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Androgen regulation of ornithine decarboxylase in human prostatic cells identified using differential display

Alan M. Betts, Ian Waite, David E. Neal, Craig N. Robson*

Department of Surgery, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK

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Abstract Androgens are essential for normal prostate physiology and have a permissive role in the development and progression of prostate cancer. Using the mRNA differential display technique, ornithine decarboxylase (ODC) was identified to be up-regulated by androgens in human prostatic LNCaP cells. On Northern analysis, the induction of ODC expression by 10 nM androgen was rapid and continued up to 48 h exposure with a maximum 6.3-fold up-regulation. The anti-androgen Casodex inhibited the androgen-induced up-regulation of ODC, whereas the protein synthesis inhibitor cycloheximide did not. Together these data suggest that regulation is mediated through the androgen receptor protein and does require secondary protein synthesis, respectively. The kinetics of induction of ODC were almost identical to those of prostate specific antigen. Taken together these data suggest that ODC is directly regulated by androgens in LNCaP cells.

Key words: Differential display; Ornithine decarboxylase; Androgen; Prostate specific antigen; Mibolerone

1. Introduction

In American and European males prostate cancer is the second most common cause of cancer-related death, with more than 317 000 new cases diagnosed and over 41 000 deaths occurring every year in the USA [1]. As androgens regulate normal growth and differentiation of the prostate, androgen regulated genes are likely to contribute to the development and progression of prostate cancer. Androgen responsive genes such as prostate specific antigen (PSA) [2] and prostate specific membrane antigen [3] have been characterized and PSA is commonly used as a clinical marker for prostate cancer [4].

Despite the fact that the human prostate is highly androgen responsive, few androgen responsive genes have been cloned. We have used the differential display (DD) technique [5,6] to identify androgen responsive genes in human prostatic cells. Using this strategy, ornithine decarboxylase (ODC) was identified as an androgen responsive gene. Ornithine decarboxylase is the first, rate-controlling enzyme in the polyamine biosynthesis pathway and catalyses the conversion of ornithine to putrescine. Polyamines are found in high concentrations in the prostate and may influence the growth and progression of prostate cancer. For instance, levels of the polyamine spermine are elevated in prostatic hyperplasia and spermine may inhibit the growth of malignant prostatic cells [7]. In addition, ODC activity is highly responsive to stimuli affecting cell growth and differentiation [8,9].

Regulation of ODC activity can occur via transcription, translation and post-translational mechanisms in a complex cell-specific manner. Its mRNA levels are elevated in animal tumours, certain transformed cell lines and human tumour models in the colon, skin and breast [10]. Disruption of ODC function results in the arrest of cell proliferation in tissue culture and in animal tumors in vivo [11] and overexpression of ODC in NIH/3T3 cells gives rise to loss of contact inhibition, anchorage independent growth and increased tumourigenesis [12,13]. ODC mRNA levels are up-regulated 5–10-fold by androgens in the rodent kidney, prostate and accessory sex organs [14], anti-estrogens in the MCF-7 cell line [15] and the 'Wilms tumour suppressor WT-1 in HCT 116, NIH/3T3 and HepG2 cells [16].

Characterization of the androgen regulated expression of ODC was investigated using the anti-androgen Casodex and the protein synthesis inhibitor cycloheximide. These studies demonstrated that the androgen-induced up-regulation of ODC was mediated through the androgen receptor and was independent of secondary protein synthesis. Thus, ODC may be an example of a key cellular metabolism gene that is directly regulated by androgens.

2. Materials and methods

2.1. Materials

LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD), RPMI 1640 from GibcoBRL (Paisley, UK), and foetal calf serum (FCS) from Sigma (Poole, UK). Radioisotopes were from ICN (Irvine, CA) and enzymes from NBL (Cramlington, UK). The synthetic androgen mibolerone was purchased from New England Nuclear-DuPont (Stevenage, UK). Casodex was generously provided by Zeneca (Macclesfield, UK). All other reagents were from Sigma unless otherwise stated.

2.2. Cell culture

LNCaP cells (passage 33–38) were routinely maintained in RPMI 1640 containing L-glutamine (20 mM) and supplemented with 10% FCS, Penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}). For androgen exposures the cells were plated out in complete growth medium and allowed to adhere for 24–48 h. Cultures were washed twice with phosphate-buffered saline then exposed to RPMI 1640 medium supplemented with 10% steroid-depleted, dextran-coated charcoal-treated FCS (DCC medium) for 72 h. Fresh DCC medium was added containing 10 nM mibolerone and the cells were incubated at 37°C for periods of up to 48 h.

2.3. Differential display

Differential display was performed essentially as described [5], with the following modifications. Total RNA was extracted from cells as previously described [17]. PolyA$^+$ RNA was purified using Dynabeads Oligo (dT_{25}) according to the manufacturer's protocol (Dynal). For each DD reaction, 0.25 μg of polyA$^+$ RNA was reverse transcribed using $T_{12}VC$ as a primer and AMV reverse transcriptase (400 U ml^{-1};

*Corresponding author. Fax: (44) (191) 222 8514.
E-mail: c.n.robson@ncl.ac.uk

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Life Sciences). Differential display was performed in duplicate using primers $T_{12}VC$ and 1016 (5′-AGCCAGCGAA-3′), [α-^{32}P]dATP (500 μCi ml^{-1}) and AmpliTaq DNA polymerase (50 U ml^{-1}; Perkin Elmer Cetus). Differential display reactions were subjected to 30 cycles of PCR comprising 94°C for 30 s, 40°C for 20 s and 72°C for 30 s. Products of the PCR were electrophoresed on 6% non-denaturing polyacrylamide gels at 400 V for 20 h. DNA from bands thought to be differentially expressed was extracted by elution into 200 μl of water and the cDNA recovered by ethanol precipitation. Eluted cDNA species were re-amplified using 15 cycles of PCR with the appropriate primers and cloned into the pCRII vector (Invitrogen) according to the manufacturer's protocol. The DNA sequences of the differential display clones were determined using the Thermo Sequenase cycle sequencing kit (Amersham Life Sciences) and homologies to known genes determined using the Genbank database.

2.4. Northern blotting

RNA samples were electrophoresed as previously described [18]. Briefly, 5 μg of each RNA was fractionated on agarose gels, transferred to a nylon membrane (Hybond N+; Amersham), fixed by heating at 80°C for 2 h and stained with methylene blue to assess the integrity of the RNA. Probes were generated from cloned cDNAs by restriction endonuclease digestion. The PSA probe was an 805 bp cDNA generated from LNCaP RNA using reverse transcriptase-PCR as previously described [19]. Probes were radiolabelled with [α-^{32}P]dATP using random-primed labelling mixture according to the manufacturer's protocol (Boehringer-Mannheim). RNA blots were hybridized at 65°C to denatured, radiolabelled cDNAs; hybridization conditions and washing procedures were carried out as previously described [20]. Blots were analyzed after exposure to a phosphor storage screen using a Phosphorimager (Molecular Dynamics). Blots were subsequently re-probed with radiolabelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as a control for RNA loading. The expression of ODC and PSA was calculated relative to GAPDH and untreated control cells (such that untreated cells have a relative mRNA expression value of 1).

3. Results

3.1. Identification of ODC as an androgen responsive gene

Differential display was performed in duplicate on polyA$^+$ RNA from untreated LNCaP cells and cells exposed to 10 nM mibolerone for 8 or 48 h. A representative result using a single primer set is shown in Fig. 1. The band patterns were at least 90% identical between duplicate samples. However, several mRNAs were reproducibly differentially expressed, of which one was up-regulated after 8 and 48 h androgen exposure when compared to untreated control cells (labelled ODC). This band was excised from the gel, cloned into the PCRII vector and four clones were fully sequenced (data not shown). All four clones contained a 96 bp cDNA insert with the correct flanking primers ($T_{12}VC$ and 1016). A search of the GenBank database revealed an 89% homology with the 3′ end of the human ornithine decarboxylase gene (Fig. 2). The DNA alignment included the first 12 bases of the polyA tail and an adjacent putative polyadenylation signal.

3.2. Temporal changes in ODC mRNA levels in response to androgen

To confirm the putative androgen regulated expression of ODC and to determine the temporal pattern of ODC induction, LNCaP cells were treated with 10 nM mibolerone for varying lengths of time and the levels of ODC assessed using Northern analysis. The ODC and PSA probes identified species of 2.3 and 1.6 kb, respectively, as previously reported [2,21]. ODC and PSA were up-regulated between 4 and 48 h exposure to mibolerone to a maximum of 6.3- and 5.1-fold, respectively (Fig. 3). Both genes showed a rapid response to androgen treatment, with ODC up-regulated 1.2-fold and PSA up-regulated 1.3-fold after 4 h exposure to mibolerone.

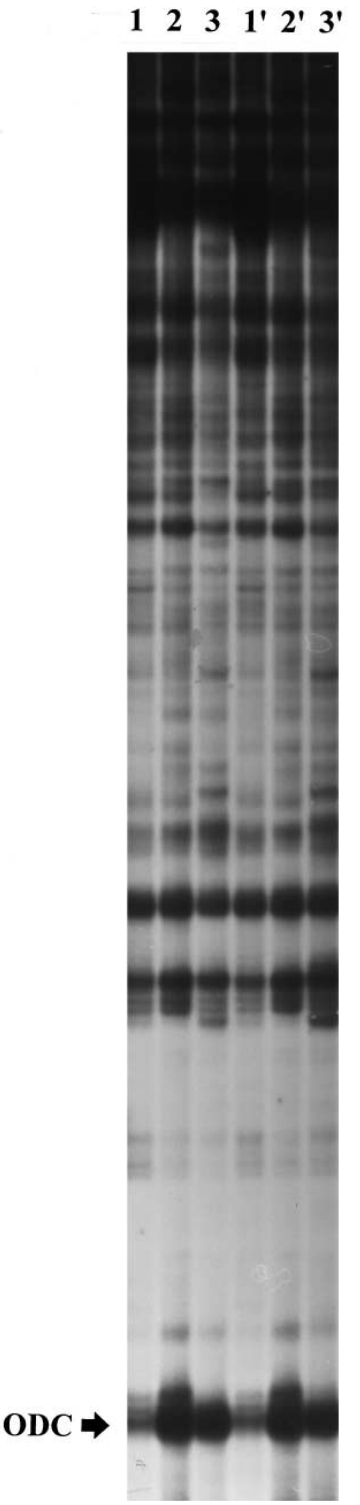

Fig. 1. Differential display of mibolerone-treated LNCaP Cells. DD was performed, in duplicate, on polyA$^+$ RNA from untreated LNCaP cells (lane 1) and cells exposed to 10 nM mibolerone for 8 (lane 2) or 48 (lane 3) h as described in Section 2. A representative experiment carried out in duplicate (marked with primed numbers) using primers $T_{12}VC$ and 1016 (lanes 1–3) is shown. A closed arrow indicates a clone that was subsequently confirmed as differentially expressed (ODC).

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DD :AGCCAGCGAA CTACCTATTG TAGCTTGTAC  30
ODC:---T--T--- -A-G-AT--- ----------  7896

DD :CATGGCAGA ATGGGCCAAAA GCTTAGTGTT  60
ODC:A-------- ----------- ----------  7926

DD : GTGACCCGTT TTTAAAATAA AGCAAAAAA  90
ODC: --C---T--- --A------- ---------  7956

DD : AAAAAA  96
ODC: ------  7962
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Fig. 2. Nucleotide sequence homology of a DD derived clone with human ODC. The nucleotide sequence of a DD derived clone and homology with the 3′ untranslated region of human ODC mRNA is shown. Dashes represent identical bases, flanking primer sequences are in boldface letters (1016 and $T_{12}VC$), the polyadenylation signal is underlined and base numbering correlates with previous references [21].

The kinetics of induction of ODC and PSA were very similar and the expression of both genes was decreased when cultured in DCC medium alone when compared to cells cultured in standard culture medium. This suggests androgen withdrawal decreases ODC and PSA expression and that their expression may be androgen dependent.

3.3. The effect of Casodex on ODC expression

LNCaP cells were exposed to the non-steroidal anti-androgen Casodex [22] for 24 h, followed by addition of 10 nM mibolerone, in the presence of Casodex, for a further 12–48 h. The expression of ODC was assessed by Northern analysis. There was no significant change in ODC expression when mibolerone was added in the presence of Casodex (Fig. 4). The abolishment of the mibolerone-induced increase in ODC expression by Casodex confirms that this effect is mediated through the androgen receptor. Casodex treatment alone did not affect ODC expression.

3.4. The effect of cycloheximide on ODC expression

LNCaP cells were exposed to the protein synthesis inhibitor, cycloheximide (Cx) for 1 h before exposure to 10 nM mibolerone for between 4 and 48 h. The expression of ODC was examined using Northern analysis. The expression of ODC was increased between 4 and 8 h exposure to mibolerone in the presence of Cx (Fig. 5). Interestingly, ODC expression appeared to be slightly augmented by Cx at these exposures. The expression of ODC reached a maximum of 2.6-fold above control levels after 8 h exposure to mibolerone in the presence of Cx, compared to a 2.1-fold induction of ODC by mibolerone alone (see Fig. 3). Between 8 and 24 h of mibolerone exposure, ODC expression remained elevated; after 48 h exposure, ODC expression fell to 1.6-fold above control levels, suggesting that the mibolerone-induced up-regulation of ODC expression is a primary response to androgen and does not require de novo protein synthesis. Identical experiments were performed examining PSA gene expression as a control. The mibolerone-induced up-regulation of PSA expression was unaffected by Cx and showed almost identical kinetics of induction to that seen by ODC (data not shown).

4. Discussion

Differential display is a powerful technique to identify genes that are present/absent or differentially expressed in one population of cells relative to another. We have used the DD procedure to identify elevated levels of ODC mRNA in response to androgen treatment of human prostatic cells. The rapid induction of ODC detected using DD was confirmed using Northern analysis, indicating that DD can detect temporal changes in gene expression. The kinetics of induction of ODC expression by androgens in LNCaP cells were almost identical to those of PSA when using Northern analysis [2], indicating that ODC is highly androgen responsive.

The up-regulation of ODC by androgen is mediated via the activated androgen receptor as Casodex, a competitive inhibitor of the androgen receptor, completely abolishes the mibo-

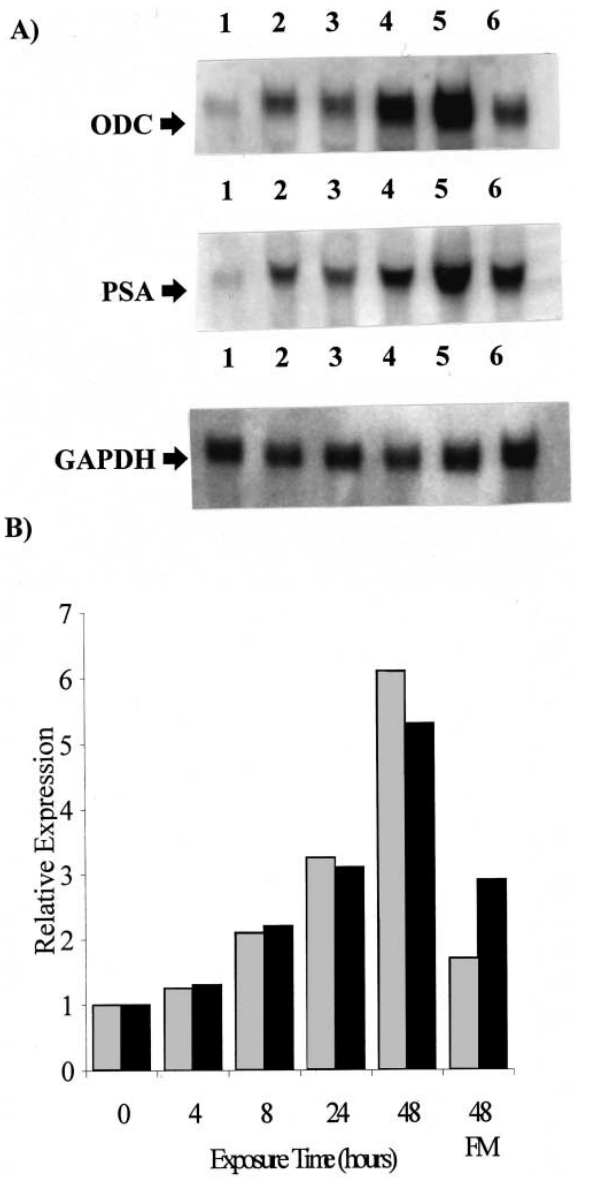

Fig. 3. Effects of 10 nM mibolerone on ODC and PSA expression. Northern analysis was performed using total RNA from untreated LNCaP cells (lane 1), cells exposed to 10 nM mibolerone for 4, 8, 24 and 48 h (lanes 2–5, respectively) and cells exposed to full medium for 48 h (lane 6/FM). (A) Blots were probed with an ODC cDNA then stripped and reprobed with a PSA cDNA. Blots were again stripped and reprobed with a GAPDH cDNA as a control for gel loading. (B) The expression of ODC (hatched bars) and PSA (solid bars) was normalised to GAPDH and the expression relative to untreated control cells was calculated.

lerone-induced up-regulation of ODC. Similar methods were used to implicate the androgen receptor in the androgen regulated expression of proliferating cell nuclear antigen [23]. The androgen receptor of LNCaP cells has a mutation at amino acid 868, which can cause certain anti-androgens to have a mildly androgenic effect [22]. However, Casodex is a highly specific anti-androgen and does not have any androgenic effects in LNCaP cells [22].

The androgen-induced up-regulation of ODC does not involve de novo protein synthesis, as cycloheximide failed to prevent the initial up-regulation of ODC. This suggests that ODC is directly regulated by the existing activated androgen receptor protein. Similar results have previously been reported for PSA [2]. However, ODC mRNA levels were reduced be-

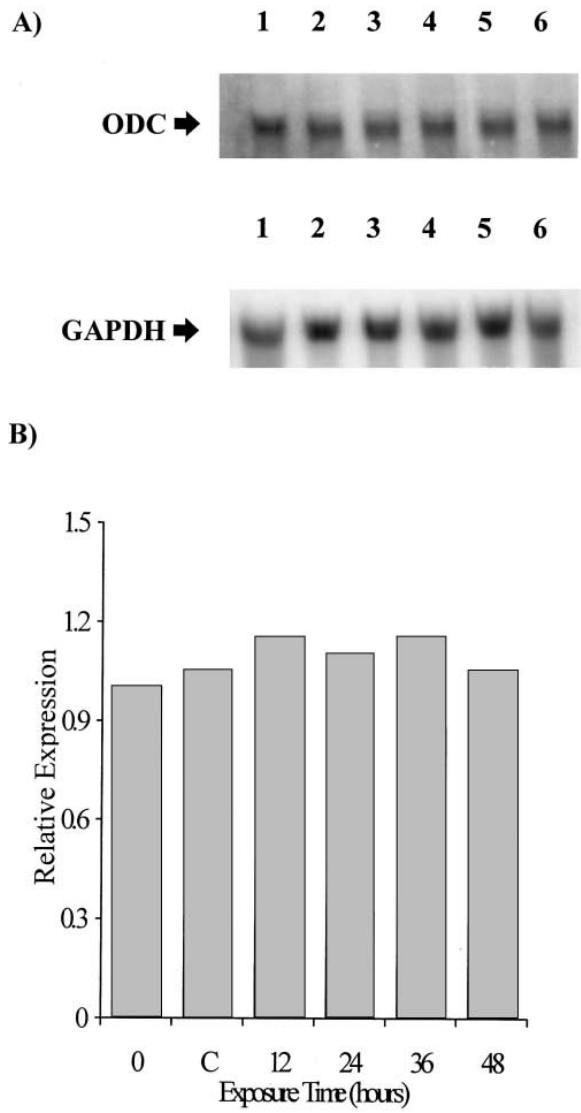

Fig. 4. Effects of Casodex on the mibolerone-induced expression of ODC. Northern analysis was performed using total RNA from untreated LNCaP cells (lane 1), cells exposed to 10 μM Casodex for 24 h (lane 2) and cells exposed to 10 μM Casodex for 24 h followed by 10 nM mibolerone for 12, 24, 36 and 48 h (lanes 3–6, respectively). (A) Blots were probed with an ODC cDNA, then stripped and reprobed with a GAPDH cDNA as a control for gel loading. (B) The expression of ODC was normalised to GAPDH and the expression relative to Casodex treated control cells (labelled 'C') was calculated.

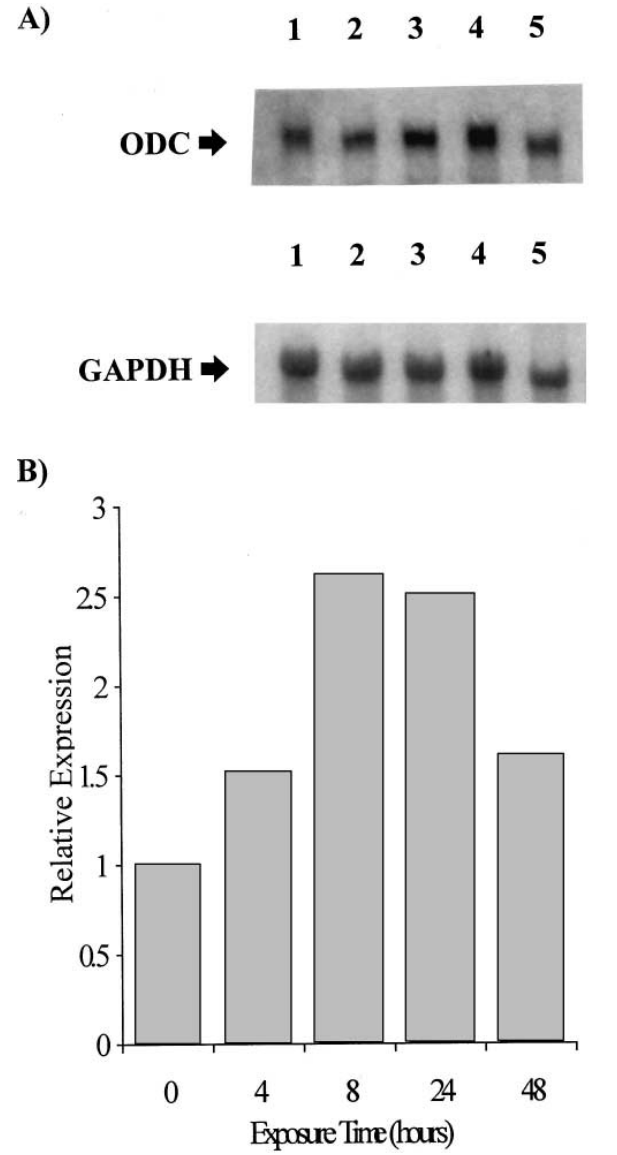

Fig. 5. Effects of cycloheximide on ODC expression. Northern analysis was performed using total RNA from LNCaP cells exposed to cycloheximide for 1 h (lane 1) and cells exposed to cycloheximide for 1 h followed by 10 nM mibolerone for 4, 8, 24 and 48 h (lanes 2–5 respectively). (A) Blots were probed with an ODC cDNA then stripped and reprobed with a GAPDH cDNA as a control for gel loading. (B) The expression of ODC was normalised to GAPDH and the expression relative to cycloheximide treated control cells was calculated.

tween 24 and 48 h exposure to mibolerone in the presence of cycloheximide. This could be due to either a breakdown in the cellular transcription machinery caused by inhibition of protein synthesis, or degradation of the ODC mRNA by virtue of its natural half life.

The androgen regulated expression of PSA has recently been characterized in detail [24,25]. The activated androgen receptor can bind to several androgen response elements and enhancer regions within the promoter region leading to transcription of the target gene. Thus, the androgenic regulation of PSA expression is directly influenced by the activated androgen receptor. However, although the expression of ODC has previously been shown to be directly regulated by andro-

gens in the rodent model system [14], the mechanism of androgen regulation of the human gene has not been elucidated. The 5′ untranslated region of the human gene contains sequences similar to steroid hormone responsive elements [21], although no functional androgen responsive elements have been found in the first 8 kb of the gene promoter region [26]. This suggests that either an androgen responsive element may be further upstream than 8 kb or androgenic regulation of ODC may not involve androgen receptor-DNA interaction, the latter of which has previously been reported for androgen receptor mediated transcriptional regulation [27].

The regulation of ODC by androgen has important implications in the androgenic control of cell growth and may in part explain the androgen dependent nature of certain tissues and cell lines. Similar genes that are influenced by androgens and are involved in cellular proliferation and progression of the cell cycle, such as early growth response gene α and proliferating cell nuclear antigen, have been previously reported [23,28]. This suggests that androgens may directly influence key cellular processes to regulate cell growth and differentiation. Further study into the regulation of ODC in the prostate will clearly give interesting insights into the mechanism of androgen dependent prostate cell growth.

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