FoxA1 specifies unique androgen and glucocorticoid receptor binding events in prostate cancer cells

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Abstract

The forkhead protein FoxA1 has functions other than a pioneer factor, in that its depletion brings about a significant redistribution in the androgen (AR) and glucocorticoid receptor (GR) cistromes. In this study, we found a novel function for FoxA1 in defining the cell-type specificity of AR and GR binding events in a distinct fashion, namely, for AR in LNCaP-1F5 cells and for GR in VCaP cells. We also found different, cell-type and receptor-specific compilations of cis-elements enriched adjacent to the AR- and GR-binding sites. The AR pathway is central in prostate cancer biology, but the role of GR is poorly known. We find that AR and GR cistromes and transcription programs exhibit significant overlap and that GR regulates a large number of genes considered as AR pathway-specific. This raises questions about the role of GR in maintaining the AR pathway under androgen-deprived conditions in castration-resistant prostate cancer patients. However, in the presence of androgen, ligand-occupied GR acts as a partial anti-androgen and attenuates the AR-dependent transcription program.

PRÉCIS

The findings of this study raise questions about the precise specificity of accepted androgen receptor pathways in castration-resistant prostate tumors under androgen-deprived states.
Introduction

The majority of prostate cancers are initially androgen-dependent, and the first line treatment is androgen deprivation (1, 2). Administration of non-steroidal antiandrogens, such as bicalutamide and flutamide, is commonly used in prostate cancer therapy (3). Bicalutamide was thought to be a pure antiandrogen (4) but has androgen agonistic activity in presence of high cellular androgen receptor (AR) content (5). Cyproterone acetate (CPA) was one of the first antiandrogens used for prostate cancer treatment (6) but has also androgen agonistic actions (7). Mifepristone (RU486) is an antiprogestin and antiglucocorticoid, but has androgenic/antiandrogenic properties as well (8, 9). All these compounds antagonize AR function by competing with T or DHT for binding to the ligand-binding domain (LBD) of AR.

Recent reports have delineated genome-wide AR-binding sites (ARBs) and androgen target genes in LNCaP cells, or clones derived from it, using chromatin immunoprecipitation (ChIP) combined with microarray (ChIP-on-chip) or massively parallel sequencing (ChIP-seq) (10-15). Only DHT or the synthetic androgen R1881 has been used as ligands, and information on other compounds such as those with androgen agonistic/antagonistic properties is lacking. Likewise, there is paucity of information on the mechanisms that underlie cell-type specificity in AR cistromes and androgen-regulated transcription programs among prostate cancer cells.

Glucocorticoid receptor (GR) plays an important role in inflammation and cancer progression, dexamethasone has been used in treatment of castration-resistant prostate cancer (16), and GR signaling exhibits tumor suppressor activity in prostate cancer cells (17, 18). AR and GR can bind to the same androgen/glucocorticoid response element (ARE/GRE) when examined by using naked DNA in vitro or transient expression conditions (19), or even on chromatin (12, 20), but there is very
little information on the factors that ensure receptor specificity in a genuine chromatin environment.

Here, we have addressed several outstanding issues in androgen action biology by examining AR and GR cistromes and transcription programs in two androgen-responsive prostate cancer cell lines, the LNCaP-1F5 and VCaP cells. The specific questions included the role of the ligand in guiding AR binding to chromatin sites, mechanisms that define AR-binding events in a cell-type specific fashion, and the interplay and cross-talk between AR- and GR-binding events and transcription programs in prostate cancer cells.

Materials and methods

Cell culture. LNCaP-1F5 cells, engineered to express rat GR (21), were obtained from Dr. Jan Trapman (Erasmus Medical Center, Rotterdam, The Netherlands) and grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) with antibiotics. The cells were tested on a regular basis for AR and GR levels by immunoblotting as well as expression of androgen-regulated transcripts, such as PSA and TMPRSS2 mRNAs, by RT-qPCR. VCaP cells were purchased from American Type Culture Collection, used within six months after receipt, and grown in DMEM supplemented with FBS and antibiotics. Both cell lines were tested to be Mycoplasma free.

Chromatin immunoprecipitation (ChIP). ChIP assays were performed as described previously (12). Cells were cultured in stripped medium (10% FBS treated with dextran-coated charcoal, DC-FBS) for 4 days and then exposed to the ligands (100 nM DHT, 1 μM CPA, 1 μM RU486 and 1 μM bicalutamide, 100 nM Dex, 100 nM DHT + Dex, or vehicle) for 2 h. The antibodies were: AR (8), rodent GR (22), human GR
[BuGR (GR32L), Millipore, and Mab-010-050, Diagenode], FoxA1 (ab23738, Abcam), RNA Pol II (sc-899x, Santa Cruz), H3K4me2 (07-030, Millipore), H2A.Z (07-594, Millipore), normal rabbit IgG (sc-2027, Santa Cruz), normal mouse IgG (sc-2025, Santa Cruz). Primer sequences are shown in Table S1.

**ChIP-seq.** The samples were processed as described in (12). Statistics for aligned reads are shown in Table S2. All experiments were performed in biological duplicates.

**FoxA1 depletion by RNA interference**

LNCaP-1F5 and VCaP cells were transfected with control siRNA (parental cells) or siRNA targeting FoxA1 mRNA (ON-TARGETplus™ SMARTpool siRNA, Dharmaco, ThermoScientific) and cultured for 72, after which they were exposed to DHT or Dex (12).

**Formaldehyde-assisted isolation of regulatory elements (FAIRE).** FAIRE was performed as described in (23) with minor modifications.

**Gene expression profiling.** Biological triplicate or duplicate RNA samples were isolated using RNAeasy kit (Qiagen) and were hybridized to Illumina HumanHT-12 v3 Expression BeadChip Kits. Data analysis was performed and heat maps generated by using Anduril software (24) together with “R” software (http://www.r-project.org/) and Bioconductor “lumi” package (http://www.bioconductor.org) as previously described (12). ChIP-seq and gene expression data have been deposited in the Gene Expression Omnibus database with accession number GSE39880.
Bioinformatics analyses. De novo motif analysis and motif over-representation in ChIP-seq binding sites were calculated against genomic background and the significance tested as previously described (12, 13). The overlap analysis, CEAS analysis, genome-wide correlation, motif analyses, and tag density maps were performed using the Cistrome (25).

Results

The ligand as regulator of the AR cistrome and transcription program. GR binding occurs largely to cis-elements at constitutively open chromatin sites (20, 26–28), and the same applies to DHT-occupied AR (12). Enhancers that bind AR exhibit nucleosome depleted regions already in the absence of androgen (29). Together, these findings raise the question as to the role of the ligand in instructing the AR to bind to appropriate chromatin loci.

For ChIP-seq, LNCaP-1F5 cells were exposed to 100 nM DHT, 1 μM cyproterone acetate (CPA), 1 μM mifepristone (RU486), 1 μM bicalutamide or vehicle for 2 h. By using two independent biological replicates, we identified 8,603 ARBs for the DHT-occupied AR (false discovery rate, FDR < 2%). Comparison of these ARBs to those reported on the parental LNCaP line, either for DHT-occupied AR analyzed by ChIP-on-chip (14) or R1881-occupied AR analyzed by ChIP-seq (11), indicated a ~60% overlap of LNCaP-1F5 ARBs with these data sets (Fig. S1A).

Occupancy of AR by DHT yielded the highest number of ARBs followed by CPA-AR and RU486-AR complexes. ARBs present at regulatory regions within the kallikrein locus revealed only quantitative differences, in that DHT-occupied AR was loaded onto these sites more efficiently than the AR occupied either by CPA or RU486 (Fig. 1A). DHT-occupied ARBs possessed twice the number of tags to those of CPA-
or RU486-occupied ARBs (Fig. 1B), and the tag density maps show less intense binding of CPA- and RU486-occupied ARs to the ARBs specified by the DHT-bound AR (Fig. 1C). Distribution of AR-binding events between distal enhancer, proximal promoter and intronic regions is independent of the ligand bound to the AR (Fig. S2A).

**Gene expression profiles after exposure to different AR ligands.** Gene expression profiles in LNCaP-1F5 cells were examined after a 24-h exposure to 100 nM DHT, 1 μM CPA or 1 μM RU486. This time point was chosen to achieve maximal response (12, 30). Exposure to DHT treatment yielded the most robust transcriptional response, as judged by the number of up-regulated and down-regulated genes (cut-off, ≥1.7 or ≤ 1.7-fold change), followed by CPA and RU486 (Fig. 1D and Dataset S1). Since CPA and RU486 are partial androgen agonists/antagonists, these compounds brought about transcriptional responses only partially similar to those of DHT (Figs. 1D and S2B). The main differences were seen among three pathways: metabolic pathways and pathways in cancer (categories 1, 2 and 3 in Fig. 1D), ribosome function (category 4), and cap junctions (category 3). We also compared all ligand-dependent and ligand-independent (=stably expressed) genes associated with AR-binding sites within a window of ±100 kb of TSSs of the genes. Both up-regulated and down-regulated genes could be mapped to the nearest ARBs significantly more often than stably expressed genes after the exposure to DHT, CPA or RU486 (Fig. 1E).

The ARBs connected to up-regulated genes were enriched for 15-bp canonical ARE-like sequences that were similar for the DHT-AR, CPA-AR, and RU486-AR complexes. For down-regulated genes, a canonical ARE-like cis-element was found only for those down-regulated by DHT (Figs. S2C and S2D). Of note, a cis-element for FoxA proteins was highly enriched adjacent to the ARBs irrespective of the ligand.
bound to the receptor in LNCaP-1F5 cells. FoxA1 cistrome in LNCaP-1F5 cells (12) exhibit ~80% overlap with that in LNCaP cells (31, 32) (Fig. S1B).

**AR cistromes in LNCaP-1F5 and VCaP prostate cancer cells.** The VCaP cells harbor AR amplification and express ~10-fold higher wild-type AR level than LNCaP cells harboring a mutant AR (T877A) (30) (Fig. S3C). We identified 44,879 ARBs (FDR < 2%) for the DHT-occupied AR in VCaP cells, a number ~5 times higher than in LNCaP-1F5 cells. The DHT-occupied ARBs exhibited ~74% overlap with the R1881-occupied ARBs (11) in VCaP cells (Fig. S1C).

The majority (~85%) of the ARBs in LNCaP-1F5 cells are present in VCaP cells (Fig. 2A), and the number of tags on VCaP ARBs was 4-fold to that on LNCaP-1F5 ARBs (Fig. 2B). *De novo* motif search analyses revealed that a *cis*-element composed of a forkhead protein family motif flanked by an ARE half-site-like element (Fig. 2C) was highly enriched among the ARBs unique to LNCaP-1F5 cells (p<10^-102). All these loci also contained a *cis*-element for FoxA proteins, but *de novo* motif search failed to identify a canonical ARE-like motif. AR binding to these chromatin sites may occur *via* tethering of the receptor to FoxA1, as in one-half of the AR- and FoxA1-binding events, the distance between the peak summits was less than 30 nt (median = 36 nt) (Fig. S3A). The importance of FoxA1 for AR binding was verified by experiments showing that FoxA1 depletion (Fig. S4A) abolished almost completely AR loading onto the LNCaP-1F5 unique sites (Fig. S4B) and that FoxA1 was bound to these sites already prior to androgen exposure (Fig. S4C). AR binding in VCaP cells was marginal at the LNCaP-1F5 unique loci, despite a much higher AR level in VCaP cells (Fig. S4D). Importantly, FoxA1 binding to the ARBs unique to LNCaP-1F5 cells
was almost nonexistent in VCaP cells (Fig. S4E), even though FoxA1 protein level in VCaP cells was much higher than in LNCaP-1F5 cells (Fig. S3B).

LNCaP-1F5 unique ARBs are located less frequently in an accessible chromatin environment in VCaP than in LNCaP-1F5 cells, as judged by FAIRE (Fig. S5A). Concomitant binding of FoxA1 and AR is associated with eviction of the central nucleosome and marked by decreased H3K4 dimethylation (H3K4me2) and destabilization of histone H2A.Z variant (33). Direct ChIP assays for H3K4me2 and H2A.Z with primers bracketing the summits of AR-binding peaks by 100–150 nt revealed that the H3K4me2 marks and H2A.Z levels were significantly higher in VCaP than LNCaP-1F5 cell chromatin, suggesting that the central nucleosome in VCaP cells is present and potentially occludes AR/FoxA1-binding sites (Figs. S5B and S5C). Although the presence of H2A.Z renders the central nucleosome unstable (33), it is not evicted in VCaP cells which correlates with higher H3K4me2 marks and lack of FoxA1 binding. Our genome-wide maps (12) indicated that decreased H3K4me2 signal is a salient feature in LNCaP-1F5 cells for concomitant FoxA1- and AR-binding events, whereas there is no AR binding at the same loci in VCaP cells (Fig. S6).

Localization of ARBs in regulatory regions of the androgen-dependent genes KLK3, KLK2 and TMPRSS2 exemplifies the similarity of AR-binding events shared by LNCaP-1F5 and VCaP cells (Fig. 2D). De novo motif search revealed that a canonical ARE is highly significantly enriched among these sites (Fig. 2E). The other highly enriched cis-element is the FoxA1 motif (Fig. 2F). The most highly enriched cis-element among the ARBs unique to VCaP cells is very similar to the canonical ARE (Fig. 2G). Of note, cis-elements for the ETS family members are highly over-represented adjacent to ARBs unique to VCaP cells (z-score < -20; p<10^-70), but not to AR-binding events unique to LNCaP-1F5 cells (see below).
Comparison of AR and GR cistromes and signaling pathways in LNCaP-1F5 cells.

LNCaP-1F5 cells express rat GR to a level ~4 times higher than that of AR in these cells (21). After a 2-h exposure to dexamethasone (Dex), 14,103 GRBs were identified (FDR < 2%). One-half of the AR cistrome overlaps with the GR cistrome (Fig. 3A). Loading of AR and GR occurred in a number of instances onto the same loci, as illustrated for \textit{FKBP5} and \textit{ELL2}, the two genes that are up regulated by both androgen and glucocorticoid in LNCaP-1F5 cells (Fig. 3B). GRB tag numbers were almost 2-fold higher than those for ARBs (Fig. 3C). There are binding events unique to AR as well as ARBs that are shared by the Dex-occupied GRBs. Likewise, there are sites unique to the Dex-occupied GRBs and those shared by ARBs and GRBs bound to their cognate ligands (Fig. 3D). Of note, under the conditions where AR and GR binding events were examined, Dex was unable to load AR and DHT unable to load GR onto any of the binding sites examined (Fig. S7).

More genes were differentially expressed in response to Dex [(437 up-regulated and 112 down-regulated, cut-off $\geq$ or $\leq$ 1.7-fold), Dataset S2] than to DHT in LNCaP-1F5 cells (243 up-regulated and 132 down-regulated), and approx. one-third of the DHT-regulated genes were also regulated by Dex (Dataset S2). There are both shared and unique gene expression pathways regulated by androgen and glucocorticoid (Fig. 3E). For example, both steroids regulate metabolic pathways (categories 1 and 2 in Fig. 3E) and drug and xenobiotic metabolism (category 6), but there are marked differences between androgen and glucocorticoid regulation of pathways related to focal adhesion, cancer and MAPK signaling (category 3) as well as Wnt signaling and cell cycle (category 5).
The unique ARBs or GRBs were significantly associated with the androgen- and glucocorticoid-regulated genes, respectively, compared to stably expressed genes when mapped within a window of ±100 kb of TSSs (Fig. 3F). Of note, the shared AR- and GR-binding events were also significantly associated with genes regulated by both androgen and glucocorticoid, implying that, in these instances, AR and GR occupied by their cognate ligands are capable of using the same regulatory cis-elements to regulate transcription programs (Fig. 3F and Fig. S8A).

Dex-occupied GR can substitute for DHT-bound AR and regulate genes typically considered as androgen target genes, as illustrated by the localization of ARBs/GRBs and RNA Pol II occupancy along the gene body of KLK3, KLK2, KLKP1, FKBP5, and ELL2 genes (Fig. S8A). In a number of instances, however, GR and AR were loaded onto the same loci, but RNA Pol II occupancy and transcript accumulation were mainly regulated by one steroid only, as exemplified by glucocorticoid-regulated PER1, RHOB, CST3 genes (Fig. S8B) and androgen-regulated genes SOX4, C1orf116, and RASSF3 (Fig. S9). On some occasions (the KLK cluster in Fig. S8A), AR or GR binding and RNA Pol II occupancy did not relate directly to transcript accumulation, perhaps due to the different time intervals used (2 h vs. 24 h).

Dex acted dominantly over DHT in that combined steroid exposure yielded transcript accumulation very similar to that with Dex alone in a majority of the cases (Fig. 3E). Examination of the 100 top-most androgen up-regulated genes containing shared AR-/GR-binding sites revealed two principal patterns (Table S3, Figs. 4A and 4B). First, co-exposure to Dex inhibits accumulation of DHT-dominant transcripts, i.e., those up-regulated more by DHT alone than by Dex alone, and 49/53 transcripts shown in Table S3 exhibit this pattern. Second, DHT is unable to inhibit, but often increases accumulation of transcripts up-regulated equally well by DHT or Dex alone (Table S3,
Figs. 4A and 4B). Thus, Dex-occupied GR should be considered as a partial AR agonist/antagonist. Intriguingly, the inhibition elicited by Dex-occupied GR on the function of DHT-bound AR was not due to competition for chromatin binding sites. By contrast, AR binding was increased by simultaneous exposure to the two hormones; DHT and Dex (Fig. 4C and 4D), a phenomenon called assisted loading (34). A similar phenomenon was also seen in many instances in GR binding events (Fig. 4C and 4D).

Approx. one-seventh of Dex up-regulated and down-regulated genes in LNCaP-1F5 cells overlapped with differentially regulated genes in GR+ prostate cancer samples in the data set of Tomlins et al. (35). Comparison of gene expression profiles of 10 GR+ vs. 10 GR- prostate cancer samples identified 824 genes differentially regulated in GR+ samples (p < 0.05). This led us to identify a core set of GR-dependent genes that are highly Dex-regulated in LNCaP-1F5 cells (Table S4). Most of these genes are Dex-dominant in LNCaP-1F5 cells, but ligand occupied-GR may also up-regulate DHT-dominant genes under androgen-deprived conditions (Table S4). The core set comprises known oncogenes, such as ACSL3, LIFR, NFIB, BTG1, and other genes that are overexpressed in prostate cancer (Fig. S10).

DNA sequence in and of itself acts as an allosteric ligand (36), and it was, therefore, pertinent to examine the nature of ARBs and GRBs that were either shared or unique to one of the two receptors. The 15-bp consensus ARE/GRE-like sequence was identified by de novo motif search for the shared ARBs/GRBs (Fig. 5A), and a very similar cis-element was also found for the unique GRBs not overlapping with the AR cistrome (Fig. 5B). Of note, the unique ARBs – those not overlapping with the GR cistrome – were highly enriched (p < 10^{-154}) for a 20-bp cis-element similar to that found to specify ARBs unique to LNCaP-1F5 cells (cf., Fig. 2C and 5C). This composite cis-element was not present among sites shared by AR and GR or those
unique to GR. Validation of randomly selected sites in the above three categories by direct ChIP assays confirmed that the shared sites bound both receptors (cf., Figs. 5A and 5D) and that sites unique to GR (Figs. 5B and 5E) or AR (Figs. 5C and 5F) were quite specific for one receptor only. That FoxA1 is indeed important for the formation of the unique ARBs is further supported by the fact that ~70% of the sites belong to the AR-binding events that are lost upon FoxA1 depletion in LNCaP-1F5 cells (12).

**Comparison of AR and GR cistromes in VCaP cells.** The monoclonal GR antibody used for LNCaP-1F5 cells (22) does not recognize the human GR and, therefore, other GR antibodies were employed for ChIP-seq (see Materials and methods). There are unique ARBs and GRBs in VCaP cells as well as binding loci shared by the two receptors; these latter sites represent approx. 58% of the GRBs in VCaP cells (Fig. 6A). Unexpectedly, the unique GR-binding events in VCaP cells were not enriched for a canonical GRE/ARE motif; rather, *de novo* motif search analyses revealed the presence of a FoxA-like *cis*-element (Fig. 6B). The importance of FoxA1 for unique GR-binding events in VCaP cells was validated by direct ChIP-qPCR assays showing that, at ten randomly selected loci, FoxA1 depletion resulted in a corresponding decrease in GR loading onto the GR sites unique to these cells (Fig. S11). The binding sites shared by AR and GR were enriched for both FoxA and GRE/ARE *cis*-elements (Fig. 6C and 6D), and the unique ARBs in VCaP cells possessed a typical ARE motif (Fig. 6E). Only one-fourth of GR-binding sites in VCaP cells were shared with those in LNCaP-1F5 cells (Fig. 6F), and the GRBs unique to VCaP cells were enriched for a FoxA1 *cis*-element (Fig. 6G and Fig. S12). By contrast, *de novo* motif search for the GRBs shared by LNCaP-1F5 and VCaP cells identified both a GRE and a FoxA1 motif (Figs. 6H and 6I), and the GRE was the most enriched element for GR-binding events unique to
LNCaP-1F5 cells (Fig. 6J). The relatively poor overlap of GRBs between LNCaP-1F5 and VCaP cells (Fig. 6F) could be, at least in part, due to different antibodies used. However, direct ChIP assays on randomly selected 14 GRBs unique to LNCaP-1F5 cells with a monoclonal antibody (BuGR) present in the antibody cocktail used for GR-binding events in VCaP cells validated the presence of all these sites in LNCaP-1F5 cells (Fig. S13).

FoxA1 has been shown to be involved in cell lineage-specific regulation of nuclear receptor (ER and AR) binding to chromatin (37, 38). An important novel feature of this work is that FoxA1 specifies unique chromatin binding events of two different receptors in a dissimilar fashion in LNCaP-1F5 and VCaP cells: for AR in LNCaP-1F5 and GR in VCaP cells. Motif enrichment analyses for the cis-elements clustered adjacent to AR- and GR-binding sites in LNCaP-1F5 and VCaP cells revealed that the clusters are almost mirror images of each other; for example, ETS family members are highly enriched adjacent to ARBs in VCaP cells as opposed to the GRBs in LNCaP-1F5 cells (Fig. 7). This appears to be particularly true for the unique AR- and GR-binding events. Both cell lines are known to express proteins of the ETS family; in VCaP cells mostly as fusion proteins expressed from the TMPRSS2 locus (15) and in LNCaP cells mainly through overexpression and/or androgen regulation of the ETV1 gene (15, 39). In agreement with our motif enrichment analyses, almost one-half of the AR cistrome has recently been shown to overlap with that of one ETS family member, ERG, in VCaP cells (15, 40).

**Discussion**

The present work shows a number of novel features for AR and GR cistromes and transcription pathways in two prostate cancer cell lines. First, the sites that AR binds to
on chromatin are not much influenced by the ligand occupying the receptor. Second, FoxA1 is important in specifying the AR-binding events unique to LNCaP-1F5 cells, and lack of FoxA1 binding in VCaP cells to these loci explains cell line specificity. Third, AR- and GR-binding events and transcription programs in LNCaP-1F5 cells exhibit features that are both overlapping and unique to one receptor only. A composite FoxA1 element is required to ensure AR binding to specific sites in LNCaP-1F5 cells. Fourth, AR and GR cistromes are partially overlapping also VCaP cells, but FoxA1 is needed in these cells to specify chromatin-binding events unique to GR rather than AR.

Our genome-wide data on ligand dependence of AR binding events and transcription programs are in agreement with previous direct ChIP results on a few loci (8, 41), in that CPA and RU486 brought about formation of ARBs that are quantitatively rather than qualitatively different from those by DHT-occupied AR. That transcriptional responses to DHT and CPA or RU486 exposure were not identical was expected, as CPA-AR and RU486-AR complexes are able to recruit, in addition to coactivators, also corepressors to the appropriate regulatory regions (8) and that binding affinity of these compounds to AR is less than that of DHT (42). The partial antiandrogenic actions of CPU and RU486 rely thus on two features; (i) as ligands, competition for binding to the LBD of AR with T or DHT, and (ii) when bound to AR, competition with AR occupied by a physiological androgen for binding to regulatory chromatin loci.

An important cellular function of FoxA proteins is to serve as pioneer factors to initiate transcriptional regulation (43, 44). This feature of FoxA1 has been examined in a genome-wide fashion for ER, AR, and GR signaling (12, 32, 38, 45-47). In addition to serving as a pioneer (or licensing) factor, FoxA1 has other steroid receptor-related functions as well, in that depletion of FoxA1 in prostate cancer cells results in
extensive redistribution of AR- and GR-binding events, generating a large number of new ARBs or GRBs not available for AR or GR binding in parental cells (12). Our present results add another important feature to FoxA1 functions; FoxA1 is a key determinant in ensuring the specificity of AR-binding events in LNCaP-1F5 cells and that of GR-binding events in VCaP cells.

One-sixth of the AR-binding events in LNCaP-1F5 cells failed to overlap with those in VCaP cells. These sites in VCaP cells are mainly located in an inaccessible chromatin region and do not bind FoxA1. In LNCaP-1F5 cells, these sites containing a 20-bp composite FoxA1 element and a canonical 10-bp FoxA1 motif exhibit signs of middle nucleosome eviction (33), binding of FoxA1 already in the absence of androgen, and androgen- and FoxA1-dependent loading of the AR. Interestingly, a composite FoxA1 element is also involved in setting apart the AR-specific binding events from those shared by AR and GR, or unique to GR, in LNCaP-1F5 cells. Most of these sites (~70%) were lost upon FoxA1 depletion (12), emphasizing the importance of FoxA1 as the specificity determinant for AR binding. Even though these results shed light into the conundrum pertaining to specificity determinants of AR and GR binding to DNA in a genuine chromatin context, it is still enigmatic as to how GR-binding specificity is insured in LNCaP-1F5 cells, as the de novo motif search identified a cis-element very similar to both consensus ARE and consensus GRE. There is a significant difference between the ARBs and GRBs in LNCaP-1F5 cells with regard to their adjacent cis-elements; ETS family motifs are highly over-represented adjacent to the GRBs but not to the ARBs. It is an intriguing possibility that, in addition to FoxA1, co-occupancy of GR and an ETS family member is required for specific GR-binding events in LNCaP-1F5 cells, but FoxA1 alone suffices for this purpose in VCaP cells. ETS family members are reported to be recruited, at least in part, to the same loci.
as AR onto VCaP cell chromatin (15), and chromatin binding of one of them, ERG, shows almost a 50% overlap with AR-binding events (40). ETS family members are not likely required for AR binding in LNCaP-1F5 cells, where FoxA1 is an important determinant.

Almost all prostate cancers express the AR protein, but the expression of GR is more variable; only ~30% of cancers express this receptor. Interestingly, the proportion of GR protein-expressing prostate cancers is increased after androgen deprivation therapies and in castration-resistant prostate cancer tissues (48, 49). Our results that there are two kinds of GR-binding events – either unique to GR or shared by AR and GR – in LNCaP-1F5 and VCaP cells and that androgen- and glucocorticoid-dependent transcription programs are partially overlapping, raise the question about the role of GR in prostate cancer progression. Since GR and AR may use the same chromatin binding sites to regulate expression of the same genes, it will be important to examine whether or not it is the GR that maintains the AR pathway in prostate cancer under castration-resistant and androgen-deprived conditions in cancers expressing the GR. On the other hand, in the presence of androgen, androgen-regulated expression of transcripts linked to sites shared by AR and GR is often – but not always – inhibited by the concomitant presence of their cognate ligands, implying that in these instances Dex-occupied GR functions to attenuate the AR pathway. Further studies are needed to elucidate the mechanism(s) by which the partial antiandrogenic action of Dex is elicited; intriguingly, it does not appear to involve direct competition between AR and GR for shared chromatin binding sites.

**Disclosure of Potential Conflict of Interest**

The authors declare that they have no conflict of interest.
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Supplementary data

Supplementary data are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
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FIGURE LEGENDS

Figure 1. Influence of the ligand on AR loading onto LNCaP-1F5 chromatin. (A) AR-binding events within the kallikrein cluster after a 2-h exposure to 100 nM DHT, 1 µM CPA, 1 µM RU486, and 1 µM bicalutamide (Bica). IgG, rabbit IgG was used for ChIP. (B) Average tag numbers of ARBs centered around the summit of the loci brought about by DHT, CPA or RU486. (C) Tag density maps for ARBs. The binding events are centralized to DHT-ARBs. (D) Unsupervised hierarchical clustering of transcripts regulated by DHT, CPA or RU486. (E) Correlation between ligand-regulated and ligand-independent genes and the incidence of binding sites unique to DHT, CPA or RU486 within a window of ±100 kb of TSSs of the genes.

Figure 2. AR cistromes in LNCaP-1F5 and VCaP cells. (A) Overlap analysis between the AR-binding sites in LNCaP-1F5 and VCaP cells. (B) Average tag numbers of all ARBs in LNCaP-1F5 and VCaP cells centered around the summit of the binding site. (C) Cis-elements identified by de novo motif search for the AR-binding sites unique to LNCaP-1F5 cells. (D) Examples of AR-binding events within regulatory regions of KLK3, KLK2, KLKP1 and TMPRSS2 genes in LNCaP-1F5 and VCaP cells. (E-G) Cis-elements identified by de novo motif search for the ARBs shared by LNCaP-1F5 and VCaP cells (E and F), and for sites unique to VCaP cells (G).

Figure 3. Comparison of AR and GR cistromes and transcriptomes in LNCaP-1F5 cells. (A) Overlap of the AR and GR cistromes. (B) Location of ARBs and GRBs in the regulatory regions of two genes (FKBP5 and ELL2) up-regulated by both steroids. The shaded numbers with arrows refer to fold increases in transcript levels, as measured by
microarray and shown on a log2 scale. (C) Average tag numbers for DHT-occupied ARBs and Dex-occupied GRBs. (D) Tag density maps for sites unique to AR or GR or shared by the two receptors. The binding events are ranked from the strongest to weakest site, and the unique sites are centered to the receptor in question. (E) Unsupervised hierarchical clustering of transcripts regulated by DHT and Dex. (F) Relationship between AR- and GR-regulated and stably expressed genes and the presence of binding events unique to AR, unique to GR and shared by AR and GR within a window of ±100 kb of TSSs.

**Figure 4.** AR- and GR-binding events and transcript levels after exposure of LNCaP-1F5 cells to DHT and Dex either alone or together. Relative mRNA levels, as measured by qRT-PCR, in the presence of 100 nM DHT, 100 nM Dex or 100 nM DHT+Dex are shown (A) DHT-dominant genes and (B) Dex-dominant genes. (Mean + SEM values, n = 3.) Snapshots of receptor binding sites at selected loci together with changes in the corresponding transcript levels are shown for (C) genes up-regulated and (D) down-regulated by androgen. Fold changes in transcript levels in shaded areas are shown using a log2 scale, and results from the combined exposure to DHT and Dex are indicated by the red text. ChIP-seq was carried out in the same run to ensure a comparable sequencing depth in all samples (~17 million reads).

**Figure 5.** Comparison of the cis-elements identified by de novo motif search for binding sites unique to AR or GR or shared by the two receptors in LNCaP-1F5 cells. (A) Top scoring cis-element for binding sites shared by AR and GR. (B) Top scoring cis-element for sites unique to GR. (C) Top scoring cis-element for sites unique to AR. (D–F) Validation by direct ChIP assays of chromatin binding sites (AG1–AG6) shared
by AR and GR (D); sites (UG1–UG6) unique to GR (E); and sites (UA1–UA6) unique to the AR (F). Mean ± SEM values for two biological replicates are shown.

**Figure 6.** Comparison of AR and GR cistromes and *cis*-elements in LNCaP-1F5 and VCaP cells. (A) Overlap of AR and GR cistromes in VCaP cells. (B) Top scoring *cis*-element for binding events unique to GR in VCaP cells. (C, D) Top scoring *cis*-elements for binding sites shared by AR and GR in VCaP cells. (E) Top scoring *cis*-element for VCaP cell binding events unique to AR. (F) Overlap of GR cistromes in LNCaP-1F5 and VCaP cells. (G) Top scoring *cis*-element for GR-binding events unique to VCaP cells. (H, I) Top scoring *cis*-elements for GRBs shared by LNCaP-1F5 and VCaP cells. (J) Top scoring *cis*-element for GRBs unique to LNCaP-1F5 cells.

**Figure 7.** Motif over-representation analysis for ARBs and GRBs in LNCaP-1F5 and VCaP cells. The analysis performed by SeqPos using a cut-off *p*<10⁻³. Each block depicts the presence of a *cis*-element adjacent to the receptor binding site, expressed as percentage and arranged in bins of 20. U = unique AR- or GR-binding sites; All = all binding sites of the receptor.
Sahu et al. Figure 1

A

Chr19: 51,345 kb 51,360 kb 51,380 kb 51,400 kb

IgG
AR-Vehicle
AR-DHT
AR-CPA
AR-RU486
AR-Bica

KLK3 KLK2 KLKP1

B

Average Profile

Distance from center (bp)

DHT CPA RU486

C

Vehicle DHT CPA RU486

D

DHT CPA RU486

Ratio of genes with a binding site

p<4.1x10^-52
p<1.2x10^-05
p<7.8x10^-25
p=0.019
p=0.041
p<5.2x10^-06

DHT CPA RU486
Sahu et al. Figure 2

A

VCaP
44,879

LNCaP-1F5
8,603

B

Average Profile

Distance from center (bp)

Graph showing average profiles for VCaP and LNCaP-1F5.

C

Bits

Graph showing bits for FoxA1:AR composite element.

D

Graph showing regions on Chr19, Chr21, and VCaP.

E

Graph showing AR cis-element – 1st significant de novo motif.

F

Graph showing FoxA1 cis-element – 2nd significant de novo motif.

G

Graph showing AR cis-element – most significant de novo motif.
Figure 3

A

Genes

ARBs
8,603

GRBs
14,103

3,992

B

Average profile

Distance from center (bp)

5

10

500

0

-500

Distance from center (bp)

Chr6: 35,540 kb 35,580 kb 35,620 kb 35,660 kb 35,700 kb

AR

GR

4.0↑

3.7↑

3.3↑

2.8↑

Chr5: 95,200 kb 95,350 kb

AR

GR

C

D

E

F

Ratio of genes with a binding site

Up-regulated
Stably expressed
Down-regulated

p<2.8x10^-50
p<0.0024
p<3.8x10^-90
p<1.9x10^-24
p<0.0039
p<0.00018

1.0

0.8

0.6

0.4

0.2

0.0

p<2.8x10^-50
p<0.0024
p<3.8x10^-90
p<1.9x10^-24
p<0.0039
p<0.00018

AR unique
GR unique
Shared by AR/GR
A  DHT-dominant genes

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B  Dex-dominant genes

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C  Genes up-regulated by androgen

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D  Genes down-regulated by androgen

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Sahu et al. Figure 5

A. AR/GR cis-element – top significant de novo motif

B. AR/GR cis-element – top significant de novo motif

C. FoxA1:AR composite element – top significant de novo motif

D. % Input

E. % Input

F. % Input
Sahu et al. Figure 6

A

GR VCaP
7,101

AR VCaP
64,255

4,114

B

FoxA1 cis-element
top significant de novo motif

12345678

0

1.0

2.0

bits

C

FoxA1 cis-element
1st significant de novo motif

12345678

0

1.0

2.0

bits

E

AR/GR cis-element
top significant de novo motif

123456789101214

0

1.0

2.0

bits

F

GR VCaP
7,101

GR LNCaP-1F5
14,103

1,858

G

FoxA1 cis-element
top significant de novo motif

12345678910

0

1.0

2.0

bits

H

GR cis-element
1st significant de novo motif

123456789101214

0

1.0

2.0

bits

J

GR cis-element
top significant de novo motif

123456789101214

0

1.0

2.0

bits

D

AR/GR cis-element
2nd significant de novo motif

123456789101214

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1.0

2.0

bits

I

FoxA1 cis-element
2nd significant de novo motif

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**Legend:**
- Not present
- 0–20%
- 21–40%
- 41–60%
- 61–80%
- 81–100%
FoxA1 specifies unique androgen and glucocorticoid receptor binding events in prostate cancer cells

Biswaajyoti Sahu, Marko Laakso, Päivi Pihlajamaa, et al.

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