

Long-range activation of *FKBP51* transcription by the androgen receptor via distal intronic enhancers

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ABSTRACT

Androgen receptor (AR) is a ligand-controlled transcription factor frequently deregulated in prostate carcinomas. Since there is scarce information on the action of AR on the chromatin level, we have elucidated the molecular mechanisms underlying the androgen-dependent regulation of immunophilin *FKBP51* in prostate cancer cells. In comparison to the canonical AR target *PSA*, *FKBP51* is more rapidly and strongly induced by androgen, with the regulation occurring merely at the transcriptional level. *FKBP51* locus harbors 13 *in silico*-predicted androgen response elements (AREs), with most of them located downstream from transcription start site (TSS) and capable of binding AR *in vitro*. Chromatin immunoprecipitation assays in VCaP and LNCaP prostate cancer cells indicate that activation of the locus by the AR relies on four major intronic sites, with the compound ARE-containing sites ≥ 90 kb downstream from the TSS playing critical roles. Binding of agonist-loaded AR onto these sites *in vivo* was accompanied with significant recruitment of RNA polymerase II and BRM-containing chromatin remodeling complexes to the *FKBP51* locus, which resulted in changes in the histone density of the locus. Our results indicate that very distal AREs act as genuine and robust enhancers, highlighting the importance of long-range regulation of transcription by the AR.

INTRODUCTION

Androgen receptor (AR) is a hormone-inducible transcription factor that belongs to the nuclear receptor superfamily (1). Many of the genes regulated by androgens and AR program the development and maintenance of the male phenotype and sexual characteristics. AR also plays a critical role in controlling the malignant growth of prostate tumors, and androgen ablation is a standard

therapy for prostate cancer. However, the therapy usually eventually fails and cancer turns into a lethal hormone-refractory state that overexpresses AR and is thereby sensitized to low androgen levels (2,3).

Transcription is regulated at many levels. DNA sequence forms platforms for binding of transcription factors that can either activate or attenuate transcription of a given gene. Androgen receptor's binding sites in DNA, androgen response elements (AREs), are essential for AR-mediated transcriptional activation, but not necessary for transcriptional repression by the receptor (4,5). However, not all AREs that are capable of binding AR *in vitro* necessarily function *in vivo*, since epigenetic regulation of chromatin structure and packaging can prevent their accessibility. Major epigenetic mechanisms that regulate chromatin structure include modifications of nucleosomes and DNA methylation (6,7).

Activation of genes by the AR requires, in addition to basal transcription machinery and RNA polymerase II, transcriptional coregulator proteins. Many coregulatory proteins harbor or recruit enzymatic activities that covalently modify histones (8). Agonist-bound nuclear receptors can interact with coactivator proteins linked to histone acetyltransferase activity, which acetylates specific lysine (K) residues in nucleosomal histones. Also histone methylation has recently been implicated in nuclear receptor signaling (9). In contrast to histone acetylation that is connected to active transcription, methylation of histones by K- or arginine (R)-specific activities have been linked to both activation and repression of transcription. The effect of these histone methylations on gene activity depends on the identity of the amino acid residue in a specific histone modified. For example, methylation of histone H3 at K9 and K27 are repressive modifications (transcriptional 'off' state), whereas methylation of H3 at K4 and K36 are needed for active transcription (transcriptional 'on' state) (6,10).

Nuclear receptors also attract chromatin remodeling complexes which utilize the energy of ATP to alter the histone-DNA association. Consequences of remodeling by SWI/SNF complexes include nucleosome sliding, alterations in nucleosome structure and histone octamer

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transfer (11–13), which can influence the accessibility of transcription factor binding sites. SWI/SNF complexes contain either BRM (SMARCA2) or BRG1 (SMARCA4) as a central ATPase subunit (14). It is not totally clear whether these ATPases are fully replaceable with each other or if there is some tissue or gene specificity in their function (15).

Perturbations in the transcriptional programs of AR are important, but poorly understood, events in prostate cancer. Knowledge of the AR action under genuine chromatin milieu is therefore essential for better understanding of the molecular mechanisms underlying prostate cancer development. However, regulation of only few AR target loci has thus far been characterized in a detailed fashion at the chromatin level. Most studies have been focusing on the prostate specific antigen (*PSA*) (16–20) of which protein product's serum concentration is used in prostate cancer diagnostics (21). Even though *PSA* has been studied as an example of 'canonical' AR target gene in prostate cells, it does not appear to be very sensitive to androgen levels *in vivo*, as *PSA* mRNA levels were not significantly suppressed in prostatectomy patients in response to androgen deprivation therapy (22). Compared to *PSA* mRNA, for example *FKBP51* (*FKBP5*) mRNA much better reflects the androgen concentration and activity in human prostate (22).

In the present study, we have focused on the androgen regulation of *FKBP51* in VCaP cells derived from a hormone-refractory prostate cancer (23). Our results show that in comparison to *PSA*, *FKBP51* mRNA is more robustly induced by androgens, with the regulation occurring merely at the level of transcription, as it is cycloheximide-independent but actinomycin D sensitive. Quantitative chromatin immunoprecipitation scans and enhancer activity analyses indicate that the androgen activation of the *FKBP51* locus occurs via several distal intronic enhancers, with the compound AREs located at ~90 and ~100 kb from the transcription start site playing the major role. Binding of AR to these distal enhancers is accompanied with chromatin remodeling throughout the locus. In LNCaP cells, that contain less AR than VCaP cells, AR occupancy and induction of the locus in response to androgen were less robust than in VCaP cells, but the pattern of the receptor loading was similar and the same intronic enhancers were important in both prostate cancer cell types. A model for long-range regulation by AR transcription complexes on the *FKBP51* locus *in vivo* is presented.

MATERIALS AND METHODS

Cell culture

VCaP and LNCaP cells were from American Type Culture Collection (ATCC). VCaP cells were maintained in DMEM containing 10% US defined FBS (HyClone, Logan, Utah, USA), 25 U/ml penicillin and 25 µg/ml streptomycin in a 5% CO₂ atmosphere at 37°C. LNCaP cells were grown in RPMI 1640 supplemented according to instructions from ATCC and FBS as above. COS-1 cells

were maintained in DMEM containing 10% FBS, penicillin (6.25 U/ml) and streptomycin (6.25 µg/ml).

DNA constructs

Selected regions of *FKBP51* were PCR cloned from human genomic DNA using Phusion DNA polymerase (Finnzymes, Espoo, Finland) and subcloned into the TATA box-containing pGL3-basic (Promega, Madison, WI, USA). Primers used were the same as listed for ChIP except for region 12 the forward primer was 5'-TCTTGCCTCCAACACTGCTG-3' (Supplementary Table S1). All sequences were verified by DNA sequencing using the ALFexpress system. pCMV encoding β-galactosidase was from Clontech (Mountain View, CA, USA).

Antibodies

Primary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): AR (sc-816), BRG1 (sc-10768), TRAP220 (sc-8998), FKBP51 (sc-13983), GAPDH (sc-25778), and normal rabbit IgG (sc-2027); from Abcam (Cambridge, UK): H3 (ab1791), H3K4me3 (ab8580) and H3K36me3 (ab9050); from Covance (Emeryville, CA, USA): PolII (MMS-126R); from UpState (Lake Placid, NY, USA): H3K9, K14ac (06-599); and from BD Biosciences (San Jose, CA, USA): BRM (610389).

Isolation of RNA and quantitative real-time RT-PCR (qRT-PCR)

VCaP and LNCaP cells were seeded onto 6-well plates (8×10^5 and 3.3×10^5 cells/well, respectively) and grown 36 h in transfection medium [DMEM containing 2.5% charcoal-stripped FBS (CCS-FBS)] devoid of steroids. Subsequently, cells were treated with or without synthetic androgen agonist R1881 (Perkin Elmer Inc., Waltham, MA, USA), 5α-dihydrotestosterone (Steraloids Inc., Newport, RI, USA) or bicalutamide (Zeneca Pharmaceuticals, Macclesfield, UK) as indicated. Total RNA was extracted using TRIzol[®] Reagent (Invitrogen Lifetechnologies, Carlsbad, CA, USA) and converted to cDNA using Transcriptor First Strand cDNA synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. cDNA was used as a template in qRT-PCR, which was carried out using MX3000P real-Time PCR System (Stratagene, La Jolla, CA, USA), FastStart SYBR Green Master (Roche) and specific primers for *FKBP51*, *PSA*, *TMPRSS2* and *GAPDH* (Supplementary Table S1). Analyzed *GAPDH* mRNA levels were used to normalize the amounts of total RNA between the samples. Fold changes were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is $\Delta Ct_{(R1881)} - \Delta Ct_{(EtOH)}$, ΔCt is $Ct_{(gene\ X)} - Ct_{(GAPDH)}$ and Ct is the cycle at which the threshold is crossed.

Electrophoretic mobility shift assay (EMSA)

For production of AR for EMSAs, COS-1 cells were transfected either with empty pSG5 or pSG5-hAR and cell extracts were prepared as described (24). Twelve micrograms of cell extract was used for 20 µl

EMSA reaction. The mock extract was treated with ethanol and AR extract with 10 nM R1881. ³²P-labeled ds-oligomer probe was added and protein–DNA complexes were allowed to form for 1 h (25). The intronic ARE 5'-AGTACGtgaTGTTCT-3' (core) of rat *prostatein peptide C3(1)* was used as a positive control. Probes contained the 27 bp native sequences (Supplementary Table S1). The complexes were separated on 4% non-denaturing PAGE. The gels were dried and detected using phosphorimager (FLA3000, Fuji, Japan).

Reporter gene assay (RGA)

VCaP and LNCaP cells were seeded onto 12-well plates (3.2×10^5 and 1.4×10^5 cells/well, respectively) and grown 4 h in transfection medium. The cells were transfected with reporter gene constructs (1.8 μ g pLUC and 0.2 μ g pCMV β /well) using jetPEITM (Polyplus-transfection SA, Illkirch, France) transfection reagent. COS-1 cells were seeded onto 12-well plates (1.4×10^5 cells/well) and grown 4 h in transfection medium. The cells were transfected with reporter gene constructs (0.46 μ g pLUC, 0.02 μ g pCMV β /well and 0.02 μ g pSG5-hAR) using TransIT-LT1 (Mirus Bio Corporation, Madison, WI, USA) transfection reagent. One day after transfection, the cells were grown for 16 h with either vehicle (ethanol) or 1 nM R1881 and lysed in Reporter Lysis Buffer (Promega, Madison, WI, USA) and LUC activity was measured with Luciferase Assay System (Promega) using Luminoskan Ascent[®] (Thermo Electron, Helsinki, Finland) luminometer and β -galactosidase activity as described (25).

Chromatin immunoprecipitation assay (ChIP)

VCaP and LNCaP cells were seeded at ~70% confluence onto 75 cm² bottles and allowed to grow in steroid-depleted transfection medium for 48 h prior to ChIP. The experiments were performed essentially as previously described (26), except for short-term assays (≤ 2 h), the cells were treated with 2.5 μ M α -amanitin (Sigma-Aldrich, St Louis, MO, USA) for 2 h to further synchronize the cell population (27) prior to addition of androgen. α -Amanitin was removed by washing twice with PBS, and R1881 was added to the medium for indicated times. Specific primers for different regions are listed in Supplementary Table S1. Quantitative PCR analyses were carried out with FastStart SYBR Green Master and MX3000P Real-Time PCR System. Results were calculated using the formula $E^{-\Delta Ct} \times 10$, where E (efficiency of target amplification) is a coefficient of DNA amplification by one PCR cycle for a particular primer pair and ΔCt is $Ct_{(\text{ChIP-template})} - Ct_{(\text{Input})}$. In Figures 5 and 6, results were presented as fold over the value of normal rabbit IgG-precipitated samples.

Immunoblotting

Cell monolayers were washed with ice-cold TBS and harvested in TBS. Cell pellets were suspended in SDS-PAGE sample buffer and lysed by sonication and lysed by sonication 2×10 s. Samples were heated for 5 min at 95°C and separated on 7.5% SDS-PAGE gels.

Proteins were transferred onto nitrocellulose membranes and visualized by indicated antibody and horseradish peroxidase-conjugated anti-rabbit antibody using the enhanced chemiluminescence detection reagents according to the manufacturer's instructions (Pierce). For protein quantification, the proteins were visualized by goat anti-rabbit IgG DyLightTM 680 conjugated secondary antibody. Quantification of the immunoblots was performed by scanning with Li-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) at 700 nm channel according to manufacturer's instructions. AR and FKBP51 protein amounts were normalized with GAPDH amounts (assessed with a specific antibody) in each sample.

RESULTS

Rapid and robust induction of *FKBP51* transcription by androgen

To study the androgen regulation of *FKBP51* of human prostate VCaP cancer cells, we isolated RNA at different periods after addition of androgen (R1881; 0, 1.5, 3, 6, 12 and 18 h) and performed qRT-PCR analysis. As shown in Figure 1A, *FKBP51* mRNA is rapidly and strongly induced after androgen exposure, with the induction being >20-fold at 3 h and peaking (≥ 60 -fold) at 12 h. Compared to the *FKBP51*, the induction of *PSA* was much slower and less impressive. Another AR target gene *TMPRSS2* showed similar induction kinetics as *FKBP51*, but its overall induction remained modest (~10-fold) in comparison to *FKBP51*. Since the *FKBP51* mRNA persisted at a high level after 12 h, we checked whether that was mainly due to new mRNA synthesis or by mRNA stabilization. To that end, we terminated mRNA synthesis by adding actinomycin D to the cells at 12 h after the androgen exposure and monitored the amount of *FKBP51* mRNA at further time points. Decline of the *FKBP51* mRNA level after addition of actinomycin D indicates that the AR-mediated transcriptional response continues for prolonged times, at least until 36 h after androgen exposure (Figure 1B, data not shown). To confirm that the gene is a direct transcriptional target for AR, the cells were treated with or without translation inhibitor cycloheximide (CHX) and qRT-PCR analyses were performed. CHX did not inhibit the androgen induction of *FKBP51* (but actually enhanced it; $p < 0.001$) or *TMPRSS2* mRNA ($p > 0.05$), whereas it nearly abolished the androgen induction of *PSA* mRNA ($p < 0.05$) (Figure 1C). These results indicate that, in contrast to *PSA*, the androgen regulation of *FKBP51* and that of *TMPRSS2* does not require *de novo* protein synthesis. The effect of CHX on the *PSA* mRNA is in line with previous findings in other prostate cancer cell types (26,28).

We next tested whether the androgen activation of *FKBP51* in VCaP cells differs from that in LNCaP cells which are often used as a model of prostate cancer cells. We first compared the AR protein levels in the two cell lines by immunoblotting with anti-AR antibody. In keeping with the amplification of AR in VCaP cells (23),

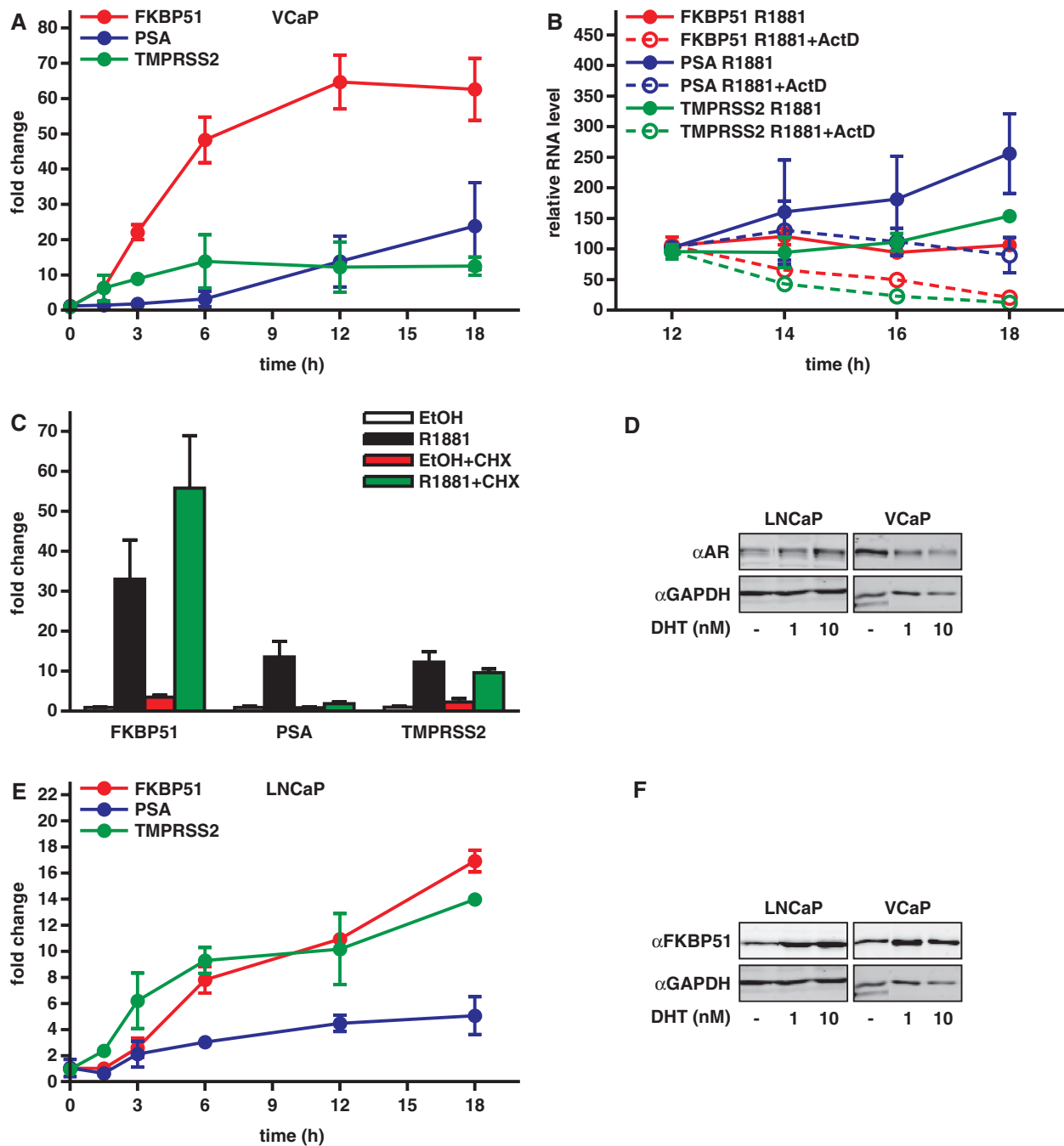


Figure 1. Rapid and robust augmentation of *FKBP51* transcription in response to androgen exposure in prostate cancer cells. (A) Accumulation of *FKBP51*, *TMPRSS2* and *PSA* mRNA in response to androgen treatment in VCaP cells. Cells were treated with 1 nM synthetic androgen R1881 for indicated times and mRNAs were analyzed by qRT-PCR analysis. (B) To test whether the *FKBP51* mRNA accumulation after 12h androgen treatment was caused by new mRNA synthesis or mRNA stabilization, cells were further grown in the presence or absence of actinomycin D (1 μ g/ml) for indicated times and qRT-PCR was performed. (C) VCaP cells were treated with vehicle (ethanol, EtOH) or R1881 for 24h in the presence and absence of translation inhibitor cycloheximide (CHX, 10 μ g/ml) and qRT-PCR analyses were performed. Total RNA levels between samples were normalized using mRNA levels of *GAPDH*. Fold changes were calculated as described in Materials and Methods. Columns represent the mean \pm SD of three independent experiments. (D) Immunoblotting analysis of AR protein levels in VCaP and LNCaP cells grown for 18h with vehicle (EtOH) or androgen (1 or 10 nM 5 α -dihydrotestosterone, DHT) as indicated. AR was detected using anti-AR antibody as described in Materials and Methods. (E) Time course of AR target gene mRNA accumulation in LNCaP cells was carried out as in panel A. (F) Immunoblotting analysis of *FKBP51* protein levels in VCaP and LNCaP cells cultured in the presence and absence of androgen as in panel D.

but not in LNCaP cells, quantification of the AR bands showed that VCaP cells contain ~3–7-fold more AR protein than LNCaP cells, with the difference being dependent on the presence of androgen in the culture medium

(Figure 1D). Interestingly, androgen exposure had a reciprocal effect on AR levels in the two cell lines; it repeatedly decreased the amount of the receptor in VCaP cells and increased it in LNCaP cells.

In comparison to VCaP cells, the fold-induction of FKBP51 mRNA by androgen was weaker and it peaked later in LNCaP cells (Figure 1E). Silencing of AR expression in VCaP cells was paralleled by a marked reduction in the androgen-induced accumulation of FKBP51 mRNA (Supplementary Figure S1), supporting the notion that the weaker induction of *FKBP51* in LNCaP cells is mainly due to the lower amount of AR in those cells. Moreover, the curve of FKBP51 mRNA accumulation in response to increasing androgen concentrations was shifted to the right compared to the situation in VCaP cells (Supplementary Figure S2). In both prostate cancer cell lines, induction of *FKBP51* by androgen was also manifested on the FKBP51 protein level (Figure 1F).

***FKBP51* locus harbors several potential AR-binding sites**

The above data strongly suggest that *FKBP51* is directly targeted by AR and mainly at the transcriptional level. Moreover, the robust androgen induction of *FKBP51* implies that there may be more than one ARE-containing region within the gene. To identify potential AREs in the *FKBP51*, we performed *in silico* analysis from -20 kb upstream of its TSS to 20 kb downstream from its last exon (exon 11) spanning ~155 kb in chromosome 6 using the *CONSITE* program (<http://asp.iu.uib.no:8090/cgi-bin/CONSITE/consite/>). The score cutoff was adjusted to 80% and the position weight matrix provided by the program was used. After program-based screening, putative AREs were further manually sifted using G at position 2 and C at position 5 in the half-site as the criterion (Figure 2B). This process resulted in identification of 13 putative AREs (AREI–AREXIII, Figure 2B) of which all, excluding AREI, were located within the introns of *FKBP51* (Figure 2A). Only two of the AREs, AREIII and AREXI, show characteristics of selective AREs consisting of two hexameric half-sites arranged as a so-called androgen direct repeat separated by a 3-nt spacer (DR3), whereas the other 11 elements resemble 3-nt-spaced inverted repeats of (IR3) of a 5'-TGTTCT-3'-like half-site (29). Interestingly, AREVIII and AREIX in the fifth intron (at ~87 kb from the TSS) are separated from each other only by 16 nt. Similarly, AREX, AREXI and AREXII are clustered within 215 nt in the 7th intron (at ~98 kb from the TSS).

To test the capability of the putative 13 AREs to bind AR *in vitro*, we performed electrophoretic mobility shift assays with recombinant human AR from COS-1 cells. AR was incubated with dsDNA oligomers containing the 15 bp core element of a given putative ARE surrounded by 6 bp native flanking sequences. The intronic ARE of the rat *prostatein peptide C3(1)* that binds efficiently AR *in vitro* was used as a positive control (30). In EMSAs, nine AREs, AREIII–VI, AREVIII–XI, and AREXIII, showed AR–DNA complex formation that was >25% of that of the positive control ARE (Figure 2C and Supplementary Figure S3). EMSAs with mutated oligomers containing A instead of G or C in the 2nd and 5th position of one half-site of AREIX (IXm) and AREXIII (XIIIIm) confirmed the specificity of AR–ARE interaction and the importance of both half-sites for the interaction

(Figure 3C, on the right). The high portion of the predicted AREs capable of efficiently interacting with the receptor indicates the biochemical relevance of our *in silico* screen.

Function of the *FKBP51* AREs as enhancers

The above EMSA results suggest that there are several potential AREs within the *FKBP51* locus. We next tested their function as androgen-regulated enhancers in prostate cancer cells. Approximately 300-nt regions covering the AREs (cf. Figure 2A) were cloned in front of a TATA-box containing luciferase plasmid. VCaP cells were transfected with the plasmids and treated with or without androgen. Empty TATA-box containing luciferase plasmid served as a negative control. As shown in Figure 2D, region 11 harboring the AREVIII and AREIX and region 12 harboring the AREX, AREXI and AREXII were the only regions conferring significant androgen induction in VCaP cells, with the region 12 showing the strongest activity and androgen induction (~10-fold). These two *FKBP51* regions displayed strong androgen induction also in COS-1 cells cotransfected with an AR expression vector. In addition, region 7 that showed only a modest induction (1.7-fold) in VCaP cells conferred in COS-1 cells a strong androgen induction (Figure 2E). Androgen induction of the regions 11 and 12 was weaker in LNCaP than in VCaP cells (cf. Figure 2D and F). Ectopic expression of AR to some extent strengthened the induction through the region 12 in LNCaP cells (Figure 2G), suggesting that the difference in the effect of androgen between the two prostate cell lines reflects the amount of AR in these cell lines. However, the region 12 harboring three AREs conferred the strongest androgen induction in all studied cell types. Taken together, these results suggest that there are two major distally located enhancers (≥87 kb from the TTS) that function in the androgen regulation of the *FKBP51* in prostate cancer cells.

Loading of AR onto *FKBP51* chromatin in response to androgen

We next analyzed the interaction of AR with the *FKBP51* locus *in vivo* by performing chromatin immunoprecipitations (ChIP) in VCaP cells with anti-AR antibody followed by real-time PCR analysis. Altogether 14 regions of *FKBP51* were scanned. In addition to the regions harboring the *in silico*-predicted AREs, these assays included four regions without predicted AREs, two on both sides of the TSS (regions 2 and 3), one within first intron (region 8) and one downstream (region 14) the gene (Figure 2A). *PSA* upstream (~4 kb) enhancer and promoter, and *TMPRSS2* upstream (~13.5 kb) enhancer regions served as positive controls (20,26), and a region located in the middle of non-androgen responsive *DSCAM* gene (in chromosome 22) served as a negative control. VCaP cells were exposed to androgen for various time points (0–120 min at 20 min intervals) prior to ChIPs. Loading of AR was clearly detectable onto several regions within the *FKBP51* locus at the 20 min time point (Figure 3A). Regions 11 and 12 were the two *FKBP51* regions showing

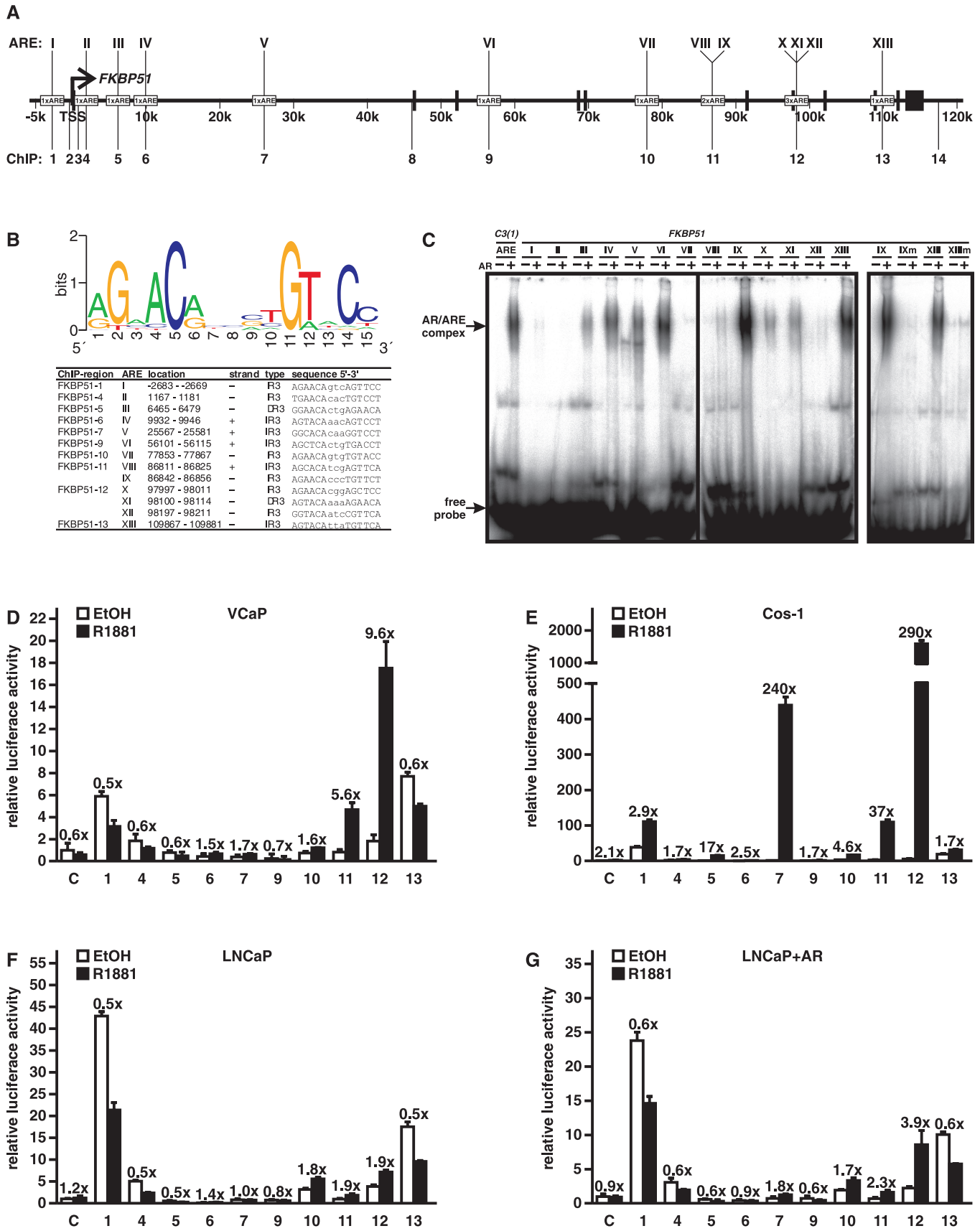


Figure 2. Binding of AR to putative *FKBP51* AREs *in vitro* and their function as enhancers. (A) Schematic locations of the AREs and regions analyzed in reporter and ChIP assays. Bars depict the positions of exons and an arrow indicates the transcription start site. Roman numerals depict

the most abundant occupancy of holo-AR, but androgen induced marked enrichment of the receptor (≥ 10 -fold) also onto regions 7 and 10. Binding of holo-AR peaked at the 60 min (region 11) or 80 min time point (regions 7, 10 and 12). Similar kinetics of AR loading was observed with the PSA and TMPRSS2 enhancers. Thus, AR can *in vivo* interact with several separated sites that are very distal and downstream from the *FKBP51* TSS. However, the occupancy level of holo-AR on those sites in chromatin did not directly correlate with their isolated enhancer activity in VCaP cells, in that the region 11 showed less activity than the region 12 as an enhancer and the interaction of AR with regions 7 and 10 in the chromatin was more prominent than expected on the basis of their very weak function as isolated androgen-regulated enhancers. Interestingly, there was also a delayed loading of holo-AR at the 100 min time point at region 3 that does not contain any potential ARE.

Since actinomycin D experiments (Figure 1B) indicate that the *FKBP51* is still actively transcribed after 12 h of the androgen addition, we also monitored the loading of AR onto the region 11 at later time points. ChIP assays showed that the amount of AR bound to the *FKBP51* decline after 6 h, i.e. earlier than the observed peak of the *FKBP51* mRNA accumulation (cf. Figures 3B and 1A). These results suggest that after the initial peaking of AR binding to the *FKBP51* and activation of transcription, declining amounts of AR are still sufficient to maintain the maximal androgen-activated transcription of the locus.

Histone marks and density reflect active chromatin structure in *FKBP51* locus

Since the above ChIP assays indicate that most of the AR binding to the *FKBP51* locus occurs to sites that are very distal to the TSS, it was of interest to monitor the occupancy and recruitment of RNA polymerase II (PolII) within the locus. In keeping with the augmenting effect of androgen on the *FKBP51* transcription, addition of androgen was accompanied with a clear increase in the occupancy of PolII throughout the locus as assessed by ChIP using an antibody against the largest subunit of the PolII in VCaP cells (Figure 4B). The relative occupancy of PolII was higher just downstream from the TSS, in the middle of the locus, and at the 3' end of the gene than other gene regions. Thus, PolII occupancy does not mirror the loading of AR to the locus, suggesting that chromatin

loop formation is involved in the recruitment of PolII by the holo-AR.

We next investigated possible changes in chromatin structure of the *FKBP51* locus upon androgen induction. Covalent modifications of core histones reflect the chromatin activity, i.e. whether it is in 'closed' or 'open' conformation. For example, acetylation (ac) of histone 3 K9 and K14 and trimethylation (me3) of both H3K4 and H3K36 are linked to active chromatin conformation (31–34). We scanned the occurrence of the above modifications by using H3 modification specific antibodies in ChIP analyses. As shown in Figure 4D and E, both H3K9,14ac and H3K4me3 levels clearly peaked around and immediately downstream the TSS, whereas H3K36me3 marks were elevated more downstream from the TSS and remained elevated throughout the reading frame of the gene. Since the androgen-induced changes in these histone marks at most sites were modest, we next studied whether androgen induction also brings about changes in histone density. Even though the total amount of H3 in the absence of androgen was similar at most sites in the locus, the occupancy of H3 around the TSS was lower than in other parts of the gene. Interestingly, androgen induction resulted in a decrease in the H3 occupancy at many sites in the locus, with the greatest drop around regions 5 and 6 (Figure 4C). Similar changes were seen in H2B levels, strongly suggesting that the changes in the H3 mirror nucleosome density in general (data not shown).

Androgen-induced recruitment of BRM-containing chromatin-remodeling complexes onto *FKBP51* locus

The above changes in the histone density suggest that chromatin remodeling complexes may be involved in the androgen activation of the *FKBP51* locus. Since SWI/SNF complexes containing BRM or BRG1 as the major ATPase subunit can catalyze nucleosome sliding and histone transfer (12,13), we investigated whether androgen induction is linked to any changes in the occupancy of BRM- or BRG1-containing SWI/SNF complexes in the locus. ChIP assays with antibodies against BRM showed that androgen treatment of VCaP cells brings about very robust recruitment of BRM onto regions 5 and 6, whereas the occupancy of BRG1 in the locus remained very low after the androgen exposure (Figure 5). Interestingly, the regions around ~ 10 kb from the TSS occupying most BRM were the same ones showing the most prominent

the AREs, and Arabic numerals indicate the regions amplified for enhancer analyses and ChIP assays. (B) Sequences of the potential AREs in *FKBP51* gene. (Top) Position weight matrix used for identification of putative AREs. The frequency of nucleotides at each of the 15 positions in the ARE in the matrix is shown. (Lower) List of the *in silico*-identified AREs, their localization in the *FKBP51* gene, sequences and type of ARE. (C) Electrophoretic mobility shift assays were performed in the presence of AR-containing COS-1 cell extracts (AR+) or extracts from empty vector-transfected cells (AR-), and 32 P-labeled dsAREs or mutated (m) versions of AREIX and AREXIII. Protein-DNA complexes were resolved on non-denaturing 4% polyacrylamide gels. The intronic ARE of *prostatein peptide C3(1)* was used as a positive control. (C, D) Transcriptional activity of the ARE-containing 300-bp *FKBP51* fragments was assessed by reporter gene assays. VCaP (D), COS-1 (E), LNCaP (F and G) cells were transfected with TATA-LUC constructs driven by indicated regions of *FKBP51* as described in Materials and Methods. For COS-1 (E) and LNCaP + AR (G) analyses, pSG5-hAR (0.02 or 0.2 μ g/well, respectively) was cotransfected with the reporter constructs. Cotransfection of pCMV β and β -galactosidase activity was used for normalization of transfection efficiency. The cells were treated with vehicle (EtOH) or 1 nM R1881 for 16 h before harvesting the cells for reporter analyses. Results are shown as relative LUC activity and fold inductions of androgen-treated samples in the relation to the activity of ethanol-treated samples are shown above the columns. Columns represent the mean \pm SD of three independent experiments.

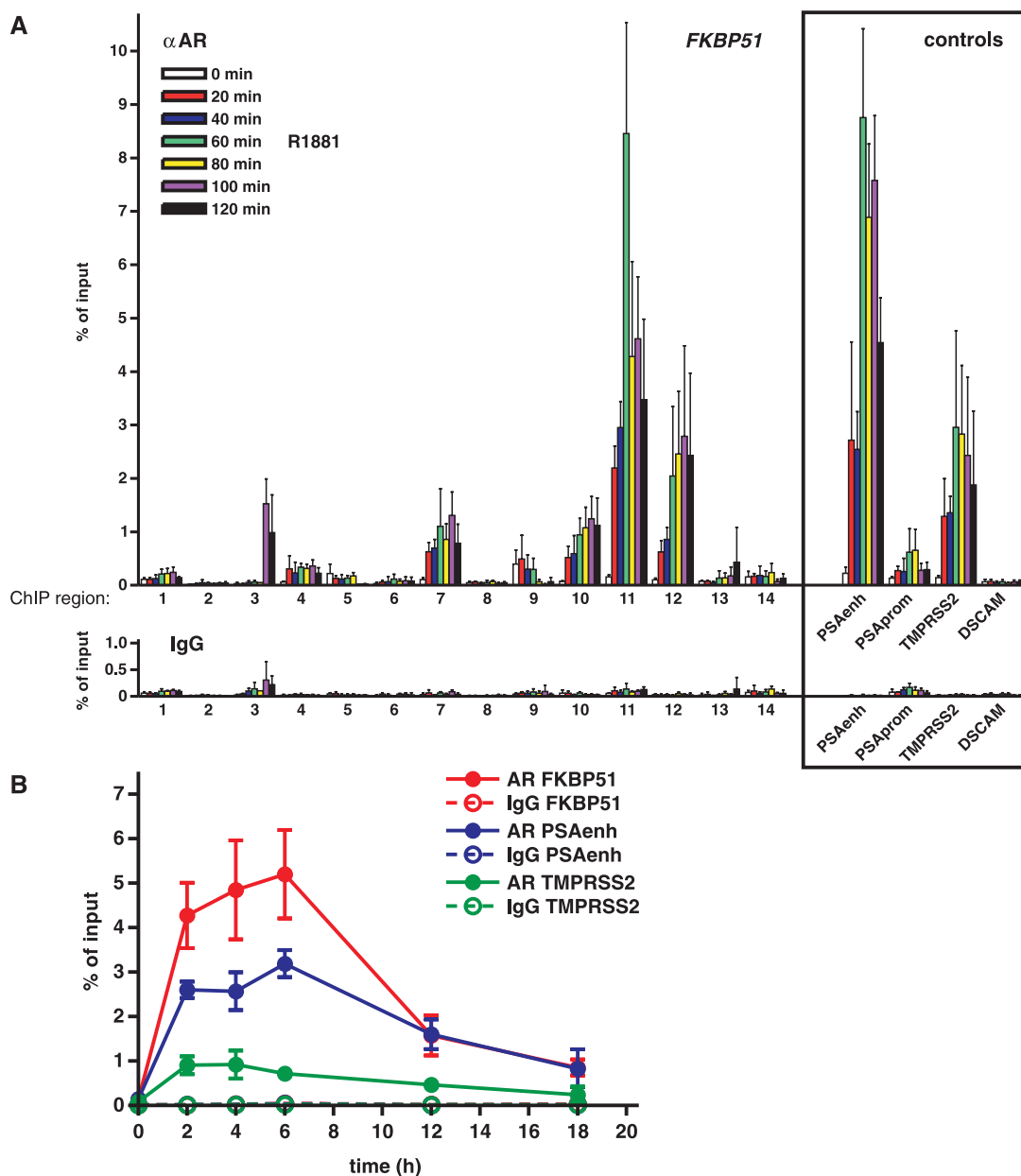


Figure 3. Spatial and temporal loading of AR onto the *FKBP51* locus in response to androgen exposure in VCaP cells. **(A)** Cells were treated with 1 nM R1881 for indicated times and chromatin immunoprecipitations with anti-AR antibody followed by real-time PCR were carried out as described in Materials and Methods section to investigate the occupancy of AR within 14 different regions of *FKBP51* gene. Enhancer and promoter regions of *PSA* and distal enhancer region of *TMPRSS2* served as known positive references, and a region in the middle of *DSCAM* was as a negative control. ChIP assays with normal IgG monitored nonspecific binding. **(B)** Unsynchronized VCaP cells were treated with R1881 for indicated times and ChIP samples were analyzed by ChIP with anti-AR or nonspecific IgG. The precipitated DNAs were used as templates for qPCR. Results are shown as percentages of the input samples. Columns represent the mean \pm SD of three experiments.

decrease in H3 density in response to androgen exposure. These results suggest that AR influences to the chromatin structure of *FKBP51* via recruitment of BRM-containing complexes to the locus.

Effect of antiandrogen on *FKBP51* transcription and AR loading

Since increased AR protein levels have been reported to convert antiandrogens, such as bicalutamide, to agonists (35), it was of interest to investigate whether the difference

in the amount of AR between VCaP and LNCaP cells is manifested in a differential response to the pure antagonist bicalutamide (BIC). However, BIC alone displayed no or minimal agonistic activity on *FKBP51* mRNA accumulation in LNCaP and VCaP cells (Figure 6A). BIC was also capable of inhibiting R1881-induced accumulation of *FKBP51* mRNA in both cell lines, albeit the pure antagonist was somewhat more potent in LNCaP cells than in VCaP cells ($\geq 90\%$ versus $\sim 80\%$ inhibition). As in the case of pure agonists, the pure antagonist down-regulated the AR protein in VCaP cells but not in LNCaP

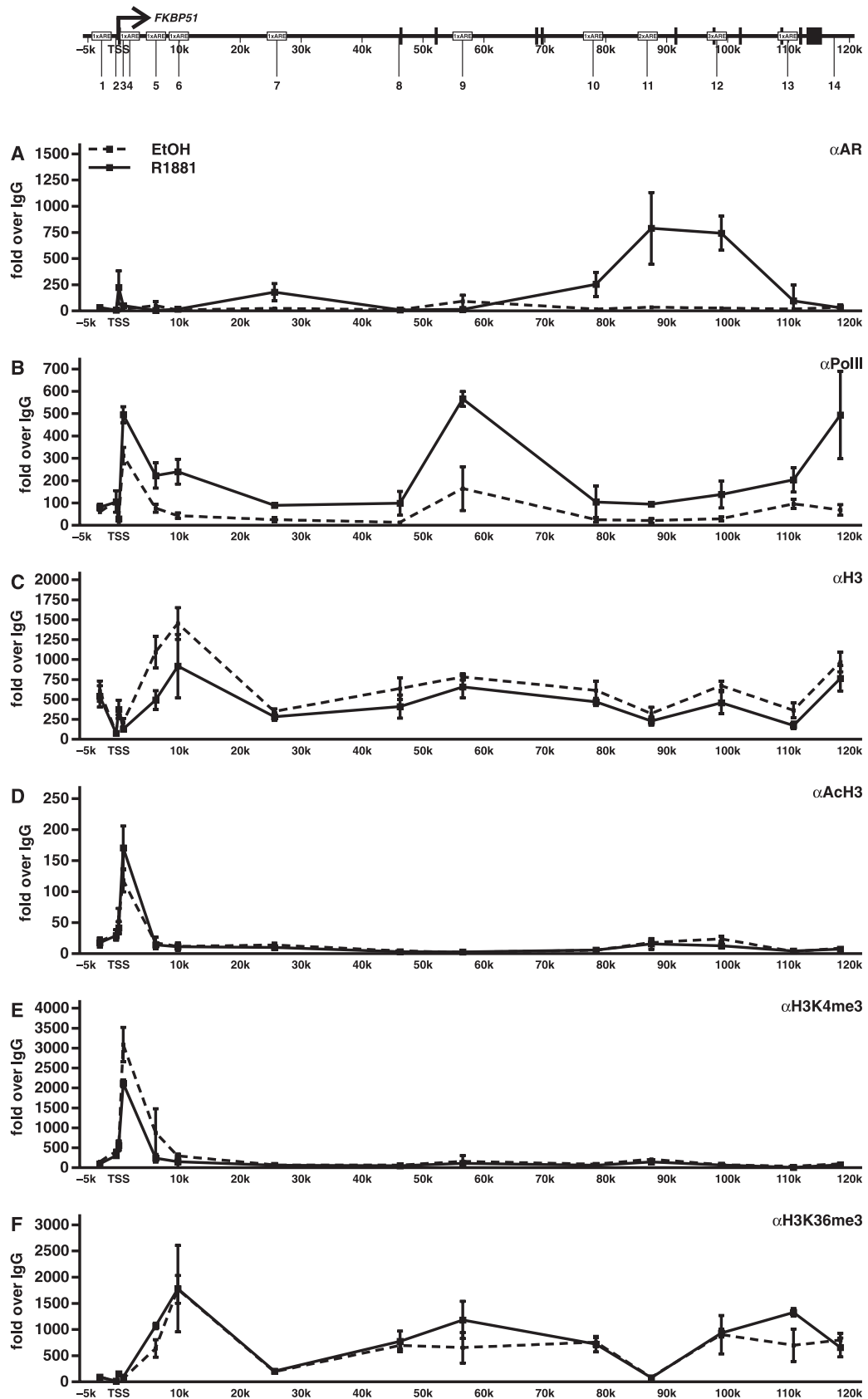


Figure 4. Occupancy of RNA Polymerase II and occurrence of H3 modifications within the *FKBP51* locus in VCaP cells. The cells were treated with R1881 or vehicle (EtOH) for 2 h and ChIP assays were performed using antibodies against AR (A), PolII (B), H3 (C), AchH3 (H3K9, K14ac) (D), H3K4me3 (E) or H3K36me3 (F). ChIP samples were used as templates in quantitative PCR. Results are shown as fold over the normal rabbit IgG-precipitated samples and represent the mean \pm SD of three experiments.

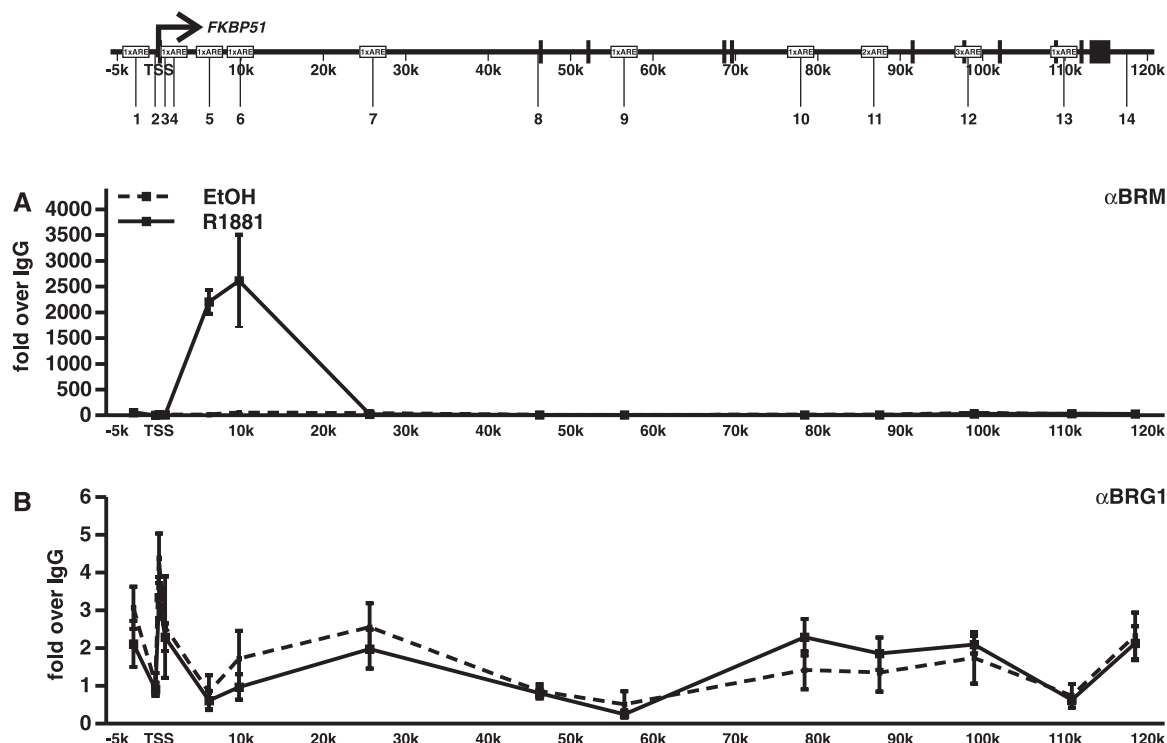


Figure 5. Androgen-induced recruitment of BRM-containing complexes onto the *FKBP51* locus. VCaP cells were treated with R1881 or vehicle as in Figure 4 and ChIP assays were performed using antibodies against BRM (A) or BRG1 (B) followed by quantitative PCR analysis. Results are shown as fold over the normal rabbit IgG-precipitated samples and represent the mean \pm SD of three experiments.

cells (Figure 6B). We next compared the loading of AR to the *FKBP51* chromatin in response to R1881 and BIC in LNCaP and VCaP cells by ChIP and qPCR analysis. As shown in Figure 6B, the overall pattern of agonist-induced loading of AR to the locus was very similar in both cell lines, but the level of AR occupancy was lower in LNCaP cells than in VCaP cells. The latter finding very likely reflects the difference in the amount of AR between the cell lines. Interestingly, BIC alone was capable of promoting binding of AR to the *FKBP51* locus in VCaP cells but not in LNCaP cells (Figure 6C). However, in agreement with the minimal mRNA induction of *FKBP51* upon BIC exposure in VCaP cells, the BIC treatment did not bring about significant changes in the occupancy of PolIII in the locus (Supplementary Figure S4).

DISCUSSION

Knowledge of the molecular mechanisms of AR-dependent transcription is essential for better understanding of its role in prostate cancer development. On chromatin and target locus level, however, detailed analyses of AR-targeted loci have been thus far limited to the *PSA* gene (16–20). Moreover, only one model cell line, LNCaP, has been employed in most of these studies. Here, we have gathered molecular insight into the AR action by investigating the regulation of *FKBP51* transcription using VCaP and LNCaP cells as model systems. The VCaP cells are derived from a bone metastasis and express

amplified levels of wild-type AR, thus perhaps better resembling the situation in hormone-refractory prostate cancer than the LNCaP cells that correspond to a lymph node metastasis with a ligand-binding domain T877A-mutated version of the receptor (23). The rationale for choosing the *FKBP51* as a model gene is based on the recent finding that the *FKBP51* expression is more sensitive to depletion of intraprostatic androgens than any other AR target gene (22). Response of *FKBP51* mRNA to androgens has previously been observed also in LNCaP cell and CWR22-R xenograft models (36–38). *FKBP51* expression is also upregulated by glucocorticoids and progestins in lymphocytes and breast cancer cells, respectively (39,40). However, the molecular mechanisms of the activation of *FKBP51* chromatin by AR or glucocorticoid receptor (GR) have not been addressed in detail.

FKBP51 (*FKBP5*) encodes a 51 kDa FK506-binding protein that belongs to the immunophilin protein family (41). The exact role of *FKBP51* is not known, but it appears to play a role in protein folding and trafficking (41). *FKBP51* was discovered as a human homolog for avian *FKBP54* (42) that in turn was originally found in progesterone receptor (PR) complexes (43). AR can interact with *FKBP51* and an increase in the immunophilin level may augment the activity of AR (37). Interestingly, *FKBP51* protein levels are elevated in prostate cancer, which may sensitize AR signaling (37,38). Also our statistical analysis of the microarray data retrieved from the Oncomine website (www.oncomine.org) indicates significantly increased expression of *FKBP51* in prostate cancer

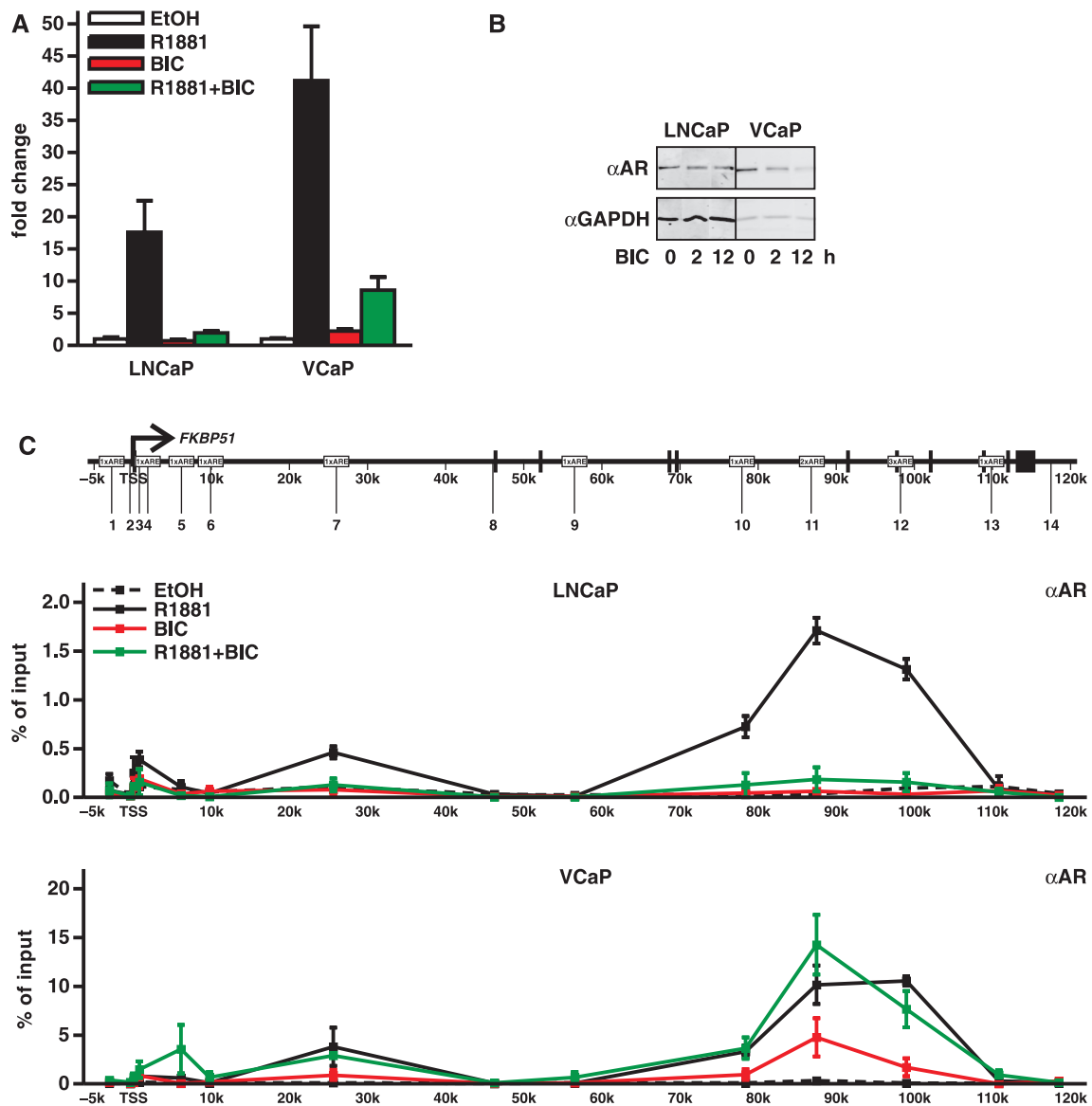


Figure 6. Effect of androgen and antiandrogen on *FKBP51* transcription and AR loading in VCaP and LNCaP cells. (A) Accumulation of *FKBP51* mRNA in response to exposure of VCaP and LNCaP cell to R1881 (1 nM) or bicalutamide (BIC, 10 μ M) alone or their combination for 12 h. Messenger RNAs were analyzed by qRT-PCR analysis as described in Figure 1. (B) Immunoblotting analysis of AR protein levels in VCaP and LNCaP cells in the presence and absence of BIC for 2 or 12 h as indicated. (C) Loading of AR onto the *FKBP51* locus in response to androgen or antiandrogen exposure for 2 h in VCaP cells and LNCaP cells. Cells were treated with vehicle, R1881 or BIC (10 μ M) as in Figure 4 and ChIP assays were performed using antibody against AR followed by quantitative PCR analysis. Results are shown as percentages of the input samples and represent the mean \pm SD of three experiments.

in comparison to non-malignant prostate (Supplementary Figure S5).

The reason for the fast and robust induction of *FKBP51* by AR, in comparison to *PSA*, appears to be twofold. Firstly, the *FKBP51* is regulated purely at the level of transcription, whereas a regulatory component requiring new protein synthesis is involved in the activation of *PSA* in VCaP cells and LNCaP cells (26). Secondly, several AR-binding sites appear to synergize in the activation of the *FKBP51*. Our *in vitro* testing of the potential AREs together with quantitative ChIP scans revealed the presence of at least seven functional AREs within four distinct

enhancer regions located in the introns of the gene. Interestingly, the two major enhancer regions harboring multiple AREs are located very distal from the TSS, at \sim 87 kb in the 5th intron (two AREs) and \sim 98 kb in the 7th intron (three AREs), with the farthest acting as the most active AR-regulated enhancer region of *FKBP51* in isolation. Even though the single ARE-containing regions at \sim 25 kb (1st intron) and \sim 77 kb (5th intron) possessed in isolation only weak enhancer activity, they showed significant androgen-induced loading of AR both in VCaP and LNCaP cells and are thus likely to cooperate with the compound AREs *in vivo*.

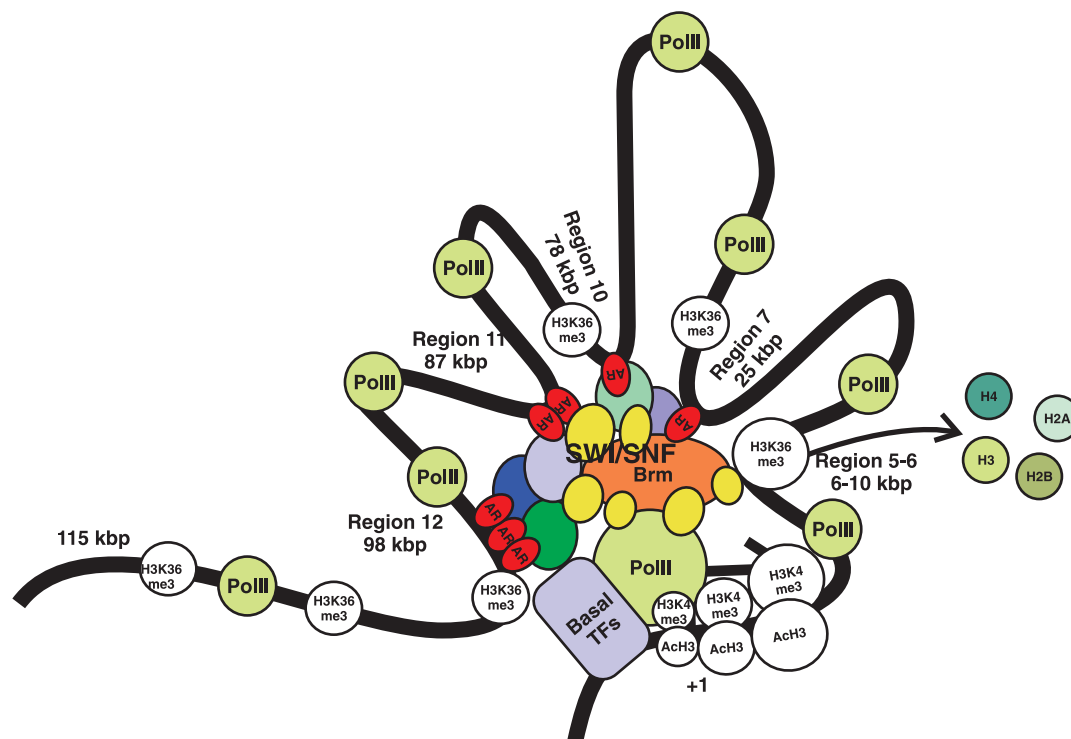


Figure 7. A spatial model of loop formation in the long-range regulation of the *FKBP51* transcription by the AR transcription complex. ARs are first primarily loaded onto two very distal regions at ~98 and ~87 kb downstream from the TSS. These two compound-ARE-containing enhancers interact via coregulatory proteins, including BRM-containing SWI/SNF complexes, and cooperate with AR-occupying single-ARE regions at ~78 and ~25 kb, which brings about further decrease in nucleosome density and recruitment of PolIII tracking through the locus.

Interestingly, the AREVIII and the AREIX in the 5th intron of *FKBP51* are conserved in the corresponding intron of mouse gene (at ~65 kb from TSS), and the region was recently shown to occupy AR in mouse prostate but not GR in mouse thymus (44). The lack of GR occupancy in the 5th intron of *Fkbp51* in the GR's target tissue thymus *in vivo* is somewhat unexpected, since both the GR and the AR (and progesterone receptor, PR) recognize *in vitro* with a similar affinity the non-selective regulatory elements that are composed of 3-nt-spaced inverted repeats of a 5'-TGTTCT-3'-type half-site (29,45). The AREs in the 5th intron as most of the other elements of the *FKBP51* correspond to the latter, non-selective-type response elements, which is in keeping with the regulation of *FKBP51* by several steroid hormones. Two of the three AREs in the 7th intron of human *FKBP51* that were found and validated in this study are conserved in the corresponding intron of the mouse gene, but their function as AREs or GREs has not been addressed in the previous studies. In contrast to the role of distal elements, ChIP assays have recently revealed that the regulation of *Fkbp51* by progestins occurs in breast cancer cells via an intronic enhancer that resides relatively close (~1.2 kb) to the TSS (46). In transfection-based reporter gene assays however, the 5th intronic region of the human gene has been reported to confer progesterone response in breast cancer cells (39). These results thus emphasize the importance of validation of the putative steroid response elements under chromatin

environment by ChIP assays. Thus, even though AR, GR and PR all recognize similar DNA elements *in vitro* and often regulate same genes, such as *FKBP51*, these steroid receptors seem to employ alternative binding sites in chromatin to activate transcription *in vivo*. Since the above analyses were carried out in different cell types, it is likely that differences in chromatin milieu significantly contributed to the observed differences *in vivo*. The role of other 'pioneering' transcription factors binding close to steroid receptor-occupied sites may also be important (20,47), as GATA2 was shown to cooperate with the PR in the regulation of *Fkbp51* (46). However, according to our unpublished data, GATA2 or FoxA1 do not play a major role in the androgen regulation of *FKBP51* in VCaP cells (Makkonen, H., unpublished results).

Scanning of the chromatin structure of *FKBP51* in VCaP cells by ChIP showed a considerable enrichment of acetylated H3 as well as trimethylated H3K4 nearby the TSS, and K36 trimethylated H3 was prevalent throughout the whole coding region of the gene. These marks along their locations are typical to genes in active chromatin (6,10). However, the above H3 modifications were only modestly influenced by androgens, suggesting that the *FKBP51* locus existed in an active conformation already prior to AR binding and the receptor was targeting pre-opened chromatin in prostate cancer cells. Similar results have been reported for the *PSA* locus in LNCaP cells (16). In keeping with the key role of AR in the activation and elongation of *FKBP51* transcription, androgen

induction brought about a clear increase in the occupancy of PolII throughout the gene, whereas antiandrogen bicalutamide-bound receptor failed to do so, even though it was in VCaP cells capable of promoting AR binding to the locus. Androgen induction also resulted in a decrease in histone density within the locus, which is likely to be at least in part caused by recruitment of BRM-containing chromatin remodeling complexes. The temporal and spatial correlation between AR loading and recruitment of BRM is in agreement with the previous reports that AR predominantly employs a subfamily of SWI/SNF complexes that depend on the BRM, but not on the BRG1, ATPase (48,49).

The very distal enhancer elements are thought to communicate with the general transcription machinery at the TSS through chromatin looping. However, the challenges associated with testing the existence of such loops and the coordination of interactions of several AREs spanning ~100 kb in *FKBP51* are daunting. In our schematic model (Figure 7), we propose that intricate cooperation between multiple AREs orchestrate recruitment of coregulatory complexes and PolII to the *FKBP51* locus, which leads to enhanced transcriptional rate of the PolII. Mediator proteins, such as TRAP220, may potentially bridge AR to PolII (50,51). However, our ChIP assays with anti-TRAP220 antibody do not support a prominent role for the mediator subunit as a bridging factor, which may not be surprising, since there appears to be a lot of functional redundancy among the AR coregulators (19).

Classic models of steroid receptor action view steroid receptor-binding sites as regulatory elements in gene promoters or their proximity as upstream enhancer elements. However, recent wider screens for their binding sites imply that steroid-regulated promoters in most cases lack proximal response elements. For example, genome-wide screening of estrogen receptor binding sites by ChIP combined with tiled microarray analysis indicated that only a minority of estrogen receptor-binding sites localize within 1 kb promoter-proximal regions (52). The same appears to hold true for AR-regulated genes, as most of the AR-binding sites in chromosomes 21 and 22 as well as among a selected sample of target genes seem to reside >10 kb from TSSs, often downstream of them (20,53). Our current validated binding and functional data of the *FKBP51* locus also highlight the emerging biological importance of very long-range regulation via distal enhancers by AR.

The nuclear organization of genome, i.e. how transcription factors, their cognate binding sites and chromatin-modifying complexes are organized with respect to each other within the nucleus, is critical for the proper function of our genome (54). Notably prostate cancers show striking genomic aberrations (55). The capability of AR to regulate promoters from a long distance in chromatin should thus be considered when evaluating the role of AR in targeting novel genes and gene programs, especially in malignant cells. Moreover, the issue concerning the insulating mechanisms that should prevent distally bound steroid receptors from promiscuously activating other than their genuine target genes warrants emphasis in further studies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Gao, W., Bohl, C.E. and Dalton, J.T. (2005) Chemistry and structural biology of androgen receptor. *Chem. Rev.*, **105**, 3352–3370.
- Culig, Z., Klocker, H., Bartsch, G. and Hobisch, A. (2002) Androgen receptors in prostate cancer. *Endocr. Relat. Cancer*, **9**, 155–170.
- Heinlein, C.A. and Chang, C. (2004) Androgen receptor in prostate cancer. *Endocr. Rev.*, **25**, 276–308.
- Kallio, P.J., Poukka, H., Moilanen, A., Jänne, O.A. and Palvimo, J.J. (1995) Androgen receptor-mediated transcriptional regulation in the absence of direct interaction with a specific DNA element. *Mol. Endocrinol.*, **9**, 1017–1028.
- Palvimo, J.J., Reinikainen, P., Ikonen, T., Kallio, P.J., Moilanen, A. and Jänne, O.A. (1996) Mutual transcriptional interference between RelA and androgen receptor. *J. Biol. Chem.*, **271**, 24151–24156.
- Li, B., Carey, M. and Workman, J.L. (2007) The role of chromatin during transcription. *Cell*, **128**, 707–719.
- Metivier, R., Gallais, R., Tiffocche, C., Le Peron, C., Jurkowska, R.Z., Carmouche, R.P., Ibberson, D., Barath, P., Demay, F., Reid, G. *et al.* (2008) Cyclical DNA methylation of a transcriptionally active promoter. *Nature*, **452**, 45–50.
- Rosenfeld, M.G., Lunyak, V.V. and Glass, C.K. (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.*, **20**, 1405–1428.
- Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempst, P., Wong, J. and Zhang, Y. (2006) JHDM2A, a JmJc-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell*, **125**, 483–495.
- Kouzarides, T. (2007) Chromatin modifications and their function. *Cell*, **128**, 693–705.
- Farrants, A.K. (2008) Chromatin remodelling and actin organisation. *FEBS Lett.*, **582**, 2041–2050.
- Kwon, C.S. and Wagner, D. (2007) Unwinding chromatin for development and growth: a few genes at a time. *Trends Genet.*, **23**, 403–412.
- Racki, L.R. and Narlikar, G.J. (2008) ATP-dependent chromatin remodeling enzymes: two heads are not better, just different. *Curr. Opin. Genet. Dev.*, **18**, 137–144.
- Trotter, K.W. and Archer, T.K. (2007) Nuclear receptors and chromatin remodeling machinery. *Mol. Cell Endocrinol.*, **265–266**, 162–167.
- Mohrmann, L. and Verrijzer, C.P. (2005) Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. *Biochim. Biophys. Acta*, **1681**, 59–73.
- Jia, L., Shen, H.C., Wantroba, M., Khalid, O., Liang, G., Wang, Q., Gentschein, E., Pinski, J.K., Stanczyk, F.Z., Jones, P.A. *et al.* (2006) Locus-wide chromatin remodeling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells. *Mol. Cell Biol.*, **26**, 7331–7341.
- Kang, Z., Jänne, O.A. and Palvimo, J.J. (2004) Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. *Mol. Endocrinol.*, **18**, 2633–2648.

18. Shang, Y., Myers, M. and Brown, M. (2002) Formation of the androgen receptor transcription complex. *Mol. Cell*, **9**, 601–610.
19. Wang, Q., Carroll, J.S. and Brown, M. (2005) Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol. Cell*, **19**, 631–642.
20. Wang, Q., Li, W., Liu, X.S., Carroll, J.S., Jänne, O.A., Keeton, E.K., Chinnaiyan, A.M., Pienta, K.J. and Brown, M. (2007) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol. Cell*, **27**, 380–392.
21. Balk, S.P., Ko, Y.J. and Bubley, G.J. (2003) Biology of prostate-specific antigen. *J. Clin. Oncol.*, **21**, 383–391.
22. Mostaghel, E.A., Page, S.T., Lin, D.W., Fazli, L., Coleman, I.M., True, L.D., Knudsen, B., Hess, D.L., Nelson, C.C., Matsumoto, A.M. *et al.* (2007) Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. *Cancer Res.*, **67**, 5033–5041.
23. Korenchuk, S., Lehr, J.E., MClean, L., Lee, Y.G., Whitney, S., Vessella, R., Lin, D.L. and Pienta, K.J. (2001) VCaP, a cell-based model system of human prostate cancer. *In Vivo*, **15**, 163–168.
24. Thompson, J., Saatcioglu, F., Jänne, O.A. and Palvimo, J.J. (2001) Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity. *Mol. Endocrinol.*, **15**, 923–935.
25. Palvimo, J.J., Kallio, P.J., Ikonen, T., Mehto, M. and Jänne, O.A. (1993) Dominant negative regulation of trans-activation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation. *Mol. Endocrinol.*, **7**, 1399–1407.
26. Makkonen, H., Jaaskelainen, T., Pitkanen-Arsiola, T., Rytinki, M., Waltering, K.K., Matto, M., Visakorpi, T. and Palvimo, J.J. (2008) Identification of ETS-like transcription factor 4 as a novel androgen receptor target in prostate cancer cells. *Oncogene*, **27**, 4865–4876.
27. Metivier, R., Penot, G., Hubner, M.R., Reid, G., Brand, H., Kos, M. and Gannon, F. (2003) Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell*, **115**, 751–763.
28. Prescott, J., Jariwala, U., Jia, L., Cogan, J.P., Barski, A., Pregizer, S., Shen, H.C., Arasheben, A., Neilson, J.J., Frenkel, B. *et al.* (2007) Androgen receptor-mediated repression of novel target genes. *Prostate*, **67**, 1371–1383.
29. Shaffer, P.L., Jivan, A., Dollins, D.E., Claessens, F. and Gewirth, D.T. (2004) Structural basis of androgen receptor binding to selective androgen response elements. *Proc. Natl Acad. Sci. USA*, **101**, 4758–4763.
30. Kallio, P.J., Palvimo, J.J., Mehto, M. and Jänne, O.A. (1994) Analysis of androgen receptor-DNA interactions with receptor proteins produced in insect cells. *J. Biol. Chem.*, **269**, 11514–11522.
31. Li, B., Howe, L., Anderson, S., Yates, J.R. 3rd. and Workman, J.L. (2003) The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. *J. Biol. Chem.*, **278**, 8897–8903.
32. Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J. and Kouzarides, T. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature*, **419**, 407–411.
33. van Leeuwen, F., Gafken, P.R. and Gottschling, D.E. (2002) Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell*, **109**, 745–756.
34. Verdone, L., Agricola, E., Caserta, M. and Di Mauro, E. (2006) Histone acetylation in gene regulation. *Brief Funct. Genomic Proteomic*, **5**, 209–221.
35. Chen, C.D., Welsbie, D.S., Tran, C., Baek, S.H., Chen, R., Vessella, R., Rosenfeld, M.G. and Sawyers, C.L. (2004) Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.*, **10**, 33–39.
36. Amler, L.C., Agus, D.B., LeDuc, C., Sapinoso, M.L., Fox, W.D., Kern, S., Lee, D., Wang, V., Leysens, M., Higgins, B. *et al.* (2000) Dysregulated expression of androgen-responsive and nonresponsive genes in the androgen-independent prostate cancer xenograft model CWR22-R1. *Cancer Res.*, **60**, 6134–6141.
37. Febbo, P.G., Lowenberg, M., Thorner, A.R., Brown, M., Loda, M. and Golub, T.R. (2005) Androgen mediated regulation and functional implications of fkbp51 expression in prostate cancer. *J. Urol.*, **173**, 1772–1777.
38. Velasco, A.M., Gillis, K.A., Li, Y., Brown, E.L., Sadler, T.M., Achilleos, M., Greenberger, L.M., Frost, P., Bai, W. and Zhang, Y. (2004) Identification and validation of novel androgen-regulated genes in prostate cancer. *Endocrinology*, **145**, 3913–3924.
39. Hubler, T.R. and Scammell, J.G. (2004) Intronic hormone response elements mediate regulation of FKBP5 by progestins and glucocorticoids. *Cell Stress Chaperones*, **9**, 243–252.
40. Vermeer, H., Hendriks-Stegeman, B.I., van der Burg, B., van Buul-Offers, S.C. and Jansen, M. (2003) Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: A potential marker for glucocorticoid sensitivity, potency, and bioavailability. *J. Clin. Endocrinol. Metab.*, **88**, 277–284.
41. Pratt, W.B. and Toft, D.O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.*, **18**, 306–360.
42. Nair, S.C., Rimerman, R.A., Toran, E.J., Chen, S., Prapapanich, V., Butts, R.N. and Smith, D.F. (1997) Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. *Mol. Cell Biol.*, **17**, 594–603.
43. Smith, D.F., Faber, L.E. and Toft, D.O. (1990) Purification of unactivated progesterone receptor and identification of novel receptor-associated proteins. *J. Biol. Chem.*, **265**, 3996–4003.
44. Magee, J.A., Chang, L.W., Stormo, G.D. and Milbrandt, J. (2006) Direct, androgen receptor-mediated regulation of the FKBP5 gene via a distal enhancer element. *Endocrinology*, **147**, 590–598.
45. Claessens, F., Verrijdt, G., Schoenmakers, E., Haelens, A., Peeters, B., Verhoeven, G. and Rombauts, W. (2001) Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. *J. Steroid Biochem. Mol. Biol.*, **76**, 23–30.
46. Magklara, A. and Smith, C.L. (2009) A composite intronic element directs dynamic binding of the progesterone receptor and GATA-2. *Mol. Endocrinol.*, **23**, 61–73.
47. Lupien, M., Eeckhoute, J., Meyer, C.A., Wang, Q., Zhang, Y., Li, W., Carroll, J.S., Liu, X.S. and Brown, M. (2008) FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell*, **132**, 958–970.
48. Link, K.A., Balasubramaniam, S., Sharma, A., Comstock, C.E., Godoy-Tundidor, S., Powers, N., Cao, K.H., Haelens, A., Claessens, F., Revelo, M.P. *et al.* (2008) Targeting the BAF57 SWI/SNF subunit in prostate cancer: a novel platform to control androgen receptor activity. *Cancer Res.*, **68**, 4551–4558.
49. Marshall, T.W., Link, K.A., Petre-Draviam, C.E. and Knudsen, K.E. (2003) Differential requirement of SWI/SNF for androgen receptor activity. *J. Biol. Chem.*, **278**, 30605–30613.
50. Wang, Q., Sharma, D., Ren, Y. and Fondell, J.D. (2002) A coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression. *J. Biol. Chem.*, **277**, 42852–42858.
51. Vijayvargia, R., May, M.S. and Fondell, J.D. (2007) A coregulatory role for the mediator complex in prostate cancer cell proliferation and gene expression. *Cancer Res.*, **67**, 4034–4041.
52. Carroll, J.S., Meyer, C.A., Song, J., Li, W., Geistlinger, T.R., Eeckhoute, J., Brodsky, A.S., Keeton, E.K., Fertuck, K.C., Hall, G.F. *et al.* (2006) Genome-wide analysis of estrogen receptor binding sites. *Nat. Genet.*, **38**, 1289–1297.
53. Bolton, E.C., So, A.Y., Chaivorapol, C., Haqq, C.M., Li, H. and Yamamoto, K.R. (2007) Cell- and gene-specific regulation of primary target genes by the androgen receptor. *Genes Dev.*, **21**, 2005–2017.
54. Fraser, P. and Bickmore, W. (2007) Nuclear organization of the genome and the potential for gene regulation. *Nature*, **447**, 413–417.
55. Morris, D.S., Tomlins, S.A., Montie, J.E. and Chinnaiyan, A.M. (2008) The discovery and application of gene fusions in prostate cancer. *BJU Int.*, **102**, 276–282.