Cancer Research

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 receptor (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 GR regulates a large number of genes considered to be 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 antiandrogen and attenuates the AR-dependent transcription program. *Cancer Res; 73(5); 1570–80. ©2012 AACR.*

Introduction

The majority of prostate cancers are initially androgen-dependent, and the first-line treatment is androgen deprivation (1, 2). Administration of nonsteroidal 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 the 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 also has 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 testosterone (T) or dihydrotestosterone (DHT) for binding to the ligand-binding domain (LBD) of AR.

Recent reports have delineated genome-wide AR-binding sites (ARB) 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; refs. 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.

The glucocorticoid receptor (GR) plays an important role in inflammation and cancer progression; dexamethasone (Dex) 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% 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*

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and *TMPRSS2* mRNAs, by reverse transcription quantitative PCR (RT-qPCR). VCaP cells were purchased from the American Type Culture Collection, used within 6 months after receipt, and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with FBS and antibiotics. Both cell lines were authenticated using short tandem repeats by FIMM Technology Center (University of Helsinki, Helsinki, Finland), and were tested to be *Mycoplasma* free.

Chromatin immunoprecipitation

ChIP assays were conducted 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 nmol/L DHT, 1 μmol/L cyproterone acetate (CPA), 1 μmol/L RU486, 1 μmol/L bicalutamide, 100 nmol/L Dex, 100 nmol/L DHT + Dex, or vehicle) for 2 hours. 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 Biotechnology), H3K4me2 (07-030; Millipore), H2A.Z (07-594; Millipore), normal rabbit immunoglobulin G (IgG; sc-2027, Santa Cruz Biotechnology), and normal mouse IgG (sc-2025; Santa Cruz Biotechnology). Primer sequences are shown in Supplementary Table S1.

ChIP-seq

The samples were processed as described previously (12). Statistics for aligned reads are shown in Supplementary Table S2. All experiments were carried out 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, Dharmacon, ThermoScientific) and cultured for 72 hours, after which they were exposed to DHT or Dex (12).

Formaldehyde-assisted isolation of regulatory elements

Formaldehyde-assisted isolation of regulatory elements (FAIRE) was carried out as described previously (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 carried out and heat maps were 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 overrepresentation in ChIP-seq binding sites were calculated against genomic background and the significance was tested as previously described (12, 13). The overlap analysis, CEAS analysis, genome-wide correlation, motif analyses, and tag density maps were carried out 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 nmol/L DHT, 1 μmol/L CPA, 1 μmol/L mifepristone (RU486), 1 μmol/L bicalutamide, or vehicle for 2 hours. By using 2 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 an approximately 60% overlap of LNCaP-1F5 ARBs with these data sets (Supplementary 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 among distal enhancer, proximal promoter, and intronic regions is independent of the ligand bound to the AR (Supplementary Fig. S2A).

Gene expression profiles after exposure to different AR ligands

Gene expression profiles in LNCaP-1F5 cells were examined after 24-hour exposure to 100 nmol/L DHT, 1 μmol/L CPA, or 1 μmol/L RU486. This time point was selected to achieve maximal response (12, 30). Exposure to DHT treatment yielded the most robust transcriptional response, as judged by the number of upregulated and downregulated genes (cut-off, $\geq$1.7- or $\leq$1.7-fold change), followed by CPA and RU486 (Fig. 1D; Supplementary Dataset S1). Because CPA and RU486 are partial androgen agonists/antagonists, these compounds brought about transcriptional responses only partially similar to those of DHT (Fig. 1D; Supplementary Fig. 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 -independent (= stably expressed) genes associated with AR-binding sites within a window of $\pm$100 kb of TSSs of the genes. Both upregulated and downregulated genes could be mapped to the nearest ARBs

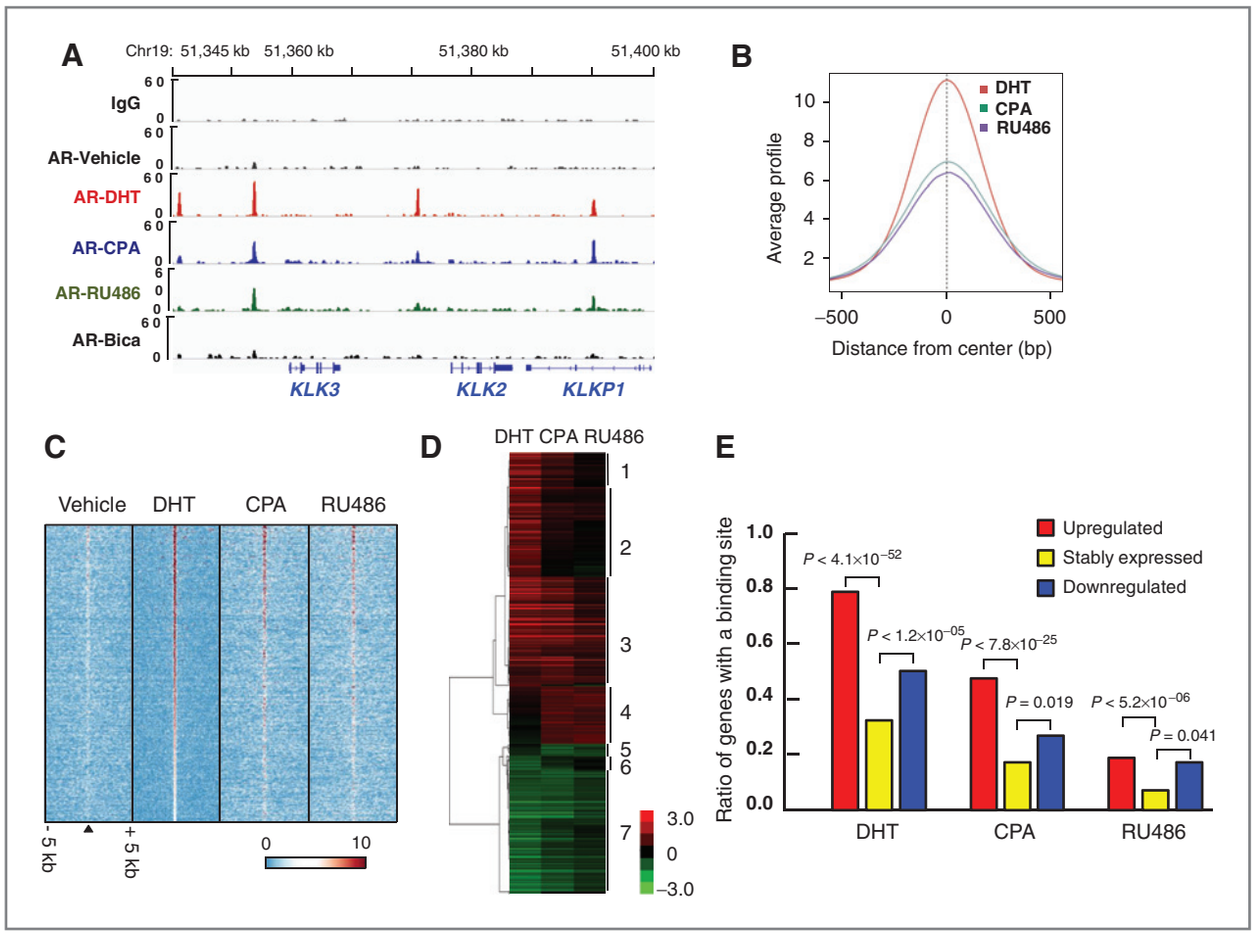

Figure 1. Influence of the ligand on AR loading onto LNCaP-1F5 chromatin. A, AR-binding events within the *kallikrein* cluster after 2-hour exposure to 100 nmol/L DHT, 1 μmol/L CPA, 1 μmol/L RU486, and 1 μmol/L bicalutamide (Bica). IgG, rabbit IgG was used for ChIP. B, average tag numbers of ARBs centered 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.

significantly more often than stably expressed genes after the exposure to DHT, CPA, or RU486 (Fig. 1E).

The ARBs connected to upregulated genes were enriched for 15-bp canonical ARE-like sequences that were similar for the DHT-AR, CPA-AR, and RU486-AR complexes. For downregulated genes, a canonical ARE-like *cis*-element was found only for those downregulated by DHT (Supplementary Fig. S2C and D). Notably, 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 approximately 80% overlap with that in LNCaP cells (Supplementary Fig. S1B; refs. 31, 32).

AR cistromes in LNCaP-1F5 and VCaP prostate cancer cells

The VCaP cells harbor *AR* amplification and express approximately 10-fold higher wild-type (WT) AR level than LNCaP cells harboring a mutant AR (T877A; refs. 30; Supplementary Fig. S3C). We identified 44,879 ARBs (FDR < 2%) for the DHT-occupied AR in VCaP cells, a number approximately 5 times higher than in LNCaP-1F5 cells. The DHT-occupied ARBs exhibited approximately 74% overlap with the R1881-occupied ARBs (11) in VCaP cells (Supplementary 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 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 nucleotides (nt; median = 36 nt; Supplementary Fig. S3A). The importance of FoxA1 for AR binding was verified by experiments showing that FoxA1 depletion (Supplementary Fig. S4A)

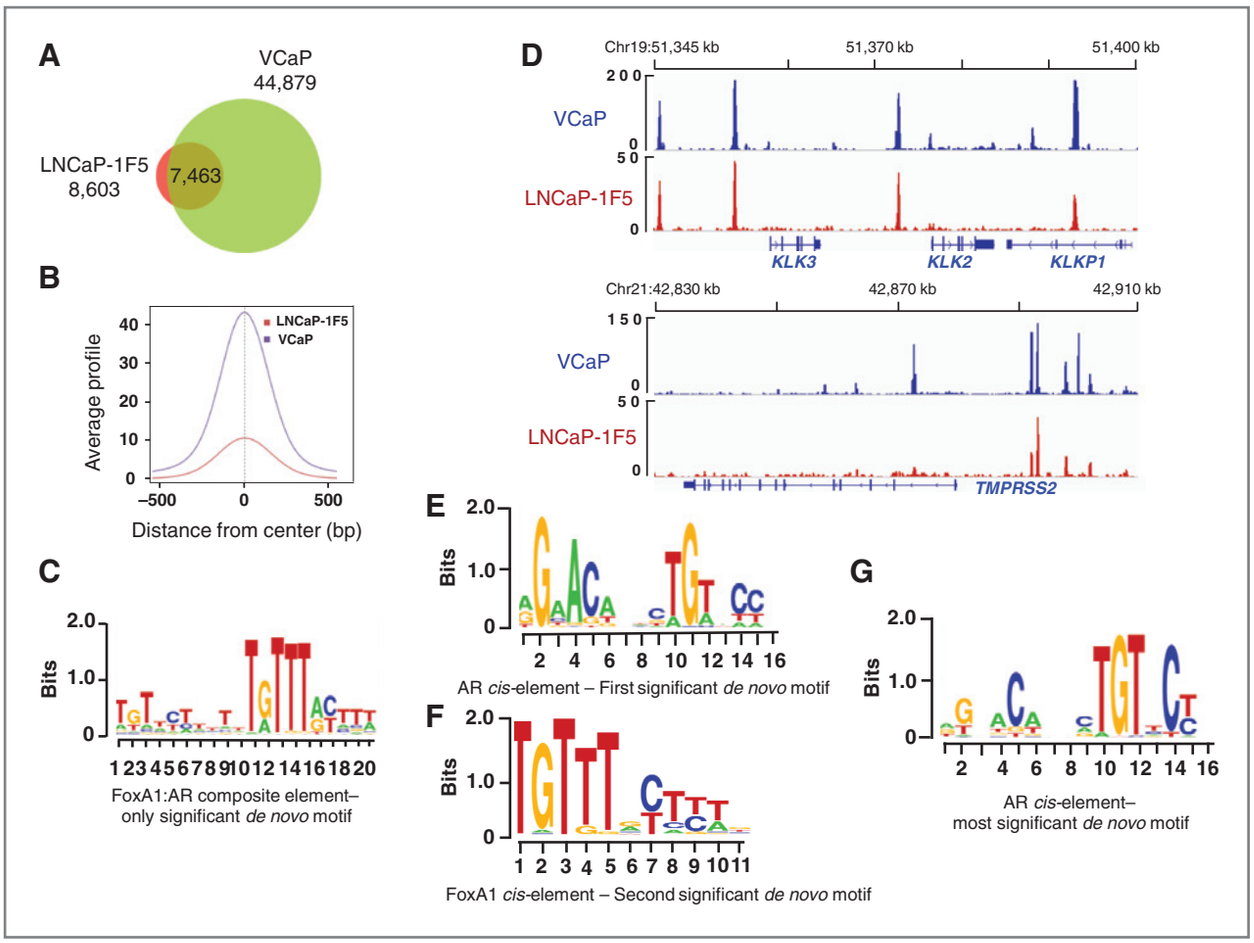

Figure 2. AR cistromes in LNCaP-1F5 and VCaP cells. A, overlap analysis between the AR-binding sites in LNCaP-1F5 and VCaP cells. B, average tag numbers of all ARBs in LNCaP-1F5 and VCaP cells centered around the summit of the binding site. C, *cis*-elements identified by *de novo* motif search for the 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).

abolished almost completely AR loading onto the LNCaP-1F5 unique sites (Supplementary Fig. S4B) and that FoxA1 was bound to these sites already before androgen exposure (Supplementary Fig. S4C). AR binding in VCaP cells was marginal at the LNCaP-1F5 unique loci, despite a much higher AR level in VCaP cells (Supplementary Fig. S4D). Importantly, FoxA1 binding to the ARBs unique to LNCaP-1F5 cells was almost nonexistent in VCaP cells (Supplementary Fig. S4E), although FoxA1 protein level in VCaP cells was much higher than in LNCaP-1F5 cells (Supplementary 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 (Supplementary 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 to 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 (Supplementary Fig. S5B and C). 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 a decreased H3K4me2 signal is a salient feature in LNCaP-1F5 cells for concomitant FoxA1- and AR-binding events, while there is no AR binding at the same loci in VCaP cells (Supplementary 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 and 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). Notably, *cis*-elements for the ETS family members are highly overrepresented adjacent to ARBs unique to VCaP cells (z-score < -20; $P < 10^{-70}$), but not to AR-binding events unique to LNCaP-1F5 cells.

Comparison of AR and GR cistromes and signaling pathways in LNCaP-1F5 cells

LNCaP-1F5 cells express rat GR to a level approximately 4 times higher than that of AR in these cells (21). After a 2-hour exposure to 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 *FKBP5* and *ELL2*, two genes that are upregulated 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). Notably, under the conditions where AR and GR binding events were examined, Dex was unable to load AR and DHT was unable to load GR onto any of the binding sites examined (Supplementary Fig. S7).

More genes were differentially expressed in response to Dex (437 upregulated and 112 downregulated, cut-off $\geq$ or $\leq$1.7-fold; Supplementary Dataset S2) than to DHT in LNCaP-1F5 cells (243 upregulated and 132 downregulated), and approximately one-third of the DHT-regulated genes were also regulated by Dex (Supplementary 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).

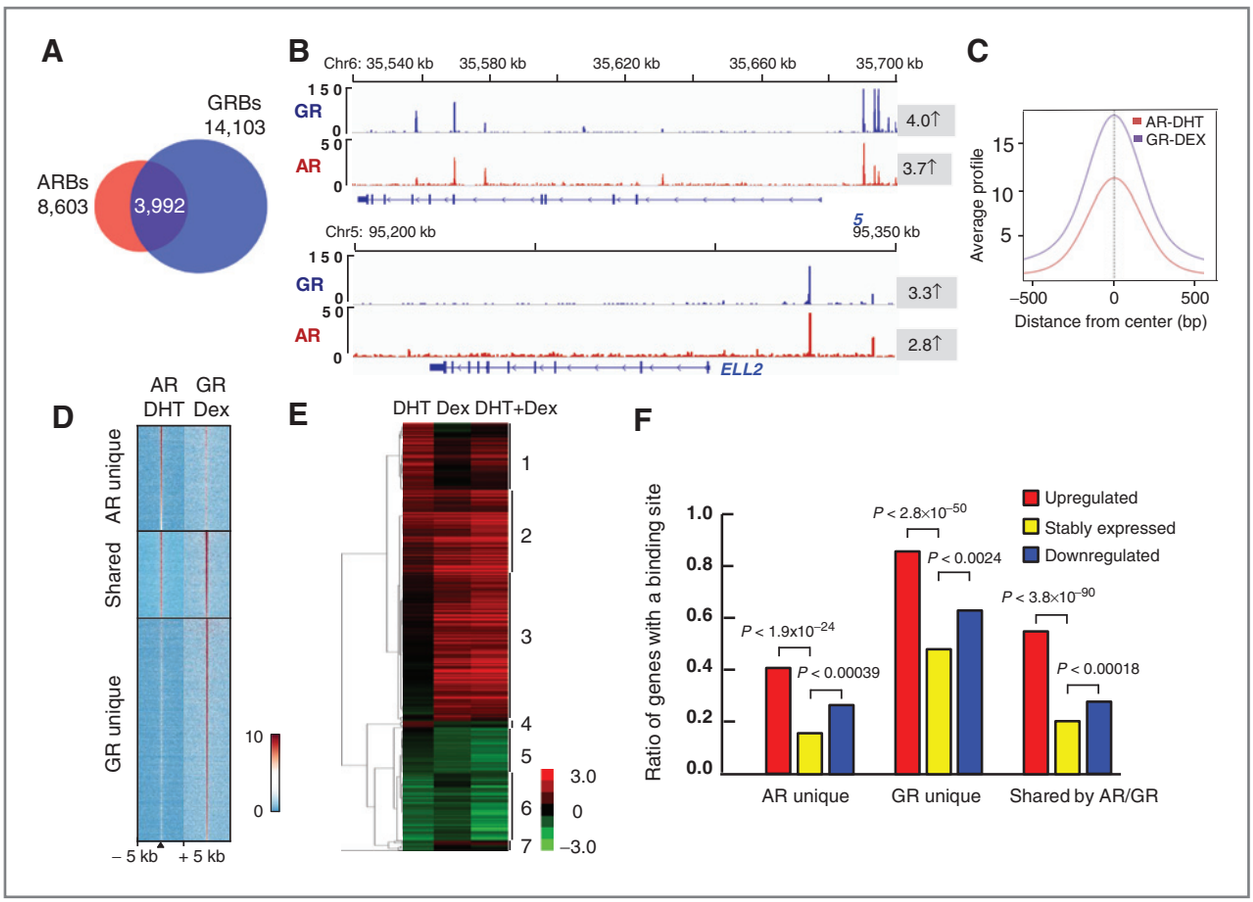

Figure 3. Comparison of AR and GR cistromes and transcriptomes in LNCaP-1F5 cells. A, overlap of the AR and GR cistromes. B, location of ARBs and GRBs in the regulatory regions of two genes (*FKBP5* and *ELL2*) upregulated by both steroids. The shaded numbers with arrows refer to fold increases in transcript levels, as measured by microarray and shown on a $\log_2$ 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 $\pm$100 kb of TSSs.

The unique ARBs or GRBs were significantly associated with the androgen- and glucocorticoid-regulated genes, respectively, compared with stably expressed genes when mapped within a window of ±100 kb of TSSs (Fig. 3F). Notably, 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; Supplementary 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 (Supplementary 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 the glucocorticoid-regulated *PER1*, *RHOB*, and *CST3* genes (Supplementary Fig. S8B) and the androgen-regulated genes *SOX4*, *C1orf116*, and *RASSF3* (Supplementary Fig. S9). On some occasions (the *KLK* cluster in Supplementary 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 vs. 24 hours).

Dex acted dominantly over DHT in that a combined steroid exposure yielded transcript accumulation very similar to that with Dex alone in a majority of the cases (Fig. 3E). Examination

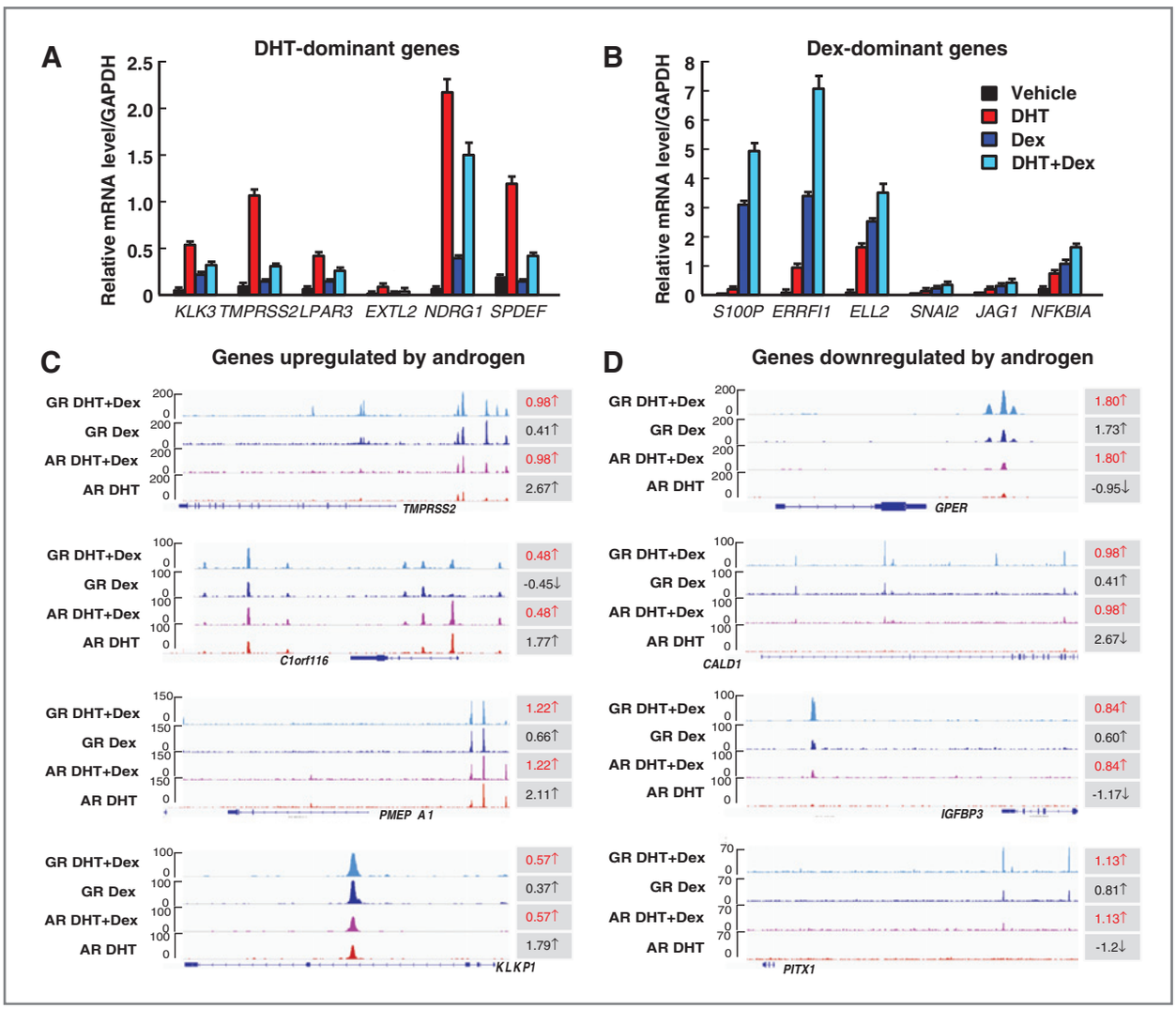

Figure 4. AR- and GR-binding events and transcript levels after exposure of LNCaP-1F5 cells to DHT and Dex, either alone or together. Relative mRNA levels, as measured by qRT-PCR, in the presence of 100 nmol/L DHT, 100 nmol/L Dex, or 100 nmol/L DHT+Dex are shown: DHT-dominant genes (A) and Dex-dominant genes (B). (Mean + SEM values, $n = 3$). Snapshots of receptor binding sites at selected loci together with changes in the corresponding transcript levels are shown for genes upregulated (C) and downregulated (D) by androgen. Fold-changes in transcript levels in shaded areas are shown using a $\log_2$ 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).

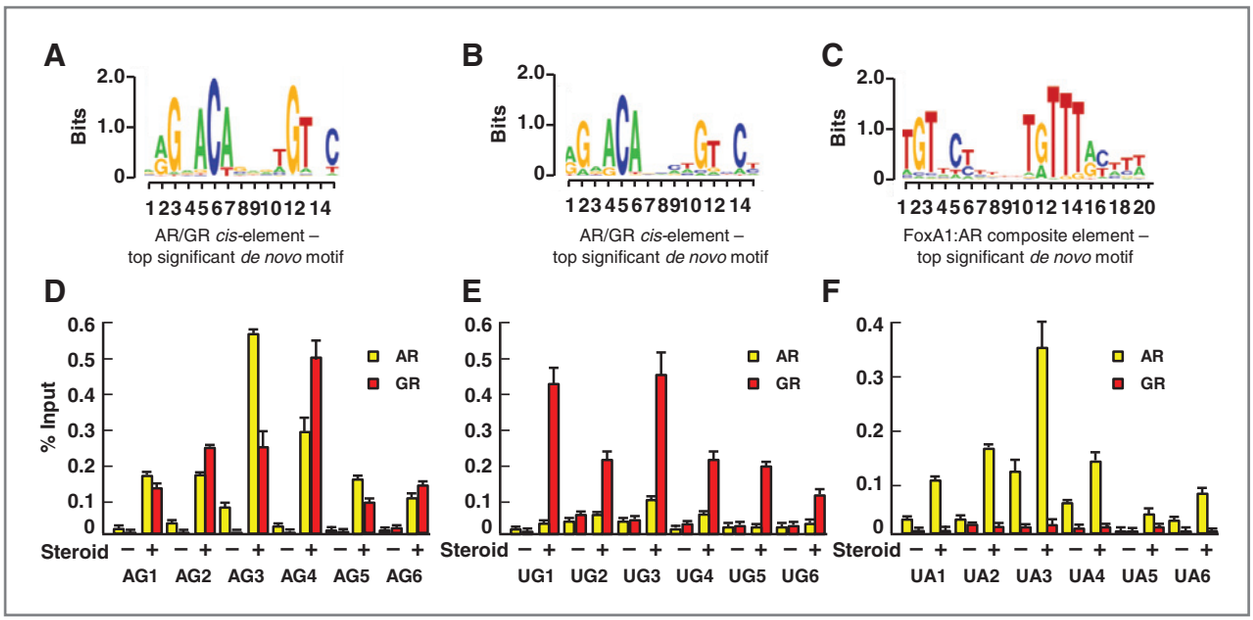

Figure 5. Comparison of the *cis*-elements identified by *de novo* motif search for binding sites unique to AR or GR or shared by the 2 receptors in LNCaP-1F5 cells. A, top-scoring *cis*-element for binding sites shared by AR and GR. B, top-scoring *cis*-element for sites unique to GR. C, top-scoring *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 2 biological replicates are shown.

of the 100 top-most androgen upregulated genes containing shared AR-/GR-binding sites revealed two principal patterns (Supplementary Table S3; Fig. 4A and B). First, coexposure to Dex inhibits accumulation of DHT-dominant transcripts, that is, those upregulated more by DHT alone than by Dex alone, and 49/53 transcripts shown in Supplementary Table S3 exhibit this pattern. Second, DHT is unable to inhibit, but often increases, accumulation of transcripts upregulated equally well by DHT or Dex alone (Supplementary Table S3; Fig. 4A and B). 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 D), a phenomenon called assisted loading (34). A similar phenomenon was also seen in many instances in GR-binding events (Fig. 4C and D).

Approximately one-seventh of Dex upregulated and downregulated genes in LNCaP-1F5 cells overlapped with differentially regulated genes in GR+ prostate cancer samples in the data set of Tomlins and colleagues (35). Comparison of gene expression profiles of 10 GR+ with 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 (Supplementary Table S4). Most of these genes are Dex-dominant in LNCaP-1F5 cells, but ligand-occupied GR may also upregulate DHT-dominant genes under androgen-deprived conditions (Supplementary Table S4). The core set comprises known oncogenes, such as *ACSL3*, *LIFR*, *NFIB*, *BTG1*, and other genes that are overexpressed in prostate cancer (Supplementary 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). Notably, 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 (compare 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 3 categories by direct ChIP assays confirmed that the shared sites bound both receptors (compare Fig. 5A and D) and sites unique to GR (Fig. 5B and E) or AR (Fig. 5C and F) 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 approximately 70% of the sites belong to the AR-binding events that are lost on 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 2 receptors; these latter sites represent approximately 58% of the GRBs in

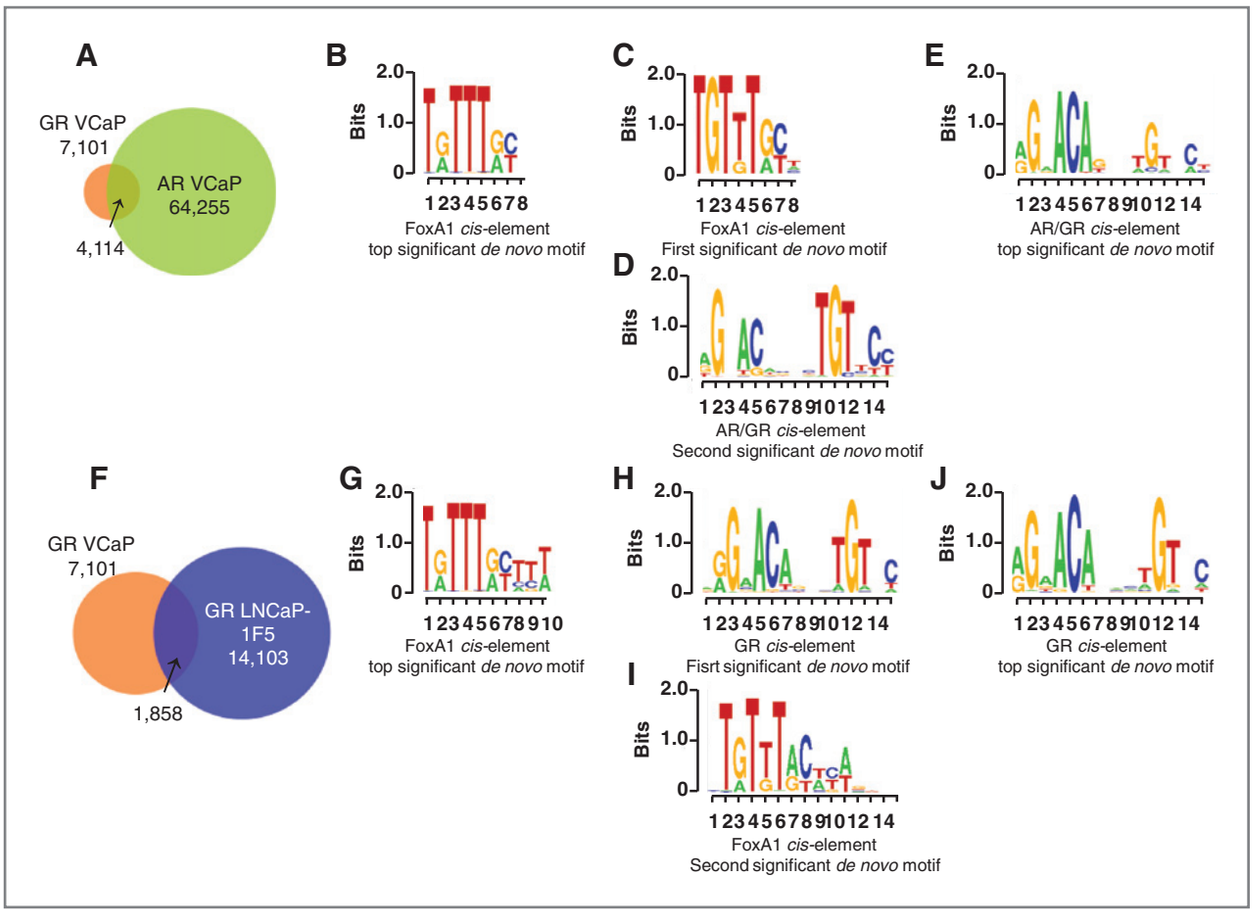

Figure 6. Comparison of AR and GR cistromes and *cis*-elements in LNCaP-1F5 and VCaP cells. A, overlap of AR and GR cistromes in VCaP cells. B, top-scoring *cis*-element for binding events unique to GR in VCaP cells. C and D, top-scoring *cis*-elements for binding sites shared by AR and GR in VCaP cells. 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 and 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.

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 10 randomly selected loci, FoxA1 depletion resulted in a corresponding decrease in GR loading onto the GR sites unique to these cells (Supplementary Fig. S11). The binding sites shared by AR and GR were enriched for both FoxA and GRE/ARE *cis*-elements (Fig. 6C and D), 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; Supplementary 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 (Fig. 6H and I), 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 (Supplementary 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

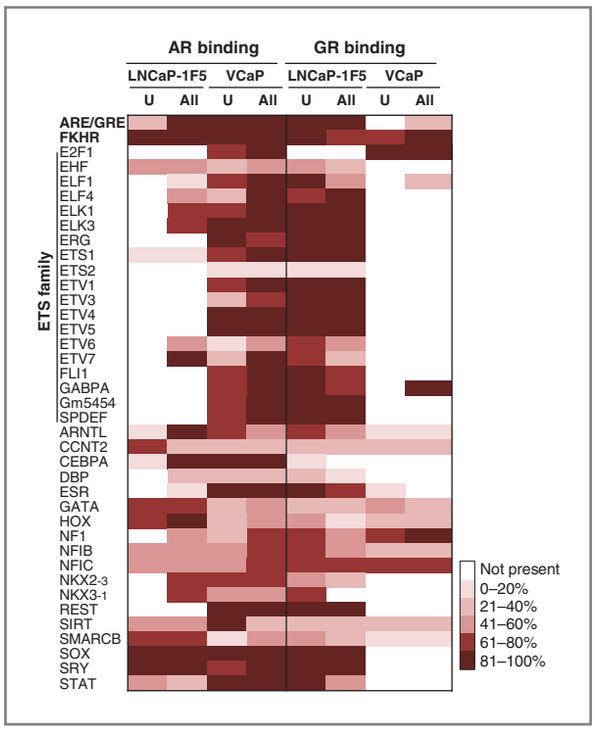

Figure 7. Motif overrepresentation analysis for ARBs and GRBs in LNCaP-1F5 and VCaP cells. The analysis was carried out by SeqPos using a cut-off *P* less than 10^{-3}. Each block depicts the presence of a *cis*-element adjacent to the receptor binding site, expressed as percentage and arranged in bins of 20. U, unique AR- or GR-binding sites; All, all binding sites of the receptor.

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 1 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 physiologic 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 on FoxA1 depletion (12), emphasizing the importance of FoxA1 as the specificity determinant for AR binding. Although 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 overrepresented 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 approximately 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 types 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. Because 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 Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Sahu, M. Laakso K. Ovaska, S. Hautaniemi, O.A. Jänne
Writing, review, and/or revision of the manuscript: B. Sahu, P. Pihlajamaa, S. Hautaniemi, O.A. Jänne
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Sahu, M. Laakso, P. Pihlajamaa, O.A. Jänne
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