The androgen receptor (AR), when complexed with 5α-dihydrotestosterone (DHT), supports the survival and proliferation of prostate cells, a process critical for normal development, benign prostatic hypertrophy, and tumorigenesis. However, the androgen-responsive genetic pathways that control prostate cell division and differentiation are largely unknown. To identify such pathways, we examined gene expression in the ventral prostate 6 and 24 h after DHT administration to androgen-depleted rats. 234 transcripts were expressed significantly differently from controls (p < 0.05) at both time points and were subjected to extensive data mining. Functional clustering of the data reveals that the majority of these genes can be classified as participating in induction of secretory activity, metabolic activation, and intracellular signaling/signal transduction, indicating that AR rapidly modulates the expression of genes involved in proliferation and differentiation in the prostate. Notably AR represses the expression of several key cell cycle inhibitors, while modulating members of the Wnt and notch signaling pathways, multiple growth factors, and peptide hormone signaling systems, and genes involved in MAP kinase and calcium signaling. Analysis of these data also suggested that p53 activity is negatively regulated by AR activation even though p53 RNA was unchanged. Experiments in LNCaP prostate cancer cells reveal that AR inhibits p53 protein accumulation in the nucleus, providing a post-transcriptional mechanism by which androgens control prostate cell growth and survival. In summary these data provide a comprehensive view of the earliest events in AR-mediated prostate cell proliferation in vivo, and suggest that nuclear exclusion of p53 is a critical step in prostate growth.

The development, maintenance, and function of the prostate gland requires the activity of the androgen receptor (AR) [(ref. 1)](http://www.jbc.org). AR is a steroid hormone receptor that when activated by its high-affinity ligand 5α-dihydrotestosterone (DHT) directs the expression of target genes by acting as a DNA-binding transcription factor. AR interacts with specific DNA sequences or with other proteins in the regulatory regions of target genes, and recruits cofactors that either enhance or inhibit the rate of transcription, depending on the nature of the regulatory element ([2]). Intracellular signaling pathways, cell cycle proteins, and other factors can modulate AR activity by modifying the receptor or interacting with it physically, providing a means for cross-talk between hormonal and growth factor signals ([3, 4]). Thus AR activity in the prostate is a complex sum of ligand concentration, input from signal transducing systems, and relative activity of transcriptional cofactors ([5]).

The importance of AR activity for prostate function and in prostatic disease progression such as in benign prostatic hyperplasia and prostate cancer (PCa) has been established ([reviewed in Ref. 6]). AR is expressed in the secretory epithelial cells, the surrounding periacinar stromal cells, and in perivascular smooth muscle ([1]). If AR activity is inhibited by any number of mechanisms, then the prostate undergoes involution because of apoptosis and atrophy in both the stromal and epithelial layers and to regression of the vasculature ([1]). This atrophy of prostate cells is due at least in part to a reduction in proliferation and -survival factors secreted by the stromal cells ([7]). Furthermore, many genes encoding seminal proteins are under direct regulation by AR in the secretory epithelial cells ([8]). Restoration of AR signaling rapidly promotes the survival, proliferation, and differentiation of prostate cells, leading ultimately to complete reformation of the functional organ and production of seminal proteins ([9]). At that time, unknown growth-limiting mechanisms make the organ refractory to further stimulation ([10]). In benign prostatic hyperplasia and PCa, these growth control mechanisms are often corrupted ([6]). Both diseases can be treated with anti-androgen therapy, demonstrating the critical role for AR in disease progression. Unfortunately, androgen blockade is not curative for PCa, and hormone-independent disease typically develops ([6]).
DHT Regulation of Prostate Gene Expression

Given the necessity for AR activity in prostate development, function, and pathogenesis, it will be important to determine the molecular events downstream of AR that promote growth and survival. However, few direct targets of AR are known, and the genes immediately downstream of AR in the prostate that regulate proliferation and differentiation remain largely uncharacterized. Because AR acts primarily as a transcription factor, differential gene expression studies are an ideal means to study the effects of AR in prostate cells. Several large-scale gene expression studies in prostate cells have been reported (e.g. Ref. 11), and these have increased the number of androgen-responsive genes known in the prostate. However, none have focused exclusively on identifying genes immediately downstream of AR that coordinate the multitude of physiological changes under androgenic control.

In this study we sought to identify genes that rapidly respond to DHT activation of the AR in the rat ventral prostate gland. We focused on gene regulation occurring before growth and organogenesis, reasoning that genes responding to DHT within that time would be enriched for direct targets of the AR that promote the survival and function of the prostate. Using large-scale gene expression analysis of ventral prostate samples collected 6 and 24 h after DHT treatment to castrated animals, 234 genes are shown to be androgen-regulated prior to the onset of cell proliferation. Approximately 200 of these genes were not previously known to be androgen-responsive. These genes were extensively reviewed for their potential roles in prostate physiology, allowing for a description of the molecular events involved in the initiation of prostate cell growth. We use this data to demonstrate that p53 inhibition by AR is an early event in androgen-stimulated proliferation. These findings confirm and extend previous observations and offer novel insights into the mechanisms by which androgens regulate prostate physiology.

MATERIALS AND METHODS

Animals—All animals were treated according to Animal Care and Use Committee guidelines. 3–4-Month-old 250–300 g Sprague-Dawley rats were obtained from the vendor after orchiectomy or sham operation. Nine days following surgery, animals were injected daily with 3 mg/kg 5α-DHT (Steroids) in propylene glycol or with propylene glycol alone. Six or 24 h later, animals were euthanized by CO2 and ventral prostate (VP) was collected and weighed before being frozen in liquid nitrogen (RNA or protein analysis) or fixed in paraformaldehyde for sectioning and staining.

Microarrays—Rats were used per treatment. RNA was collected from ventral prostates using Triozol (Invitrogen) according to the manufacturer’s instructions. After treatment with Dnase I (as directed by the manufacturer, GenHunter), 5 distinct pools of RNA, each from 3 animals, were generated per group by mixing equal amounts of RNA from individual samples. These n = 3 RNA pools were each used to probe a single microarray chip, providing 3 non-redundant replicates per treatment. Hybridizations were performed as directed by Affymetrix (97). Briefly, cDNA was synthesized from total RNA by SuperScript II primed with oligo(dT) coupled to the T7 promoter. Double-stranded cDNA was captured using QIAGen columns (Qiagen) and used as the template for in vitro transcription by T7 RNA polymerase (MEGAscript T7 kit, Ambion) in the presence of biotin-labeled nucleotides (MEGAscript T7 kit, Ambion). Nuclei were captured using streptavidin-R-phycoerythrin using a fluorescence scanner (Amersham Biosciences).

Microarray Analysis—In pilot experiments we confirmed the sequence of several microarray probes by mass spectrometry, verified their specificity toward their intended transcript by BLAST search, and optimized the normalization and analysis methodologies such that a high correlation (r² = 0.91) between microarray and quantitative real-time RT-PCR (QRT-PCR) data were achieved (data not shown). Data were normalized using the hybridization intensities of multiple housekeeping genes. Then the normalized mean fluorescent intensity for each probe set (probesets consist of 16–25 oligonucleotides) was compared against the experiments using the SAFER algorithm (100). In this approach the inherent variability of each probe set is determined (in this case, based on 18 hybridizations of different VP RNA samples) and used to assign a probability that the normalized mean signal from probesets significantly varied from controls by ANOVA. For a transcript to be considered differentially expressed: 1) the hybridization signals must have exceeded the experimentally determined background, and 2) must have tested different from vehicle control (p < 0.05) at both 6 and 24 h with an absolute difference of >1.5-fold normalized fluorescent intensity units at one of those time points. Gene names were determined by BLAST search of GenBank sequences. Probesets directed toward sequences that were not ≥90% homologous to a known full-length gene were excluded.

Functional Clustering—Genes were then grouped by function. First, genes encoding enzymes involved in specific biochemical pathways (e.g. hexokinase II) were placed into a functional category annotating the metabolic process (e.g. glycolysis). Each remaining gene was analyzed extensively using the primary literature at NCBI PubMed to determine the commonly accepted function of each gene product. In cases where gene products perform multiple functions, the best documented or most prostate-relevant function was subjectively determined. Each functional category was then assigned to a general category (e.g. glycolysis is included in general metabolism), and in some cases these general categories were further grouped (e.g. lipid metabolism and general metabolism were grouped into metabolism). Signal transduction and transcription factor genes were not assigned to subcategories, because their protein products often function in multiple cellular processes.

Quantitative RT-PCR—Gene-specific primers and probes (Applied Biosystems) were (F, forward; R, reverse; P, probe; all probes labeled 5’-mix–Cy5 (MEGAscript T7 kit, Ambion) in the presence of biotin-UTP and -CTP. These biotin-cRNA transcripts were collected on RNeasy columns (Qia-gen) and then hydrolyzed to 50-nucleotide fragments by 10 mM MgCl2 at 95°C for 35 min. Ten-mg aliquots of these probes were hybridized to the Affymetrix RGU34A arrays. Arrays were sectioned, and stained with hematoxylin and eosin. Histomorphometric analyses used the Bioquant Nova V4 semiautomated image analysis system (R&M Biometrics, Nashville, TN). To determine distance from nuclei to luminal surface 10 epithelial cells from 10 glandular structures were measured from each specimen. For cell proportion 6 glan- dular structures from each specimen were analyzed, and all nuclei within the basal lamina (considered “epithelium”) and those outside the basal lamina (“stromal”). These numbers were expressed as a ratio then averaged for each section and tested for significance by ANOVA.

Cell Culture and Protein Analysis—For prostate samples, tissue was collected frozen in liquid nitrogen, and proteins were extracted in 2% SDS. LNCaP cells were obtained from the American Type Culture Collection and maintained in RPMI medium 1640 with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. Cells were treated for 24 h with 5α-DHT or OH-flutamide (Sigma), with or without 10 μM cycloheximide (Sigma), then fractionated into cytosolic or nuclear fractions by homogenization in hypotonic HEPES, HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl2, 2 mM EDTA, 0.1% Nonidet P-40, and 1× protease inhibitor mixture (Roche Diagnostics). Nuclei were collected by centrifugation at 1000 × g, then the supernatant was removed, and nuclear proteins were extracted in 20 mM Tris-HCl, pH 8.0. These solutions were added to 2× Laemmli sample buffer. All samples were boiled for 5 min and run on a 12% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h and probed overnight at 4°C with polyclonal antibodies specific for p53 (Santa Cruz). Membranes were washed in TBS-T and incubated for 1 h with horseradish peroxidases-conjugated secondary antibodies (Santa Cruz). Blots were developed with ECL (Amersham Biosciences) according to manufacturer’s instructions. 

Western Blot analyses were performed as described by others (98).
8.0, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, and protease inhibitors on ice for 1 h. Protein concentrations were determined by the BCA method (Pierce), then equal amounts of protein were mixed 1:1 with 2× Laemmli buffer. Samples were boiled for 5 min, resolved on 4–20% gradient SDS-PAGE gels (Bio-Rad), and transferred to nitrocellulose. Coomassie staining of replicate gels verified equal loading. p53 was detected by anti-p53 monoclonal (Sigma) followed by horseshadish peroxidase-linked secondary antibodies (Amersham Biosciences) and chemiluminescent detection (Pierce). Results were quantified with a densitometer (Bio-Rad) and tested for significance using ANOVA. Immunofluorescence was performed in LNCaP using standard procedures with anti-p53 (Sigma), propidium iodide, and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody.

Bioinformatics Search for Potential Androgen Response Elements—The strategy for finding putative androgen response elements (AREs) is similar to the weight matrix searching method employed by the TESS software (99). A list of 28 experimentally defined AREs was compiled (see Supplemental Materials Table II). The aligned AREs were used to generate a weight matrix of log base 2 nucleotide frequencies at each position. A pseudocount of 0.1 was added to each nucleotide occurrence. Query sequences were scored by summing the individual nucleotide positions and normalizing to the range of possible scores, such that the quality of the match was reported as a number between 0 and 100. Putative AREs were searched in 5′ upstream regions of genes of interest. For each rat gene, mouse and human orthologs were identified based on LocusLink assignments. The 5′ transcript ends were approximated from available RefSeq mRNAs available in the public databases. 5 kb of upstream sequences were generated from the University of Southern California genome assemblies, January 2003 release (rat), February 2003 (mouse), and April 2003 (human). Each 5-kb upstream sequence was scanned with the ARE weight matrix and the top 10 scoring sequence elements were compared among orthologous genes with respect to score, position relative to the transcript start, and nucleotide sequence.

RESULTS

Experimental Strategy—To identify genes that are immediately downstream of AR and thus control cell growth and differentiation, DHT or vehicle alone were given to ORX rats and RNA samples were collected after 6 and 24 h. DHT was chosen as the ligand because, unlike testosterone, it cannot be aromatized and activate estrogen receptors. This time period precedes revascularization and mitosis (12, 13), which occur 2–3 days after androgen repletion. To confirm that the VP were collected prior to rapid growth, we measured the effects of DHT on VP weighting ORX rats after 6, 24 h, and 17 days. Prostate weight was completely restored after 17 days, confirming that this dose of DHT is fully effective. However, no significant change in prostate weight was observed after 6 or 24 h of DHT treatment (Fig. 1A). Histological examination revealed no gross changes in cell proportion or vascularization (Fig. 1B), and histomorphometric analysis confirmed that the proportion of nuclei in the epithelial layer to nuclei in the stromal layer was not different (Fig. 1C). DHT did, however, produce a significant increase in the distance from the luminal surface to the epithelial nuclei (Fig. 1C). These data confirm that the VP samples were derived from similar proportions of the most abundant cell types, but also show that the DHT-treated VP were in the early stages of epithelial reorganization and differentiation.

Identification of Androgen-responsive Genes—VP were collected from 3 groups of 3 animals from each of the 4 treatment groups (vehicle 6 h, vehicle 24 h, DHT 6 h, and DHT 24 h) so that the gene expression experiments could be performed on triplicate independent RNA samples. Using high-density oligonucleotide microarrays, the relative abundance of ~7000 transcripts was then measured. The mean signal intensity of each transcript was tested for significant difference from time-matched controls using error models specific for each gene. Transcripts were considered to be regulated if the mean hybridization signals were different (p < 0.05) at both 6 and 24 h from their corresponding vehicle controls, and were at least 1.5-fold regulated at one time point. To allow functional interpretation of the data, only genes that encode for a known protein were included. 234 genes met these criteria and are presented in Supplemental Materials Table I.

Reproducibility of Gene Expression Data—To assess the reproducibility of the hybridization data, we analyzed the primary literature and tested a subset of genes in a replication experiment. First, the regulation of at least 29 of the 234 genes is supported by published data (Supplemental Materials Table I, bold type). Then 8 genes with various levels of regulation were evaluated by QRT-PCR in an independent group of animals. All 8 genes showed similar changes in expression in both experiments, and there is quantitative correlation between QRT-PCR and microarray results (Fig. 2). Thus the criteria used for data mining produced reproducibly regulated genes, with the caveat that a small fraction might falsely appear regulated because of Type I statistical error (predicted to be less than 1 transcript) or infidelity of the oligonucleotide probes.

Functional Categorization of Regulated Genes—We then mined published literature to organize the androgen-responsive genes into functional clusters so that the effects of androgens on whole genetic pathways could be studied. Each gene was extensively reviewed for its potential role in prostate physiology by analyzing human and rodent genetic data, biochemical studies, and experiments conducted in cultured prostate and other cells. After this manual data mining exercise, genes were grouped according to function (Fig. 3). To describe the behavior of genes within functional groups, genes were assigned to one of three temporal categories (maximal at 6 h, approximately equal at both 6 and 24 h, and maximal at 24 h), and scored as either induced or repressed.

This analysis allows for a characterization of the first 24 h following AR activation in the VP. Consistent with the effects of androgens on prostatic secretory activity (14), over one-third of the rapidly regulated genes function in the various steps of polypeptide synthesis, maturation, trafficking, and degradation (26.5%), are involved in vesicle trafficking, or encode secreted proteins (10.3%). The majority of these genes were up-regulated. A second major effect of DHT is the metabolic activation of the prostate. 59 (25.2%) of the genes encode proteins involved in metabolic processes; 17 of which are involved in lipid and cholesterol metabolism. Finally, 84 (34.9%) of the regulated genes were classified as intercellular ligands, receptors, signal transducers, transcription factors, and cell cycle/apoptosis effectors. In contrast to protein synthesis and secretory genes, these clusters tended to have roughly equal proportions of induced and repressed genes, perhaps reflecting that both inductive and inhibitory mechanisms are important in prostate growth (see below). Finally, only a small proportion (2.1%) of the regulated genes were classified as involved in cell adhesion/extracellular matrix, in agreement with the absence of major morphological changes (Fig. 1). Thus prior to proliferation, DHT has profound effects on the expression of genes involved in secretory activity, metabolism, and intracellular signaling/signal transduction. Each of these major findings is explored below.

Induction of Secretory Activity—AR activation in the androgen-depleted prostate induces epithelial reorganization (Fig. 1), consistent with the known androgenic control of seminal protein secretion by the prostate (15, 16). The microarray data reveals that the multiple steps in protein secretion are regulated at the level of gene expression by DHT (Supplemental Materials Table I). Nascent polypeptides targeted for secretion are accepted into the endoplasmic reticulum by the signal
sequence receptor, glycosylated by the oligosaccharide transferase complex, folded by the activity of chaperones, and transported to the Golgi for additional processing and eventually exocytosis. All of these initial steps in polypeptide secretion appear to be up-regulated by AR: DHT induced the expression of genes that code for components of the signal sequence recep-

**Fig. 1.** A, effects of DHT on rat ventral prostate weight. Following orchiectomy or sham operation (Sham), animals \( (n = 9) \) were treated for the indicated times with vehicle (CON) or vehicle + 3 mg/kg DHT and sacrificed at the indicated times. Ventral prostate wet weight is expressed as a percentage of total body weight and is shown ± S.D. *, different from all 6- and 24-h samples and 17 day control; **, different from all other groups \( (p < 0.01 \) by ANOVA). B, representative sections of rat ventral prostate from animals treated for 24 h with either vehicle (Control) or 3 mg/kg DHT, and fixed and stained with hematoxylin and eosin. C, histomorphometric analysis of ventral prostate sections. Nuclei were scored as either residing in the epithelial layer (including basal cells) or in the stromal layer in sections from animals treated for 24 h with vehicle (control) or 3 mg/kg DHT treated (DHT). The average ratio of epithelial layer to stromal layer nuclei determined for each animal, and the values were averaged by group. Error bars are ± S.D. Right, the distance between the luminal side of the epithelial nuclei and the lumen surface shown as a function of treatment group, ± S.D. *, different from control values \( (p = 0.005) \).
FIG. 2. Validation of microarray results. ORX rats of the same weight and age as those used in the microarray experiments were treated with vehicle or 3 mg/kg DHT \((n = 3\) sets of 3 animals per treatment group). 6 or 24 h later, ventral prostates were harvested and the expression of the indicated gene was analyzed relative to an internal standard using QRT-PCR. The values for time-matched vehicles were set to 1 and are omitted for clarity. Gray bars are the values generated by QRT-PCR, black are the data from the microarray experiment \((\pm \text{S.D.})\). Bottom, the values from the QRT-PCR experiments were plotted against the corresponding values from the microarray experiment, along with results of the regression analysis.
tor (such as SSRΔ), the oligosaccharide transferase complex (ribophorins-1 and -2, defender against cell death-1, and glucosidase-1), and endoplasmic reticulum-resident chaperones (e.g. calreticulin, protein-disulfide isomerase, and BiP). Many prostate secretory products undergo complex glycosylation, a process regulated by androgen levels (17). UDP-galactose 4-epimerase, UDP-galactose: N-acetylglucosamine 1,4-galactosyltransferase, and 1,3-fucosyltransferase were induced greater than 15-fold at 24 h and thus are likely to be involved in complex modification of seminal proteins. Furthermore, 17 genes involved in vesicle trafficking were induced, most of which function in transport from the endoplasmic reticulum to the Golgi. Notably these genes include the ADP-ribosylation factors Arf3, Arf4, and Arf11, as well as the Golgi protein gene p115. Because these proteins induce Golgi biogenesis (18, 19), an effect of androgens in prostate epithelial cells (15), they might function in the expansion of the secretory apparatus. These data together indicate that AR rapidly promotes the formation of an active secretory pathway at the level of gene expression.

**Metabolic Activation**—Androgens control the metabolic activity of the prostate, as androgen withdrawal affects the biosynthesis and catabolism of several classes of biomolecules (20). The gene expression data allows for several of these biochemical observations to be explained, at least in part, at the level of transcriptional regulation. For example, androgens regulate the lipid and cholesterol content of normal prostate tissue and prostate cancer cells (16, 21). This phenomenon might be related to proliferation or the abundant synthesis of cholesterol-rich vesicles (prostasomes) of the prostate that are secreted into seminal fluid (22). Studies in LNCaP cells indicate that elevation of cholesterol and lipid metabolism likely result from induction of the sterol response element-binding proteins (SREBP) and the SREBP cleavage activating protein, which control the transcription of cholesterol and lipid synthesizing genes (21, 23). This phenomenon also occurs in vivo; 12 genes encoding cholesterol synthetic or lipid modifying enzymes, as well as SREBP-1c, were induced by DHT (Supplemental Materials Table I; SREBP cleavage activating protein and SREBP-2 probesets are not on the microarray). Overall, these data point to the induction of SREBPs as an important step in prostate growth and/or differentiation.

**Glycolysis**—Androgens stimulate glycolysis in the prostate (24, 25), and prostate cancer cells exhibit elevated glucose consumption, a common feature of the transformed phenotype (26). Increased glycolysis in prostate cells can be attributed in
part to elevated pyruvate dehydrogenase (PDH) activity (27). This observation might be explained by the repression of the PDH inhibitory subunit gene PDH kinase 2 (Supplemental Materials Table I). Furthermore, hexokinase II, which initiates glycolysis by phosphorylating glucose, was induced 5.5-fold at 6 h, consistent with observations that hexokinase activity is induced by androgens (25). Thus AR activation likely promotes glucose utilization by repression of PDH kinase 2 and induction of hexokinase II. Whereas it is unclear whether PDH kinase 2 is repressed by AR directly or via other factors, hexokinase II is repressed by p53 (28), suggesting that p53 inhibition by AR could play a role in elevated glycolysis and rapid growth in prostate cells (see below).

**Cell Growth and Survival**—Next, the mechanisms by which AR initiates cell proliferation were examined. DHT induced the expression of cyclins D1 and D3, which promote DNA synthesis (29) (Fig. 4). There was concordant down-regulation of several cell cycle inhibitor genes such as retinoblastoma 1, Id-2, and BTG-2/TIS21/PC3. Error bars represent standard deviation.

**AR Suppression of p53**—These data led us to examine p53, because it inhibits prostate growth (e.g. Ref. 33), both by inducing the expression of cell cycle inhibitors and pro-apoptotic genes, and possibly by interfering with the activity of AR (34). Although p53 RNA levels were unaltered by DHT, the expression of several of its key regulators were (Fig. 5). Mdm2, a p53 target gene that induces its degradation in a feedback loop (35), was induced, whereas the Hoxa5 gene, which promotes p53 expression in breast tumors (36), was repressed. Furthermore, early growth response-1 (Egr1) RNA levels were reduced. Egr-1 is typically an anti-proliferative, pro-apoptotic protein that, in certain cell types functions by activating p53 expression (37). These data are consistent with AR repressing p53 function indirectly by altering the expression of its key regulators. In agreement with that possibility, the p53-activated genes Rb, Scyld/fractalkine, IGF-BP3 (38), and BTG-2/TIS21/PC3 were down-regulated, whereas hexokinase II, a p53-repressed gene (28), was induced (Fig. 5).

Prompted by this pattern of gene expression we tested the possibility that AR reduces p53 activity by inhibiting the accumulation of p53 protein. LNCaP nuclear extracts were analyzed by anti-p53 immunoblot 24 h after treatment with DHT or with the AR antagonist 4-hydroxyflutamide. DHT significantly decreased nuclear p53 levels, whereas 4-hydroxyflutamide did not (Fig. 5). Interestingly, 4-hydroxyflutamide acts as an agonist in LNCaP cells because of a threonine to alanine substitution in amino acid 868 in AR (39). However, DHT and 4-hydroxyflutamide are not equivalent in LNCaP cells, as has been shown before with other androgenic effects (40, and here. To confirm this pattern of gene expression we tested the possibility that AR reduces p53 activity by inhibiting the accumulation of p53 protein. LNCaP nuclear extracts were analyzed by anti-p53 immunoblot 24 h after treatment with DHT or with the AR antagonist 4-hydroxyflutamide. DHT significantly decreased nuclear p53 levels, whereas 4-hydroxyflutamide did not (Fig. 5).

**Peptide Hormones**—Neuroendocrine signaling in the prostate has important roles in normal prostate physiology and the progression of PCa (53, 54). The microarray data demonstrate that androgens regulate peptide hormone synthesis, maturation, degradation, and the expression of their receptors (Fig. 7).
In terms of peptide hormone synthesis, there was repression of both peptidylglycine α-amidating monooxygenase (pam), which activates certain peptide hormones by α-amidation (55), and its proposed trafficking co-factor PAM COOH-terminal interacting protein 1 (pcip-1) (56). Conversely the novel gene pcip-10, which promotes PAM degradation (57), was induced. These regulations would be expected to reduce active PAM protein, as previously proposed (58). Furthermore, the transcript for the peptide hormone degrading enzyme neutral endopeptidase (Nep) was up-regulated. Nep is induced in prostate epithelial cells by AR activation of its promoter (59) and is likely involved in tumor progression, as it is frequently lost in androgen-independent disease (60). Together these data suggest that during growth initiation, AR inhibits certain peptide hormone(s) by reducing PAM expression and inducing the degradative enzyme NEP.

Thus we examined the regulation of peptide hormone signaling genes in search of potential growth-limiting factors (Fig. 7). Two peptide hormone signaling system genes were repressed, prodynorphin and oxytocin. Prodynorphin, which is synthesized in enkephalinergic prostatic nerves (61), was down-regulated, in agreement with its proposed anti-proliferative effects (62). Also the oxytocin receptor was repressed 4-fold by 6 h, mirroring previous reports showing that oxytocin itself is DHT-repressed (63). Oxytocin is synthesized locally in the prostate, and has been implicated in epithelial growth and smooth muscle contractility (64). However, the data presented here and in previous studies (63) suggest that oxytocin might also be...
growth inhibitory, and its suppression could be an early event in prostate cell proliferation.

Notch and Wnt—The gene expression data supports growing evidence that the Notch and Wnt pathways, regulators of growth, differentiation, and tumorigenesis in many tissues (65), (66), might also act in the prostate (Fig. 8). In the Notch pathway, notch1 and its ligand jagged1, were repressed, and a negative regulator of Notch signaling, sel-1l (67), was induced, observations consistent with inhibition of Notch signaling. In contrast a decoy receptor for wnt signaling molecules, secreted frizzled related protein-4 (Sfrp4) was repressed, which could indicate that DHT modulates wnt signaling through membrane frizzled receptors. Several genes involved in Notch and Wnt signaling have altered expression in benign prostatic hyperplasia and/or PCa (Notch1 is expressed in the prostate epithelium and inhibits the growth of PCa cells in vitro) (68); Frizzled 1, Frizzled Related Protein B, Frizzled 7, and Sfrp4 were differentially expressed in several clinical specimens (69). β-Catenin, the major signaling protein in the canonical Wnt signaling pathway, associates with AR and is transported with it to the nucleus where it directly regulates inositol polyphosphate levels within the endoplasmic reticulum lumen (75), S100A, and calcineurin B type I. Interestingly the activity of MINPP1 is similar to PTEN, a well known regulator of prostate cell survival, and its gene may be mutated in thyroid carcinomas (76). The genetic locus containing PTEN and MINPP1 (10q23) is frequently lost in PCa, but inactivating mutations in PTEN are less frequently found (77). Although loss of a single PTEN allele could explain this observation (78), the data here and the loss of MINPP1 in thyroid cancer warrants examination of this gene in PCa.

Transcription Factors—The prostate may be similar to other tissues in that an initiating event (in this case, AR activation) induces a cascade of transcription factors that coordinate a gene expression program. Several transcription factors were regulated by DHT and thus could be components of this hypothetical transcriptional cascade (Supplemental Materials Table I). These include egr-1, Id-2, hoxa5, and SREBP-1c, which are discussed elsewhere. Other transcription factors are likely to play a more general role. For example, AP-1 regulates the expression of many genes, and there was significant repression of the AP-1 components fra-2 and c-jun. AP-1 complexes accumulate in the nuclei of prostate cells after androgen deprivation (79, 80). Thus AR appears to repress AP1 activity in part by inhibiting expression, in addition to direct inhibition by protein-protein interaction (81). Cited2, which encodes p35srj/MRG1, a co-activator for CBP/p300 and AP-2 that is required for the development of multiple tissues (82), was induced 7-fold. Interestingly, this transcription factor is a negative regulator of hypoxia inducible factor-1α, a critical effector of hypoxia-driven gene expression (83). It will be important to determine whether this gene is involved in the response of normal and cancerous prostate cells to hypoxic conditions. Members of the nuclear factor-I family facilitate AR transcriptional regu-
cell cycle control (Transcription factor HNF-3/forkhead homolog-1 (Hfh1) was repressed during prostate growth. Finally, the winged helix/forkhead signaling might have an important role in the prostate cell proliferation.

Microarray data from the ventral prostates of orchidectomized rats treated with DHT for 6 or 24 h were analyzed using manual data base mining to identify genes involved in these pathways. Shown here are the mean (n = 3) RNA expression values from the microarray (normalized to time-matched vehicle controls) for Notch1, its ligand Jagged1, the Notch cofactors presenilin-1 and Sel-1L, and the wnt decoy receptor Sfrp4. Error bars represent standard deviation.

Identification of Potential Androgen Response Elements—Biochemical studies of several androgen-responsive genes show that AR typically binds to a 6-nucleotide half-site that resembles the consensus sequence 5'-TGTTCT-3'. In most cases a partially palindromic half-site is spaced 3 nucleotides upstream of this sequence and serves as a binding site for the second AR molecule in the transcriptionally active AR homodimer. These sequences can be represented multiple times in the promoters of AR-regulated genes and can occur very close to the transcriptional start site, or can be found in enhancers several kilobase pairs upstream. The biochemically validated AR binding sites from direct transcriptional targets of AR were used to generate a weighted matrix that scores DNA sequences for similarity to these androgen response elements (AREs, see "Materials and Methods"). Then genomic DNA sequences within 5000 nucleotides of the first known transcribed nucleotide were gathered from rat, mouse, and human genomes, and high-scoring sequences were identified in all three species. Genes were selected as potential direct AR targets based on the following criteria. First, the RNA must have been induced more than 2-fold at 24 h. Second, the 5 kb upstream of the first known transcribed nucleotide must contain at least 2 AREs that score greater than 84 of a possible 100 in all three species (76 is the minimum score possessed by the bona fide AREs used to generate the matrix). Finally, reasoning that sequences conserved through evolution are more likely to be functional, both the position of the potential AREs and their nucleotide composition were required to be similar across all three species. These criteria yielded 11 genes (Fig. 9), including those involved in protein synthesis and secretion (e.g. signal sequence receptor Δ, BiP, defender against cell death-1), metabolism (e.g. farnesyl diphosphate synthase, steroid 5α-reductase, hexokinase II), and cell signaling/proliferation (e.g. prolactin receptor, SC65, and BAX). Although further experimentation is required to confirm that these conserved putative androgen receptor binding sites are functional, this analysis suggests that these genes could be direct transcriptional targets of AR and thus mediators of downstream physiological changes.

**DISCUSSION**

AR, when bound to DHT, directs the expression of genes in the prostate gland that controls growth, function, and development of benign prostatic hypertrophy and prostate cancer. The few known direct targets of AR in the prostate, such as pro-basin (86), prostate specific antigen (87), C3(1) (88), ornithine decarboxylase (89), and neutral endopeptidase (59) are unlikely to account for these multiple effects. More importantly for the understanding of prostatic disease, little is known about how androgens induce quiescent prostate epithelial cells to enter the cell cycle. Here we identify 234 previously characterized, protein-encoding genes that rapidly respond to AR activation prior to cell division, revascularization, and differentiation. Most of these genes had not been reported to be androgen-dependent, perhaps because most published gene expression studies focus either on the effects of androgens on prostate cancer cells, which differ fundamentally from normal prostate epithelial cells, or on the long-term effects of castration and testosterone repletion. The reliability of the data was ensured by two strategies: 1) using triplicate independent samples so that gene-specific error models could be used to assign p values; and 2) requiring that genes be significantly regulated at both 6 and 24 h. The regulation of at least 29 of these genes is supported by published data, and each of the genes that were tested for reproducibility in a replication experiment responded as predicted (Fig. 2). Thus we conclude that this data set is comprised of genes that are androgen-responsive in vivo, and thus expands our current knowledge of androgenic effects in the prostate by ~200 genes. However, transcript abundance could be altered by many mechanisms, including direct transcriptional regulation, changes in the rate of synthesis or degradation, changes in levels of circulating hormones or metabolites, or alterations in the proportions of specific cell types. Limiting the analysis to genes expressed differentially from controls within 24 h after dosing should limit some of these confounding factors and enrich for direct targets of AR activity. Bioinformatics strategies aimed at identifying transcription factor binding sites as described here (Fig. 9) could eventually identify regulatory circuits downstream of AR active in prostate cells in vivo.

To analyze the significance of this set of rapidly androgen-responsive genes we employed literature-based data mining methodology. Each gene was extensively reviewed to determine its best known function, protein localization patterns in various tissues, and expression in prostate and other epithelial cell carcinomas. Genes whose protein products function in distinct physiological pathways were clustered on this basis (Fig. 3). This strategy is in contrast to many gene expression studies that classify genes primarily or exclusively on their expression patterns. From this analysis we observed three major early effects of AR activation in the prostate: induction of genes involved in the secretory apparatus, metabolic activation, and regulation of intracellular communication/signal transduction/cell cycle control. Genes whose protein products function in virtually every step of the secretory pathway were up-regulated by DHT, giving insight into the hormonal control of epithelial cell secretory activity. Interestingly, there might be a temporal order to the changes in these synthetic and secretory genes. For example, genes involved in ribosomal biogenesis were maximally induced by 6 h; genes involved in amino acid synthesis and mRNA splicing were equally expressed at both 6 and 24 h, whereas genes active in translation, folding, processing, and trafficking were not maximally induced until 24 h (Fig. 3).

A second finding is that AR activation sets into motion rapid changes in the metabolic status of prostate cells (Fig. 3, Supplemental Materials Table I). At the level of gene expression,
cholesterol and lipid synthesis appears to be induced by AR indirectly via the activity of SREBP proteins, as described in LNCaP cells previously (21, 23). Rapidly growing cells, including normal and cancerous prostate cells, often increase glucose utilization (24, 25), and here we identify specific glycolysis-regulating genes (PDH kinase 2 and hexokinase II) that respond to DHT. Finally, a number of other metabolic genes are modulated by DHT prior to cell growth and differentiation, including nucleotide and amino acid biosynthesis, polyamine production, oxidative stress, the glutathione system, and steroid metabolism (Fig. 3). Each of these metabolic processes are androgen-sensitive, and several are altered in prostate cancer (90). Careful examination of the gene expression data should in turn provide molecular explanations for these effects of androgens on intermediate metabolism and potentially provide novel targets for the inhibition of prostate cell growth or survival.

The mechanisms by which AR leads to prostate cell proliferation are unclear, but likely involve the regulation of trophic factors and repression of cell cycle inhibitors such as p16 and others (13). Here we gain a glimpse into the initial stages of prostate cell proliferation in vivo. As evidenced by the induction of cyclins D1 and D3, the prostate cells examined in these experiments were most likely entering the G1 phase of the cell cycle. During this time, the cell cycle inhibitors retinoblastoma (Rb), BTG2/TIS21/PC3, and ID-2 were repressed (Fig. 4). Furthermore, the key growth regulator, tumor suppressor, and pro-apoptotic protein p53 appeared to be repressed at the level of protein abundance or activity, as the RNAs for some of its key regulators such as MDM2 were androgen regulated, and several known p53 target genes were altered (Fig. 5). This possibility was confirmed in vitro, where we show that AR induces substantial and rapid nuclear exclusion of p53. As MDM2 induces the nuclear export and degradation of p53 (91), MDM2 induction by AR might be a critical step in prostate growth regulation. Consistent with this hypothesis, MDM2 is often overexpressed in advanced prostate tumors and associated with reduced apoptosis (92, 93).

The role of p53 in prostate growth and the hormone sensitivity of PCa cells has been clearly established. Antisense inhibition of p53 in LNCaP cells induces a hormone-resistant phenotype (94), whereas overexpression of wild-type p53 into prostate cancer cells inhibits their growth (33). The interplay between p53 and AR is likely an important component of the hormone sensitivity of prostate cells. p53 inhibits AR through intermediate factors as well (34), suggesting the existence of mutual antagonism between these two proteins. These data provoke a model in which AR regulates genes involved in p53 regulation such as MDM2. p53 nuclear exclusion leads to a number of events including the reduced transcription of cell cycle inhibitors such as Rb, enhanced IGF-1 signaling by reduction in IGF-BP3 expression, increased glucose utilization through de-repression of hexokinase II, and overall greater androgen sensitivity because of de-repression of AR. Further experimentation will be required to determine whether this pathway operates in vivo and if it contributes to prostate tumorigenesis.

Paracrine and autocrine growth factor signaling is a key component of growth regulation in sex steroid-dependent s...
creatory tissues such as the prostate (1). IGF-1, epidermal growth factor, TGF-β2, and adrenomedullin were all rapidly regulated by AR activation in these experiments (Figs. 6 and 7). These data confirm the validity of the microarray strategy used here for identification of key regulators, as all of these signaling proteins play important roles in prostate growth (1). We also identify several genes whose importance in prostate homeostasis is currently less clear, including granulin/epithelin, ephrin-A1, oxytocin (via rapid down-regulation of the RNA for its receptor), and prodynorphin. It will be important to evaluate these genes in prostate cancer specimens and in animal models. Finally, data presented here (Fig. 8) in conjunction with published information (68, 70, 71) suggests a role for Notch and wnt signaling pathways in prostate development. We speculate that presenilin-1, the Alzheimer’s disease-associated gene that is also an AR-induced gene in the prostate (Fig. 8), could play a pivotal role in the androgenic modulation of these pathways because it functions in both Notch and β-catenin signaling (95). Such a function for presenilin-1 in growth modulation has previously been demonstrated in mice with tissue-selective presenilin-1 deficiency in skin, where aberrant β-catenin-mediated proliferation was observed (96).

In summary we present here a snapshot of the earliest events in the androgenic induction of prostate proliferation and differentiation. These data provide several potential tools for exploration in the management of benign prostatic hyperplasia and PCa, as well and provide molecular insights into the anabolic regulation of prostate cells within the context of the mesenchymal-epithelial interactions that are critical for normal organogenesis. Furthermore, the use of manual, function-based clustering of regulated genes described here is likely to be of use in other settings where the goal is to understand the complex relationship between transcription and physiology.

REFERENCES
DHT Regulation of Prostate Gene Expression