sug-

**Fig. 3.** S1 nuclease protection assay of androgen receptor RNA levels in prostate carcinoma cell lines (LNCaP, PC-3, and DU-145) and in a control genital skin fibroblast strain (704) by S1 nuclease protection analysis. A, a 25-µg sample of RNA from control genital skin fibroblasts (strain 704, B), and 50-µg samples of RNA from either the DU-145 or PC-3 cells (C) were 0.128, 0.035, and 0 femol of androgen receptor mRNA, respectively. These values, when corrected for the amount of RNA loaded, correspond to 25.6, 1.4, and 0 amol of androgen receptor mRNA for samples designated as A, B, and C, respectively.

**Fig. 4.** Quantitation of androgen receptor RNA levels in prostate carcinoma cell lines (LNCaP, PC-3, and DU-145) and in a control genital skin fibroblast strain (704) by S1 nuclease protection analysis. A, a 25-µg sample of RNA from control genital skin fibroblasts (strain 704, B), and 50-µg samples of RNA from either the DU-145 or PC-3 cells (C) were 0.128, 0.035, and 0 femol of androgen receptor mRNA, respectively. These values, when corrected for the amount of RNA loaded, correspond to 25.6, 1.4, and 0 amol of androgen receptor mRNA for samples designated as A, B, and C, respectively.

**Fig. 5.** Southern analysis of genomic DNA prepared from human prostate carcinoma cell lines. Samples of genomic DNA from the three cell lines were digested with the restriction endonuclease EcoRI and run on a 0.8% agarose gel. After transfer, the membranes were hybridized with labeled probes specific for each coding exon (8, 20). Exon 6 is contained on the same EcoRI restriction endonuclease fragment as exon 5.
not androgen responsive and do not express measurable androgen receptor. We have explored this heterogeneity by examining the expression of the androgen receptor in these three continuous human prostate carcinoma cell lines. Our studies indicate that the high level of specific 5α-dihydrotestosterone binding presented in the LNCaP cell line correlates with the presence of an androgen receptor protein with an apparent molecular weight of approximately 110,000, similar to that observed in the prostate and other target tissues (23, 24). These experiments also demonstrate that the absence of detectable androgen binding in the PC-3 and DU-145 cell lines is associated with the absence of detectable normal or mutant androgen receptor protein using antibodies directed at an epitope located at the amino terminus of the androgen receptor protein.

Our Western analyses were limited, however, both by their sensitivity and by the nature of the assay, which detects only a limited number of epitopes within the androgen receptor protein. To circumvent these shortcomings we extended these findings and examined androgen receptor mRNA expression within these cell lines by Northern and cDNA nuclease protection analysis using probes designed to detect two distinct regions of the androgen receptor mRNA molecule. Using these assays we demonstrated that RNA samples prepared from the LNCaP cell line contained high levels of androgen receptor mRNA. Furthermore, the mobilities of the androgen receptor mRNA species visualized in these samples, approximately 7 and 10 kilobases, are similar to those observed in preparations from other human tissues. However, using these same assays, we were unable to detect androgen receptor mRNA in the PC-3 or DU-145 cell lines. This appears to represent a complete absence of androgen receptor mRNA, inasmuch as our methods would permit the detection of androgen receptor mRNA at a level one-tenth that found in fibroblasts, which express only low levels of androgen receptor mRNA and protein.

The virtual absence of detectable androgen receptor mRNA in the PC-3 and DU-145 cell lines does not appear to be correlated with discernible alterations within the androgen receptor gene itself because no gross deletions or rearrangements are detectable by genomic Southern blotting. While this does not exclude the possibility that subtle mutations such as point mutations might be present within the androgen receptor gene of the PC-3 and DU-145 cell lines, it would appear that the androgen receptor gene is inactive in these receptor-negative prostate carcinoma cell lines.

While the relevance of these studies to the progression of human prostate cancer is not clear, the derivation and androgen-independent growth of the receptor-negative prostate carcinoma cell lines used in these studies invite comparison to androgen-independent prostate carcinoma. Further analysis of the mechanisms resulting in the absence of androgen receptor expression in the PC-3 and DU-145 cell lines should provide insight into the factors controlling androgen receptor expression and may identify processes that lead to the development of androgen-independent human prostate cancers.

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3 W. D. Tilley, M. Marcelli, and M. J. McPhaul, unpublished observations.

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