Molecular Cell, Vol. 19, 631–642, September 2, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.molcel.2005.07.018

Spatial and Temporal Recruitment of Androgen Receptor and Its Coactivators Involves Chromosomal Looping and Polymerase Tracking

Qianben Wang,[1,2,3] Jason S. Carroll,[1,2,3] and Myles Brown[1,2,3,*]
[1]Division of Molecular and Cellular Oncology
Department of Medical Oncology
Dana-Farber Cancer Institute
Boston, Massachusetts 02115
[2]Department of Medicine
Brigham and Women's Hospital and
Harvard Medical School
Boston, Massachusetts 02115

Summary

Androgen receptor (AR) plays a critical role in the development and progression of prostate cancer, where it is a key therapeutic target. Here we report that, in contrast to estrogen receptor transcription complexes which form within minutes and recycle hourly, the levels of regulatory regions bound by AR complexes rise over a 16 hr period and then slowly decline. AR regulation of the prostate specific antigen (PSA) gene involves both a promoter-proximal sequence as well as an enhancer ~4 kb upstream. Recruitment of AR and its essential coactivators at both sites creates a chromosomal loop that allows RNA polymerase II (pol II) to track from the enhancer to the promoter. Phosphorylation of the pol II C-terminal domain is required for pol II tracking but not chromosomal looping. Development of improved hormonal therapies for prostate cancer must take in account the specific spatial and temporal modes of AR-mediated gene regulation.

Introduction

The androgen receptor (AR) regulates not only development and maintenance of male reproductive functions, but myriad other sexually dimorphic processes (Quigley et al., 1995). Changes in AR signaling pathway plays a major role in the onset and progression of prostate cancer and AR is a therapeutic molecular target (Jenster, 1999). AR is a member of the nuclear hormone receptor (NR) superfamily that regulates the expression of target genes in a ligand-inducible manner (Mangelsdorf et al., 1995; Tsai and O'Malley, 1994).

NR coactivators, defined by their role in enhancing NR transcriptional activity, are generally divided into two families: those that modify chromatin structure, and those that recruit and modify RNA polymerase II (pol II) and general transcription factors (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). The p160 proteins modify chromatin through association with potent histone acetyltransferases (HAT) such as CBP/p300 and p/CAF (Bannister and Kouzarides, 1996; Hanstein et al., 1996; Ogryzko et al., 1996) and histone methyltransferases such as CARM1 and PRMT1 (Chen et al., 1999; Wang et al., 2001). The p160 coactivators interact with the activation function 2 (AF-2) domain in the ligand binding domain (LBD) of NRs through consensus LXXLL motifs. While the LXXLL motifs within p160 proteins interact strongly with the ligand-activated AF-2 domains of most nuclear receptors (Glass and Rosenfeld, 2000; Moras and Gronemeyer, 1998), they interact only weakly with AR AF-2 (He et al., 1999). In AR, a ligand-induced intramolecular interaction of the activation function 1 (AF-1) and AF-2 domains provides the primary binding surface for p160 proteins (Alen et al., 1999; He et al., 1999). The human Mediator complex was originally identified as a group of distinct nuclear proteins (termed TRAPs) that copurified with ligand-bound thyroid receptor (TR) (Fondell et al., 1996). Evidence for a broader role of the human Mediator complex in regulating transcription comes from the purification of several similar transcription cofactor complexes, including DRIP, CRSP, ARC, and the NAT (Lewis and Reinberg, 2003; Malik and Roeder, 2000). The Mediator complex is involved in direct recruitment of the general transcription factors and pol II to the promoter (Malik and Roeder, 2000). The TRAP220 subunit was demonstrated to directly contact ligand-bound NRs through its two LXXLL motifs and help to anchor the entire Mediator complex to DNA-bound NRs, including TR, estrogen receptor (ER), and AR (Lewis and Reinberg, 2003).

Prostate specific antigen (PSA) has been extensively studied as a model of AR-mediated gene transcription (Balk et al., 2003). The PSA proximal promoter contains two androgen responsive elements (AREs) at positions –170 and –400 (ARE I and ARE II) (Cleutjens et al., 1996; Riegman et al., 1991). ARE III was identified within a potent core enhancer element located 4.2 kb upstream of transcription start site (Cleutjens et al., 1997). We and others have previously shown AR, HAT, Mediator, and pol II are recruited to the PSA enhancer and/or promoter in response to short-term (0.5–4 hr) androgen stimulation (Louie et al., 2003; Shang et al., 2002; Wang et al., 2002). However, the dynamics of long-term androgen-induced recruitment of the AR coactivator complex to PSA regulatory regions is not known. Furthermore, the mechanisms by which PSA enhancer and promoter coordinate to regulate gene transcription are controversial. Finally, the relative importance of the various endogenous AR coactivators recruited to PSA regulatory regions in regulating PSA transcription is poorly known.

Here, we describe that in contrast to ER, the occupancy of the AR coactivator complex on PSA regulatory regions increases gradually after androgen exposure, peaks at 16 hr, and then gradually declines following longer stimulation. We also find that pol II is first recruited to the PSA enhancer and then tracks along the looped chromatin upstream of the PSA promoter, providing evidence for an integrated looping/tracking model for the PSA enhancer-promoter interaction. Furthermore, we show that p160 proteins are redundant

*Correspondence: myles_brown@dfci.harvard.edu
[3]Lab address: http://research.dfci.harvard.edu/brownlab

while TRAP220 is indispensable in mediating AR transcription. Collectively, these data suggest a new model for the assembly of AR coactivator complex at PSA regulatory regions.

Results

Analysis of the Temporal Pattern of AR Coactivator Recruitment to PSA Regulatory Regions in Response to Androgen Stimulation

While previous studies have shown short-term (0.5–4 hr) androgen treatment increases AR and coactivator occupancy on the PSA enhancer and/or promoter (Jia et al., 2004; Kang et al., 2004; Louie et al., 2003; Shang et al., 2002; Wang et al., 2002), the distribution of AR coactivator complex association with the PSA enhancer, promoter, and region between the enhancer and promoter is less well defined. Furthermore, the temporal dynamics of AR coactivator complex recruitment to the PSA enhancer/promoter in response to long-term androgen treatment is poorly known. To address these questions, chromatin immunoprecipitation (ChIP) assays were performed to examine AR coactivator complex formation at the PSA enhancer, promoter, and the region between the enhancer and promoter. LNCaP cells were grown in hormone-free medium for 3 days and then treated with or without 100 nM dihydrotestosterone (DHT) for 4–16 hr. The formaldehyde-crosslinked chromatin-protein complexes were immunoprecipitated from LNCaP cells using specific antibodies against AR, SRC1, GRIP1, AIB1, p300, TRAP220, CARM1, AcH3, TBP, pol II, and phosphorylated pol II. The immunoprecipitated DNA was subsequently analyzed by quantitative PCR or standard PCR using primers spanning the PSA enhancer, promoter, and the intervening middle region. The results were analyzed as either percentage of immunoprecipitated DNA versus total input DNA or fold enrichment after immunoprecipitation relative to untreated control. Consistent with previous studies (Jia et al., 2004; Louie et al., 2003), AR and associated HAT complexes were recruited to the PSA enhancer and promoter, resulting in increases in AcH3 upon short-term (4 hr) DHT treatment (Figure 1A). Interestingly, the level of recruitment to the PSA enhancer was significantly greater than to the promoter (Figure 1B). The Mediator component TRAP220 was not only recruited to the PSA enhancer (Wang et al., 2002), but also enriched on the PSA promoter in response to 4 hr DHT treatment (Figures 1A and 1B). The histone methyltransferase CARM1 has been found to be an AR coactivator (Chen et al., 1999). We found CARM1 to be recruited to the PSA enhancer but not the promoter after 4 hr DHT treatment (Figures 1A and 1B). Interestingly, the general transcription factor TATA binding protein (TBP) bound at the PSA enhancer and promoter simultaneously and androgen induced greater TBP occupancy on the enhancer than promoter (Figure 1B). When we examined the presence of AR, HATs, Mediator, CARM1, AcH3, and TBP to the middle region between the PSA enhancer and promoter (2 kb away from both the enhancer and the promoter), we did not find significant enrichment of these proteins after 4 hr DHT stimulation (Figure 1B). Strikingly, both unphosphorylated and phosphorylated pol II were recruited to the PSA enhancer and promoter after 4 hr DHT treatment, but not to an upstream region (1.5 kb away from the PSA enhancer) (Figures 1C and 1D). In contrast to AR, coactivators, and AcH3, the relative abundance of pol II was higher on the promoter than on the enhancer, in agreement with prior studies (Kang et al., 2004). While the relative abundance of pol II on the middle region was significantly lower than that on the enhancer and promoter, surprisingly there was 2- to 5-fold enrichment of pol II on the middle region between the enhancer and promoter after 4 hr DHT treatment relative to untreated control (Figure 1D).

In contrast to ER where occupancy peaks very rapidly, the recruitment of AR, HATs, Mediator, AcH3, pol II, and phosphorylated pol II but not CARM1 to the PSA enhancer and promoter gradually increased in response to long-term androgen treatment (4–16 hr) (Figure 1B). Long-term androgen treatment also led to a gradual enrichment of pol II and phosphorylated pol II on the middle region (Figures 1C and 1D). To further address the effect of long-term androgen treatment on AR coactivator complex assembly on PSA regulatory regions, LNCaP cells were treated with DHT for 24–96 hr and ChIP assays were carried out using primers spanning the PSA enhancer (Figure 2A). Treatment of LNCaP cells with DHT for greater than 16 hr caused a gradual decline in the level of AR, SRC1, p300, TRAP220, AcH3, and pol II at the PSA enhancer.

To address whether AR coactivator complex recruitment to PSA regulatory regions correlates with PSA gene transcription, quantitative RT-PCR was performed to analyze PSA mRNA and unprocessed heterogeneous nuclear PSA RNA (PSA hnRNA) levels in androgen-treated LNCaP cells. In line with the kinetics of AR coactivator complex binding at PSA regulatory regions, PSA mRNA and hnRNA levels gradually increased following androgen treatment, peaked at 16–24 hr, and then gradually declined (Figure 2B), paralleling the accumulation of proteins on PSA regulatory regions.

Pol II Tracks through the Large Chromatin Loop Upstream of the PSA Promoter

Once we had defined the temporal dynamics of AR coactivator complex recruitment to PSA regulatory regions, we next explored how the AR coactivator complex spatially assembles on the PSA enhancer and promoter. The PSA enhancer is approximately 4 kb upstream of the PSA promoter, but the mechanism by which these two elements communicate is controversial. We previously proposed a looping model in which enhancer-bound AR and promoter-bound AR share a common p160/CBP coactivator complex (Shang et al., 2002). Conversely, Louie et al. (2003) suggested a tracking model in which pol II transfers from the enhancer to the promoter to facilitate transcription. Our results now suggest that an integrated model involving both looping and sliding may better reflect the actual mechanism. In support of a looping mechanism, we find by ChIP that the AR coactivator complex is predominantly recruited to the PSA enhancer, more weakly recruited to the PSA promoter, and not at all to the middle region between (Figure 1B), suggesting AR and coactivators

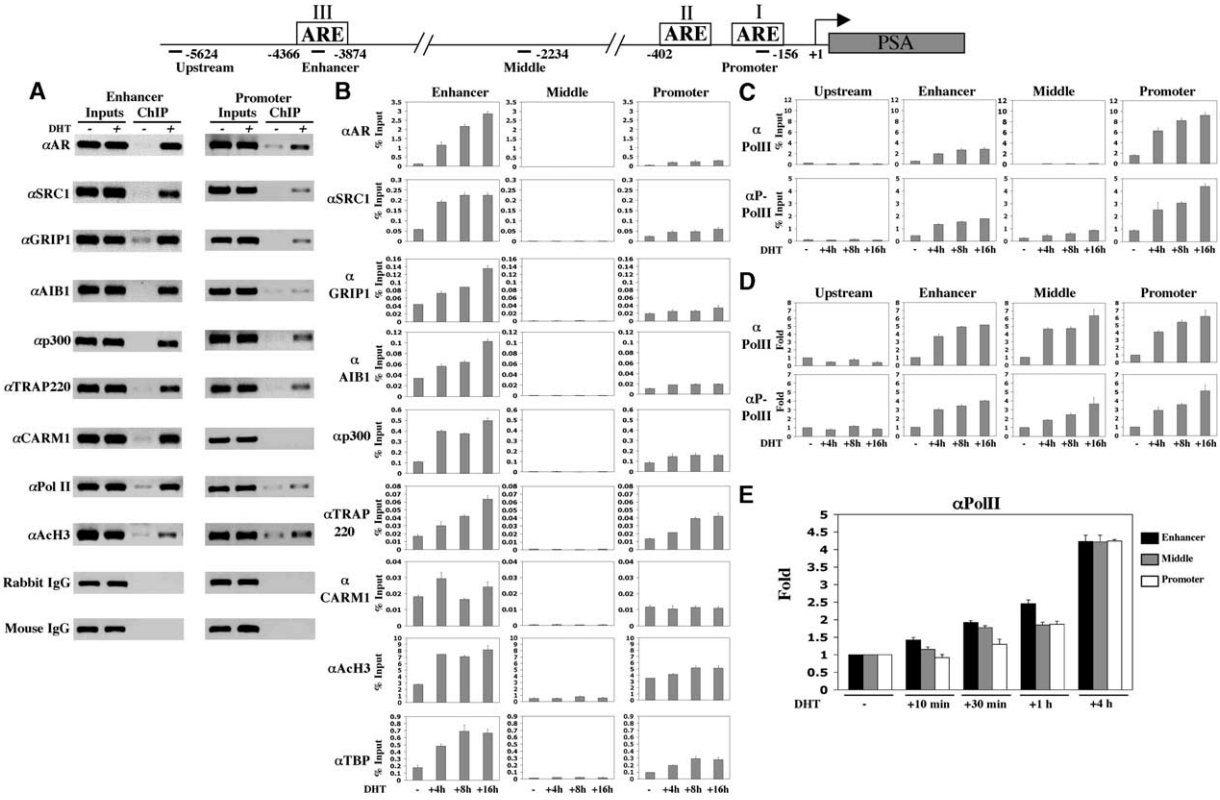

Figure 1. Long-Term Recruitment of AR Coactivator Complex to PSA Regulatory Regions

(A) LNCaP cells were treated with 100 nM DHT for 4 hr. Crosslinked chromatin was immunoprecipitated with antibodies indicated on the left. The precipitated DNA was amplified using primers spanning the PSA enhancer and promoter.

(B) LNCaP cells were treated with 100 nM DHT for 4–16 hr. ChIP assays were performed as above, except that DNA precipitates were measured by real-time PCR using primers spanning the PSA enhancer, promoter, and middle region. The results are presented as percentage to input.

(C and D) ChIP assays were carried out as described above, except that a pair of upstream primers was also used in real-time PCR analyses. The results were shown as either percentage input (C) or fold enrichment to vehicle control (D).

(E) LNCaP cells were treated with 100 nM DHT for 10 min, 30 min, 1 hr, and 4 hr. ChIP assays were performed as above using antibodies against pol II. Graphical representations of the mean ± SE from two to five independent experiments are shown in (B)–(E).

do not slide through the DNA between the enhancer and promoter. In addition, TBP, which binds to the TATA sequence located ~25 bp upstream of PSA transcription initiation site, was detected on both the PSA enhancer and promoter (Figure 1B). In contrast, pol II recruitment to the middle region followed the same pattern as its binding to the enhancer and the promoter after long-term androgen exposure, especially when the data are analyzed as fold enrichment following DHT treatment (Figure 1D). We also examined the timing of the recruitment of pol II to the enhancer, middle region, and promoter following brief exposure of LNCaP cells to DHT (10 min, 30 min, 1 hr, and 4 hr) using anti-pol II antibodies (Figure 1E). Interestingly, we find pol II is first recruited to the PSA enhancer, then to the middle region, and finally to the promoter region. The recruitment to all three regions reaches a steady state following prolonged androgen treatment (4 hr). These data support the conclusion that pol II tracks from the PSA enhancer to the promoter.

To provide further evidence that looping occurs in vivo, we performed ChIP combined chromosome conformation capture assay (ChIP-3C) (Dekker et al., 2002; Horike et al., 2005). If the distal PSA enhancer is physically located close to the proximal PSA promoter due to looping, fragments of the enhancer and promoter generated by digestion with the same restriction enzyme should be able to be ligated and detected by PCR. Hormone-depleted LNCaP cells were treated with or without DHT for 4 hr. The crosslinked chromatin was digested with BstYI, immunoprecipitated with an anti-AR antibody, and ligated. After reversing crosslinking, PCR was performed with one primer in the PSA enhancer and another in the PSA promoter (primer E+/P–). As shown in Figure 3A, the PSA enhancer and promoter interacted in a ligase-dependent manner and this interaction was enhanced by androgen treatment. The specific 612 bp primer E+/P–-amplified band was sequenced and confirmed the interaction between the PSA enhancer and promoter. In addition, several larger PCR products were seen, which are the result of incompletely digested chromatin. In contrast, E+ combined with a primer from the middle region (J–) did not give rise to a specific product, suggesting that the loop is

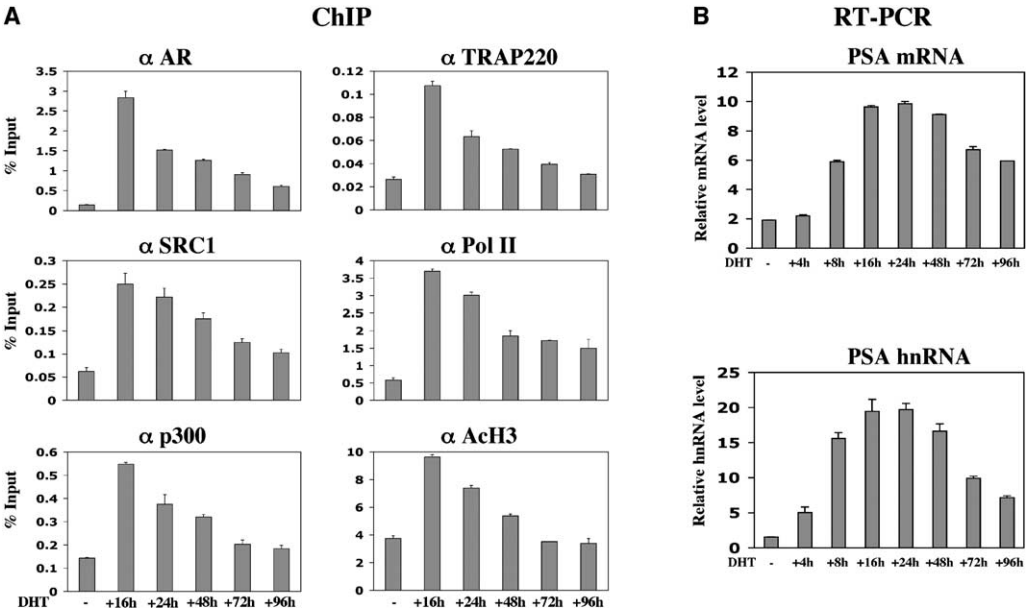

Figure 2. The Long-Term Pattern of AR Coactivator Complex Recruitment to PSA Regulatory Regions Coincides with the Long-Term PSA RNA Production Pattern

(A) ChIP analyses were performed with antibodies against indicated proteins. PSA enhancer content was assessed by real-time PCR.

(B) LNCaP cells were treated with 100 nM DHT for 4–96 hr. Total RNA was isolated and amplified with primers recognizing PSA mRNA and hnRNA by real-time RT-PCR. Values represent the mean ± SE of the two to five independent experiments.

only formed between the enhancer and the promoter. Using a PSA reporter gene containing the full-length 6 kb regulatory region as positive controls, both primer pairs produce strong PCR signals due to high molar concentration of BstYI-digested DNA fragments. These data indicate that the PSA enhancer comes in close vicinity to the PSA promoter in vivo, supporting the formation of a chromosomal loop.

To further address how the PSA enhancer functions over a long distance on the proximal promoter, we generated a series of PSA reporter constructs with chicken β-globin insulator fragments inserted at different positions. Insulators are DNA sequence elements that function by blocking the action of a distal enhancer on a promoter and/or acting as chromatin boundaries (Labrador and Corces, 2002; West et al., 2002). The chicken β-globin insulator was originally described as a 1.2 kb DNA element with strong enhancer blocking activity located at the 5′ end of the chicken β-globin locus (Chung et al., 1993). Further studies identified a 42 bp fragment (FII) within this domain that possessed all the insulator activity (Bell et al., 1999; Chung et al., 1997). While insulators usually block enhancer activity only when located between an enhancer and a promoter on chromatinized templates (Labrador and Corces, 2002; West et al., 2002), they have highest enhancer blocking activity when located on both sides of the enhancer on plasmid templates in human erythroleukemic cells (Recillas-Targa et al., 1999). Therefore, we inserted the 42 bp FII fragment between the PSA enhancer and the promoter, outside the PSA enhancer, or on both sides of the PSA enhancer (Figure 3B). These constructs were transiently transfected into LNCaP cells and luciferase was measured after 24 hr DHT stimulation. As shown in Figure 3B, the transcriptional activity was enhanced 18.7-fold from a control PSA-Luc construct (construct a), compared to 9.1-fold for a PSA-Luc construct with an FII insulator inserted between the enhancer and the promoter (construct b) and 11.7-fold for a PSA-Luc construct with an FII insulator placed outside the enhancer (construct d). As expected, transcription activation was further decreased to 6.4-fold from a PSA-Luc construct with an FII insulator on both sides of the enhancer (construct e). Two copies of FII outside the enhancer plus one copy of FII inserted between the enhancer and the promoter possessed the highest enhancer blocking activity (construct f, transactivation fold 3.7), indicating that the insulator dosage correlates with enhancer blocking activity. The sequence specificity of FII is crucial for enhancer blocking because a mutated FII inserted between the PSA enhancer and the promoter was devoid of blocking activity (construct c, transactivation fold 22.5). Insertion of an FII insulator downstream of an isolated PSA enhancer reporter gene, which can mediate a robust androgen-induced transcription activity similar to a full-length PSA promoter (construct g), also inhibited transcriptional activity to one-half (construct h). These results suggest that insulators block the transmission of signals from the PSA enhancer to the promoter and luciferase gene. Our evidence in Figure 1 showing that pol II tracks between the enhancer and promoter makes it a likely candidate to be the signal blocked by the insulator.

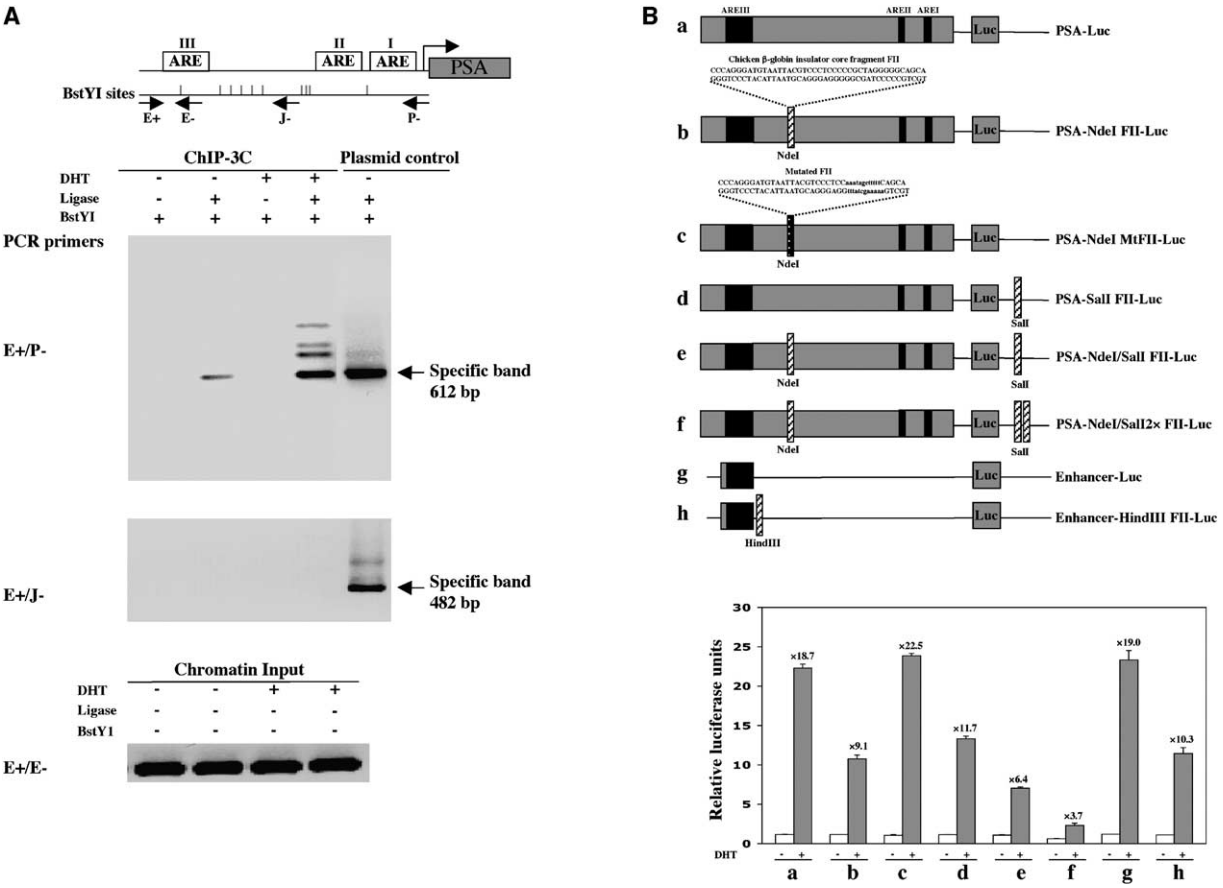

Figure 3. Spatial Communication between the PSA Enhancer and Promoter

(A) ChIP-3C was performed using crosslinked, BstYI-digested chromatin from LNCaP cells treated with DHT or vehicle for 4 hr. The ligated DNA was PCR amplified with primers as indicated. A PSA reporter plasmid containing 6 kb regulatory region after BstYI digestion and ligation was used as positive control.

(B) PSA reporter constructs with or without FII inserted at different positions were transiently transfected into LNCaP cells. Cells were stimulated with 100 nM DHT or vehicle for 24 hr. Luciferase activities were determined and results were presented as the mean ± SE of the triplicated transfections.

Mechanisms for AR Transcription Complex Assembly

In an effort to investigate the underlying mechanisms for long-term AR coactivator complex assembly, we first examined the effects of long-term androgen treatment on expression levels of AR, coactivators, and pol II. LNCaP cells were treated with either DHT or ethanol control for 4–96 hr. Western blot analyses were performed using antibodies against AR, p160 proteins, p300, TRAP220, and pol II. In agreement with a previous report (Wolf et al., 1993), DHT stimulation caused upregulation of AR protein level that did not change significantly over the 96 hr time course (Figure 4A). Interestingly, while coactivator and phosphorylated pol II levels decreased after the cells were continuously cultured for 72–96 hr, treatment of cells with androgen induced a faster downregulation of all examined coactivator and phosphorylated pol II (upper band) levels at 48 hr (Figure 4A). These results suggest that androgen-induced downregulation of coactivator and phosphorylated pol II levels accounts, at least in part, for the decline of coactivators and pol II on PSA regulatory regions after 48 hr DHT stimulation.

Pol II C-terminal domain (CTD) phosphorylation has been shown to play a pivotal role in coupling gene transcription and pre-RNA processing (Orphanides and Reinberg, 2002). We have recently shown that CTD phosphorylation is required for the rapid cycling of ER coactivator complex on ER responsive promoters (Shang et al., 2000). To investigate the effects of CTD phosphorylation on AR coactivator complex assembly at PSA regulatory regions, we pretreated LNCaP cells with the CTD kinase inhibitor 5,6-dichlorobenzimadazole riboside (DRB) (50 μM) or vehicle for 1 hr followed by exposure to DHT for 4–16 hr, and ChIP assay was performed using antibodies against AR, SRC1, p300, TRAP220, AcH3, and pol II. As shown in Figure 4B, treatment of LNCaP cells with DRB significantly reduced the enrichment of SRC1, p300, TRAP220, and AcH3 at the PSA enhancer after 4–16 hr DHT stimulation. Pol II's fold enrichment in occupancy on the enhancer, promoter, and the middle region are all decreased (Figure

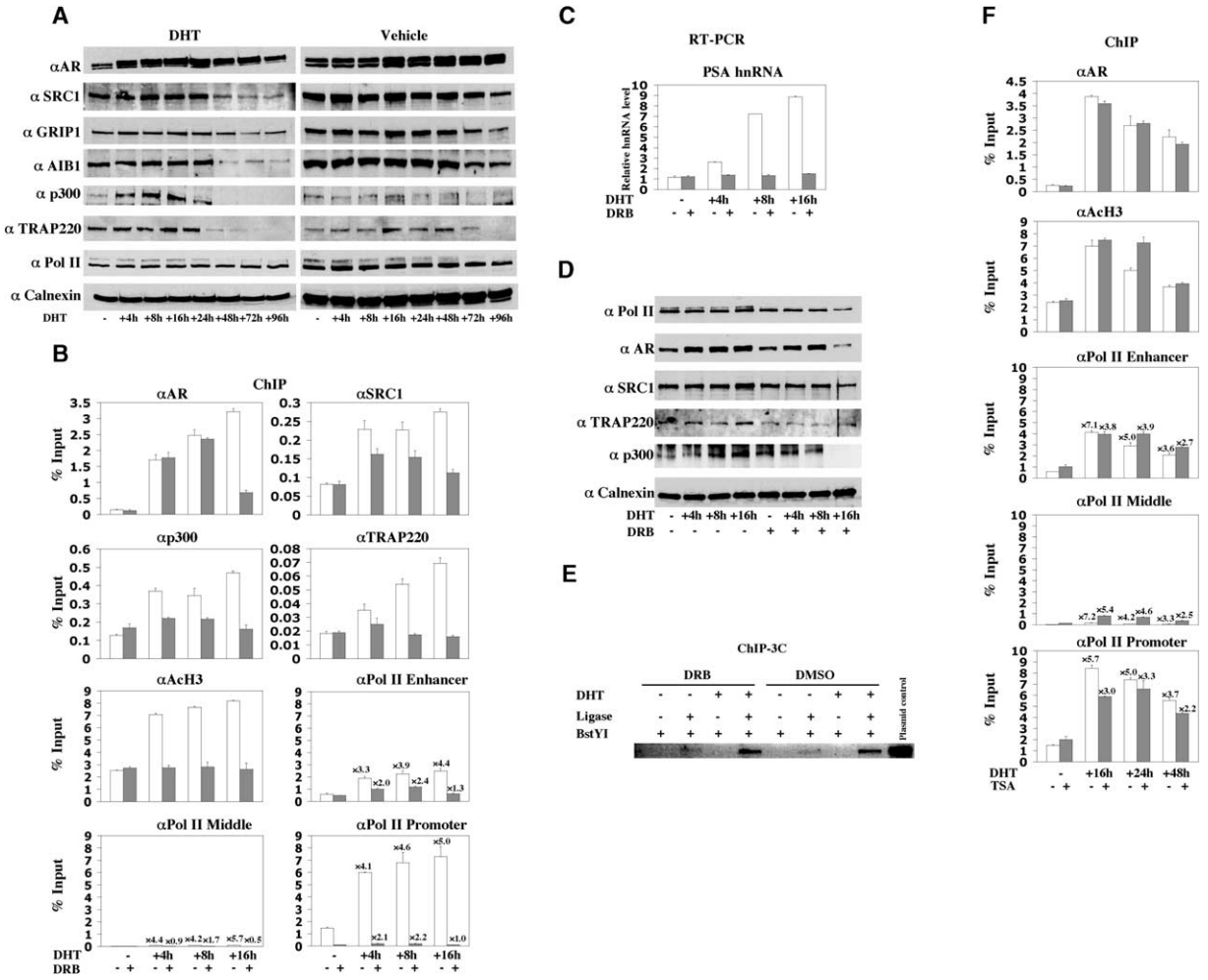

Figure 4. Mechanisms of AR Coactivator Complex Assembly

(A) Western blot analyses were performed comparing relative protein expression levels of AR, coactivators, and pol II following 4–96 hr stimulation with 100 nM DHT or vehicle.

(B) LNCaP cells were treated with 50 μM DRB or vehicle for 1 hr followed by addition of DHT for 4–16 hr, and the occupancy of AR and coactivators on the PSA enhancer and pol II on the PSA enhancer, promoter, and the middle region was assessed by ChIP.

(C) LNCaP cells were treated with DRB and DHT as described above and real-time RT-PCR was performed to measure PSA hnRNA level.

(D) LNCaP cells were treated with DRB or vehicle as described above. Western blot analyses were performed to measure relative protein levels of AR, coactivators, and pol II.

(E) LNCaP cells were treated with DRB or vehicle for 1 hr followed by addition of DHT for 4 hr, and ChIP-3C assay was performed.

(F) LNCaP cells were treated with or without 100 nM TSA for 24 hr followed by treatment with DHT for 16–48 hr. ChIP analyses were then performed. Graphical representations of the mean ± SE from two to five independent experiments are shown in (B), (C), and (F).

4B). In contrast to androgen treatment alone in which greater levels of pol II were detected at the promoter as compared with the enhancer, DRB treatment resulted in a greater decrease in pol II occupancy at the promoter than the enhancer, reversing the ratio (Figure 4B). These data suggest DRB may inhibit pol II tracking from the enhancer to the promoter, which is consistent with the report of Louie et al. (2003) showing that another CTD kinase inhibitor, flavopiridol, perturbs pol II's transfer. Consistent with the significantly decreased binding of coactivators and pol II to PSA regulatory regions, PSA RNA synthesis was completely blocked by DRB treatment (Figure 4C). Interestingly, AR recruitment was not affected by DRB treatment after 4–8 hr DHT stimulation, and it was only significantly diminished at 16 hr of DHT exposure (Figure 4B). While DRB treatment did not significantly affect AR, coactivator, and unphosphorylated pol II protein levels after 4–8 hr DHT stimulation, it reduced protein levels after 16 hr DHT treatment (Figure 4D). Thus, the reduced AR recruitment following 16 hr DHT treatment may be caused by the downregulation of AR protein levels. These results suggest that pol II CTD phosphorylation is required for efficient AR coactivator and pol II association with PSA regulatory regions but not AR itself.

Because DRB blocks pol II tracking and PSA RNA production, we next addressed whether DRB affects PSA enhancer/promoter loop formation. We pretreated

LNCaP cells with DRB or vehicle for 1 hr followed by ChIP-3C assay. As shown in Figure 4E, treatment of cells with DRB had no effect on interaction between the PSA enhancer and promoter, suggesting that tracking is not required for looping.

Histone acetylation is correlated to gene expression and histone deacetylation is linked to gene silencing (Hu and Lazar, 2000; Struhl, 1998). We have previously showed that a histone deacetylase inhibitor, trichostatin (TSA), can enhance androgen-induced transcriptional activity (Shang et al., 2002). To examine the effects of histone acetylation on long-term AR coactivator complex assembly, we treated LNCaP cells with TSA for 24 hr followed by DHT for 16–48 hr. In control cells, AcH3 and pol II recruitment to PSA regulatory regions peaked after 16 hr DHT treatment but declined after 24 hr. However, in TSA-treated cells, AcH3 and pol II peaked at PSA regulatory regions by 16–24 hr of DHT exposure and declined with delayed kinetics (48 hr) (Figure 4F), suggesting enhanced histone acetylation can promote AcH3 and pol II stability on PSA regulatory regions. No changes were observed in AR association with the PSA enhancer in the presence of TSA (Figure 4F).

Functional Recruitment of p160 Proteins and Mediator Complexes at PSA Regulatory Regions

The p160 coactivators, p300, Mediator complex, and CARM1 have all been characterized as AR coactivators by their ability to enhance AR-mediated transcription in transient overexpression assays (Heinlein and Chang, 2002; Wang et al., 2002). However, whether the endogenously expressed coactivators play functional roles and the relative importance of these coactivators in AR-mediated transcription are poorly known. To delineate the functional roles of endogenous p160 proteins (SRC1, GRIP1, and AIB1), p300, TRAP220, and CARM1 in AR-mediated transcription from PSA regulatory regions, we used short interfering RNA (siRNA) to specifically knock down the expression of each of these proteins in LNCaP cells. To verify the optimal silencing conditions, we transiently transfected increasing concentrations of each siRNA oligonucleotide (24 nM, 40 nM, and 80 nM). As shown in Figure 5A, siRNA transfection resulted in a significant suppression of each coactivator protein level at all examined concentrations. To determine the functional role of endogenous coactivators in AR transcription, we transiently transfected siRNAs (24 nM) targeting each coactivator into LNCaP cells. Forty-eight hours after siRNA transfection, cells were treated with DHT for 4–16 hr. Quantitative RT-PCR was then performed to measure PSA hnRNA levels. As shown in Figure 5B, silencing of SRC1 or GRIP1 did not attenuate PSA hnRNA production after DHT treatment for 4–16 hr when compared with a control siRNA (siLuc). Silencing of AIB1 or CARM1 had a modest effect. PSA hnRNA levels decreased significantly following 4–8 hr DHT treatment when p300 was silenced. However, after 16 hr androgen treatment, the PSA hnRNA levels in p300 silenced cells were similar to those in siLuc transfected control cells. Significantly, silencing of TRAP220 had a potent effect in reducing PSA hnRNA production following 4–16 hr androgen stimulation. These findings indicate that SRC1, GRIP1, AIB1, and CARM1 are not essential, whereas p300 (in an early phase) and TRAP220 are crucial in androgen-mediated PSA gene activation.

We next asked why silencing of SRC1 failed to inhibit AR-mediated PSA gene transcription. LNCaP cells were transiently transfected with either siSRC1 or control siLuc, cultured for 48 hr, and then treated with DHT for 16 hr. Western blot analyses showed that while SRC1 silencing had no major effect on AR and pol II expression levels, it led to a modest increase of GRIP1 and AIB1 expression levels (Figure 5C, left). We next performed ChIP assays to study the effects of SRC1 silencing on AR coactivator complex recruitment to PSA regulatory regions. As shown in Figure 5C (left and right), recruitment of SRC1 to the PSA enhancer was greatly decreased in SRC1 silenced cells as compared with the robust recruitment of SRC1 to the PSA enhancer in siLuc transfected cells. Interestingly, the association of GRIP1 and AIB1 with the PSA enhancer was modestly increased, leading to a modest increase in AcH3 at the PSA enhancer (Figure 5C, right). Pol II and TBP recruitment to the PSA enhancer and promoter in siSRC1 transfected cells were not changed compared with siLuc transfection control (Figure 5C, right). These data suggest that a compensatory elevation of GRIP1 and AIB1 recruitment to the PSA enhancer may contribute to the failure of SRC1 silencing to attenuate PSA gene transcription.

To investigate the mechanism of PSA RNA inhibition by TRAP220 silencing, we transiently transfected siTRAP220 into LNCaP cells, cultured the cells for 48 hr, and then treated cells with DHT for 16 hr. In cells transfected with siTRAP220, the expression levels of SRC1, AR, and pol II were similar to those in siLuc transfected cells (Figure 5D, left). ChIP analyses showed attenuated recruitment of TRAP220 to the PSA enhancer in siTRAP220 transfected cells as compared with the siLuc transfected control (Figure 5D, left and right). The recruitment of AR, SRC1, AcH3, and TBP to PSA enhancer in siTRAP220 transfected cells was similar to that in siLuc transfected cells (Figure 5D, right). In contrast, silencing TRAP220 led to a modest decrease in TBP association with the PSA promoter and pol II association with the PSA enhancer and middle region. Moreover, the recruitment of pol II to the PSA promoter was significantly reduced in siTRAP220 transfected cells (Figure 5D, right). These results indicate that Mediator facilitates the occupancy of pol II and TBP on PSA regulatory regions, which is essential for AR-mediated transcription.

Discussion

Transcriptional activation by NRs is a complex and orchestrated process requiring multiple coactivators. NR coactivator complexes assemble on gene promoters either sequentially or combinatorially to initiate transcription (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). For example, Metivier et al. (2003) suggested a sequential model for ER complex assembly at the ER-responsive pS2 promoter: p300 and SRC1 are recruited first to induce histone acetylation, followed

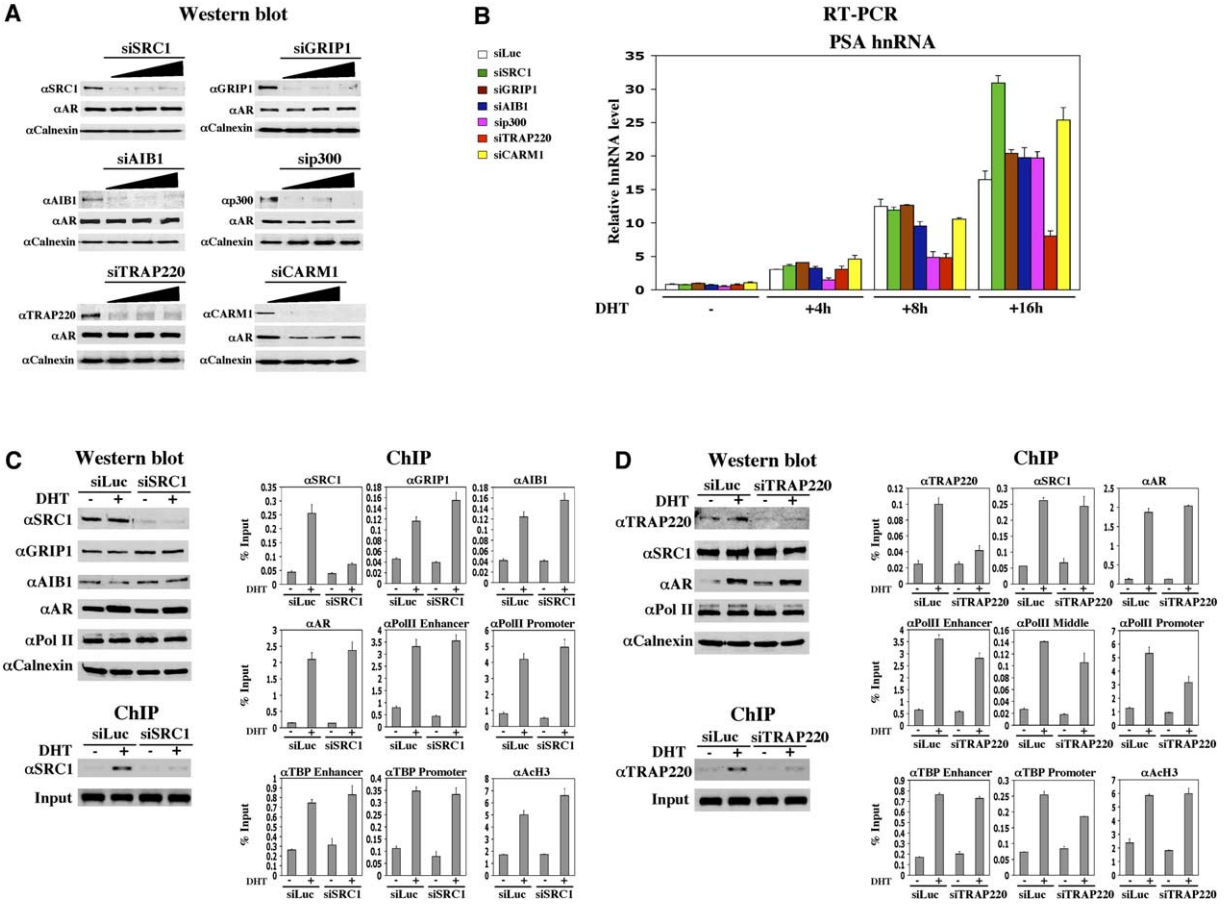

Figure 5. Functional Analyses of AR Coactivators by siRNAs

(A) LNCaP cells were transiently transfected with siRNA targeting each coactivator with increasing concentrations (24 nM, 40 nM, and 80 nM). Forty-eight hours posttransfection, protein levels were determined.

(B) LNCaP cells were cultured in hormone-free medium and transfected with 24 nM siRNA targeting each coactivator and the luciferase gene (control). Forty-eight hours after transfection, cells were treated with either vehicle or 100 nM DHT for 4–16 hr. Real-time RT-PCR was then performed.

(C) LNCaP cells were transfected with siSRC1 or siLuc (24 nM) and, 2 days posttransfection, cells were treated with or without 100 nM DHT for 16 hr. Cells were either harvested for Western blot analyses (left) or continued for ChIP assays (right).

(D) LNCaP cells were transfected with either siTRAP220 or siLuc. Western blot analyses and ChIP assays were performed as described above. Values are presented as the mean ± SE of two to five independent experiments (B)–(D).

by the recruitment of ER and the Mediator complex. Conversely, p160 proteins, p300, and TRAP220 are simultaneously recruited to another ER promoter (cathepsin D) rapidly after estrogen stimulation (Shang et al., 2000). In the case of AR, it appears that p160 proteins, CBP, and TRAP220 are combinatorially associated with PSA regulatory regions within 0.5–1 hr following DHT stimulation (Louie et al., 2003; Shang et al., 2002; Wang et al., 2002). The dynamics of the recruitment of AR coactivator complex to PSA regulatory regions after long-term androgen treatment is of particular interest considering the constant elevation of AR protein level and the potential for secondary events of transcription. In contrast, ER coactivator complex is cyclically recruited to ER-responsive elements during early time points and ER is subsequently degraded (Shang et al., 2000; Shao et al., 2004). In this study, we examined the binding dynamics of the AR coactivator complex at PSA regulatory regions following 4–96 hr androgen stimulation. Our results suggest a long-term temporal model for the association of the AR coactivator complex with PSA regulatory regions: after androgen treatment, AR coactivator complex occupancy gradually increases, reaches a maximum at 16 hr, and thereafter gradually declines (Figure 6A). The long-term pattern of AR coactivator complex recruitment to PSA regulatory regions may reflect the steady state of AR complex binding and release. It also coincided with the long-term PSA RNA production pattern (Figure 2B), consistent with a unified theory of gene expression in which transcription factor complex binding at the promoter, pre-RNA synthesis, and RNA processing are functionally connected (Orphanides and Reinberg, 2002).

Three models have been proposed to explain how a distal enhancer communicates with a proximal promoter. The looping model proposes that proteins bound

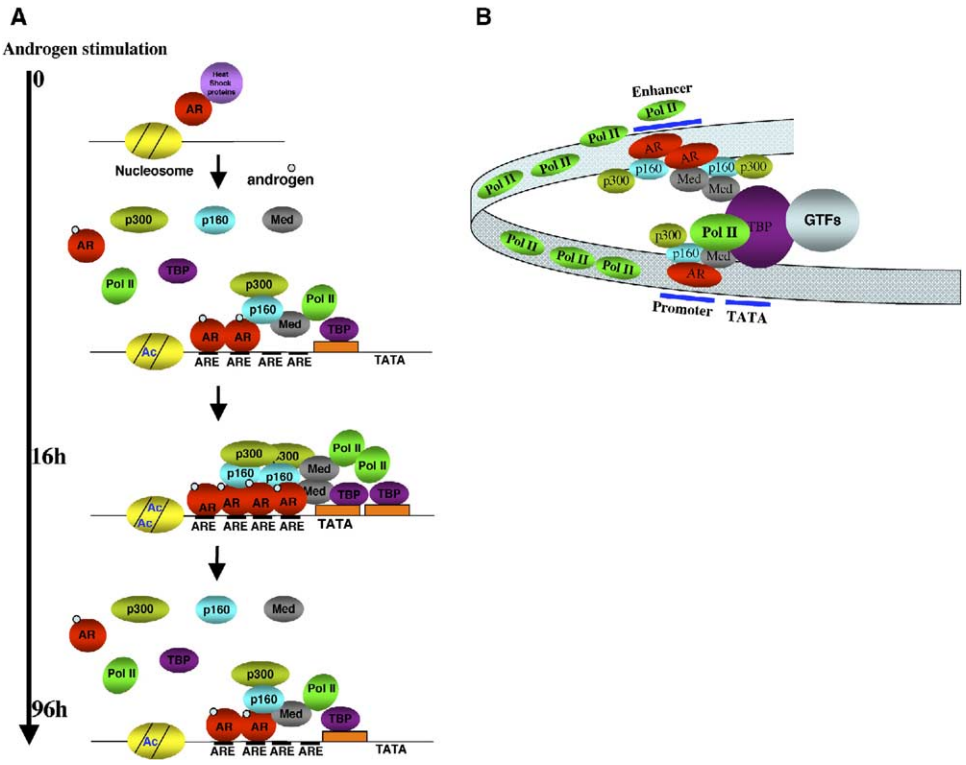

Figure 6. Models of AR Coactivator Complex Assembly on PSA Regulatory Regions

(A) From a temporal view, the level of PSA regulatory regions bound to AR complexes gradually increases after androgen treatment, peaks at 16 hr, and then gradually declines following longer treatment.

(B) From a spatial view, AR coactivator complex is predominantly recruited to the PSA enhancer, which communicates with AR transcription complex weakly associated with the PSA promoter through the 4 kb intervened DNA on which pol II tracks through.

to enhancers directly interact with the proteins bound to promoters with the intervening DNA looped out; the tracking model suggests signals recruited by enhancers slide through the DNA to promoters; the linking model proposes the looped enhancer-promoter communication involves propagation of nucleoprotein structures along the intervening DNA (Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999). With regard to the PSA enhancer-promoter communication, ChIP analyses of AR, coactivators, TBP, and pol II binding at the PSA enhancer, the promoter, and the middle region support a combined looping and tracking model. Moreover, our ChIP-3C assay provides evidence that the PSA enhancer and the promoter physically interact in vivo, supporting the looping component of the model. Conversely, in general, insulator studies have supported a tracking model. Our studies using transiently transfected LNCaP cells found that an insulator inserted far away from the PSA enhancer and promoter (2 kb from both the enhancer and promoter; construct d; Figure 3B) also could reduce AR-mediated transcription to one-half, making it unlikely that insulators act to block looping. Furthermore, we found that in a construct containing only an isolated PSA enhancer and no defined promoter element (construct h) in which loop formation is unlikely, an insulator could also suppress transcription (Figure 3B). These results suggest that insulators may block signals that track from an enhancer in both directions in transiently transfected circular plasmids. Based on our ChIP data, 3C experiments, and insulator studies, we conclude that pol II tracks through a large loop formed between the PSA enhancer and promoter (Figure 6B). Such a combined looping/tracking model is different from a facilitated tracking model that proposes transcription complexes bound to the enhancer all track to the promoter through small loops (Blackwood and Kadonaga, 1998). In addition, our combined looping/tracking model cannot exclude a linking model in which unidentified proteins (Bulger and Groudine, 1999) may facilitate PSA enhancer/promoter looping.

Recent studies found that distal enhancer-bound pol II could be phosphorylated and that this may be important for pol II tracking from the enhancer to the promoter (Johnson et al., 2001; Louie et al., 2003). We found phosphorylated pol II was recruited to the PSA enhancer, the promoter, and the middle region after androgen stimulation (Figures 1C and 1D). When pol II CTD phosphorylation was inhibited by DRB, we found that not only was pol II transfer from the enhancer to the promoter blocked but also that the recruitment of SRC1, p300, TRAP220, and AcH3 to the enhancer was decreased (Figure 4B). A possible explanation is that inhibition of pol II CTD phosphorylation may reduce HAT recruitment to the PSA enhancer, which in turn decreases Mediator recruitment and histone hyperacety-

lation. However, AR recruitment to PSA enhancer was not affected by either pol II CTD phosphorylation or histone acetylation (Figures 4B and 4F), consistent with the notion that chromatin remodeling is not crucial for some activators binding to nucleosomal DNA (Orphanides and Reinberg, 2002).

The p160 proteins play a scaffold role in forming coactivator complex involved in NR-mediated transcription (Shang et al., 2000, 2002). However, examples of p160 protein redundancy have been documented. For example, an increase in GRIP1 in SRC1 knockout mice partially compensates for the loss of SRC1 function in target tissues (Xu et al., 1998). Using a re-ChIP approach, Metivier et al. (2003) found SRC1 and AIB1 are never found on the same site of an estrogen responsive promoter, suggesting a degree of functional redundancy between these two proteins. Our finding that silencing of SRC1 failed to inhibit AR-mediated transcription and led to increased GRIP1 and AIB1 recruitment to the PSA enhancer also suggests that some degree of functional redundancy exists among the p160 proteins. Conversely, we found TRAP220 is indispensable for AR-mediated transcription. siRNA-ChIP analysis demonstrated that inhibition of TRAP220 resulted in less pol II recruitment to the PSA enhancer/promoter, which is consistent with the hypothesis that Mediator facilitates recruitment of pol II into transcription preinitiation complexes (Malik and Roeder, 2000). Given that TRAP220 anchors the Mediator complex to AR, our finding that silencing TRAP220 led to less TBP recruitment to the PSA promoter is relevant to a previous report demonstrating that occupancy of TATA by TBP is dependent on Mediator Srb4 (yeast homolog of TRAP80) in yeast (Kuras and Struhl, 1999).

In summary, our findings illuminate a comprehensive model of AR coactivator complex assembly at PSA regulatory regions. Unlike other nuclear receptors such as ER, AR activity involves sustained chromatin association and transcriptional activation of target genes for more than 96 hr. This activity of AR is dependent on phosphorylated pol II tracking from the PSA enhancer to the promoter through looped DNA and on members of the Mediator complex. Improved hormonal therapies for prostate cancer may be able to exploit these unique aspects of AR-dependent gene regulation.

Experimental Procedures

Cell Culture, Reagents, and Antibodies

LNCaP cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Dihydrotestosterone (DHT) and 5,6-dichlorobenzimadazole riboside (DRB) were purchased from Sigma (St. Louis, MO). Trichostatin A (TSA) was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies were anti-AR (441), anti-AR (N20), anti-SRC1 (M341), anti-GRIP1 (M343), anti-p300 (C20), and anti-TRAP220 (M255) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-CARM1, anti-AcH3, and anti-TBP from Upstate Biotechnology; anti-RNA pol II (8WG16) from Covance (Berkeley, CA); and anti-phosphorylated RNA pol II (Ser2 and Ser9) from Abcam (Cambridge, MA). An anti-AIB1 (Shang et al., 2000) and an anti-GRIP1 rabbit polyclonal antibody (Shao et al., 2004) have been described.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described (Shang et al., 2002) with a few modifications. Briefly, chromatin was crosslinked for 10 min at room temperature with 1% formaldehyde directly added to cell culture medium. The crosslinked chromatin was sonicated, diluted, and immunoprecipitated with specific antibodies at 4°C overnight. Protein A-Sepharose beads were added and then washed sequentially with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer. The protein-DNA complexes were eluted and the crosslinking was reversed at 65°C for 15 hr. DNA fragments were purified and analyzed by either real-time PCR or regular PCR. For real-time PCR, the SYBR Green PCR kit (Applied Biosystems, Foster City, CA) was used and the samples were amplified with the ABI Prism 7700 Sequence Detector (Applied Biosystems). The primers used in real-time PCR are listed in Figure S4 (in the Supplemental Data available with this article online). For regular PCR, PCR was performed for 25–35 cycles and the primer sequences for enhancer (E+/E–) and promoter (P+/P–) were as described in Figure S4.

Real-Time RT-PCR

Total RNA from LNCaP cells was isolated using an RNeasy kit (Qiagen, Valencia, CA). Real time RT-PCR was performed on 100 ng of RNA by using SYBR Green PCR kit and MultiScribe reverse transcriptase (Applied Biosystems) according to the manufacturer's protocol. The primers for PSA mRNA and PSA hnRNA are listed in Figure S4.

ChIP Combined Chromosome Conformation Capture Assay (ChIP-3C)

The crosslinked chromatin was sonicated, digested with specific restriction enzymes overnight, and immunoprecipitated with anti-AR antibodies coupled to protein A beads. The beads were then precipitated, resuspended in ligation buffer, and overnight ligation was performed. The beads were washed and protein-DNA complexes were eluted. The crosslinking was reversed and ligated DNA was purified. The PCR primers (E+, E–, J–, Q–, and P–) for amplifying ligated DNA were as described in Figure S4.

Plasmids

pGL3-PSA-Luc is already described (Shang et al., 2002). pGL3-PSAEnhancer-Luc was constructed by PCR amplification using the primers E2+/E2– (Figure S4) and subcloned into pGL3-Basic-Luc (Promega, Madison, WI). To generate pGL3-PSA-NdeI FII-Luc, the in vitro annealed chicken β-globin insulator core fragment FII 5′-CCC AGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCA-3′ (Bell et al., 1999) was inserted into NdeI-digested pGL3-PSA-Luc. The pGL3-PSA-NdeI MtFII-Luc was constructed by subcloning a mutant FII 5′-CCCAGGGATGTAATTACGTCCCTCCaaatagcttttCAGCA-3′ (Bell et al., 1999) into NdeI-cut pGL3-PSA-Luc. The pGL3-PSA-Enhancer-HindIII FII-Luc plasmid was constructed by subcloning annealed FII into HindIII-cut pGL3-PSAEnhancer-Luc. The pGL3-PSA-SalI FII-Luc and pGL3-PSANdeI/SalI FII-Luc were generated by inserting annealed FII into SalI-digested pGL3-PSA-Luc and pGL3-PSANdeI FII-Luc, respectively.

Reporter Gene Assays

Transfection of hormone-depleted LNCaP cells was performed using Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA). Cells were treated with DHT for 24 hr and then harvested. Luciferase activity was measured using a luciferase kit (Promega) and transfection efficiency was normalized with β-galactosidase activity.

RNA Interference

Short interfering RNA (siRNA) oligonucleotide duplexes targeting luciferase, SRC1, GRIP1, AIB1, p300, TRAP220, and CARM1 (Dharmacon, Lafayette, CO) were transfected into LNCaP cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were treated with or without androgen and harvested later. The target sequences are: luciferase (5′-AACACUUACGCUGAGUA CUUCGA-3′); SRC1 and AIB1 (from Shang and Brown, 2002); GRIP1 (5′-AAAGGAAUGUCCUGACCAACU-3′); p300 (from Debes et al., 2002); and TRAP220 and CARM1 (from SMARTpool reagents, Dharmacon).

Supplemental Data

Supplemental figures and a supplemental reference can be found with this article online at http://www.molecule.org/cgi/content/full/19/5/631/DC1/.

Acknowledgments

We would like to thank Drs. Olli Janne and Mitch Lazar for reviewing the manuscript. This work was supported by Department of Defense award W81XWH-05-01-0023 (Q.W.) and Department of Defense award W81XWH-04-1-0512 (J.S.C.).

Received: April 8, 2005
Revised: June 6, 2005
Accepted: July 15, 2005
Published: September 1, 2005

References

Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., and Peeters, B. (1999). The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol. Cell. Biol. *19*, 6085–6097.

Balk, S.P., Ko, Y.J., and Bubley, G.J. (2003). Biology of prostate-specific antigen. J. Clin. Oncol. *21*, 383–391.

Bannister, A.J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. Nature *384*, 641–643.

Bell, A.C., West, A.G., and Felsenfeld, G. (1999). The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell *98*, 387–396.

Blackwood, E.M., and Kadonaga, J.T. (1998). Going the distance: a current view of enhancer action. Science *281*, 61–63.

Bulger, M., and Groudine, M. (1999). Looping versus linking: toward a model for long-distance gene activation. Genes Dev. *13*, 2465–2477.

Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., and Stallcup, M.R. (1999). Regulation of transcription by a protein methyltransferase. Science *284*, 2174–2177.

Chung, J.H., Whiteley, M., and Felsenfeld, G. (1993). A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell *74*, 505–514.

Chung, J.H., Bell, A.C., and Felsenfeld, G. (1997). Characterization of the chicken β-globin insulator. Proc. Natl. Acad. Sci. USA *94*, 575–580.

Cleutjens, K.B., van Eekelen, C.C., van der Korput, H.A., Brinkmann, A.O., and Trapman, J. (1996). Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J. Biol. Chem. *271*, 6379–6388.

Cleutjens, K.B., van der Korput, H.A., van Eekelen, C.C., van Rooij, H.C., Faber, P.W., and Trapman, J. (1997). An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol. Endocrinol. *11*, 148–161.

Debes, J.D., Schmidt, L.J., Huang, H., and Tindall, D.J. (2002). p300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6. Cancer Res. *62*, 5632–5636.

Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002). Capturing chromosome conformation. Science *295*, 1306–1311.

Fondell, J.D., Ge, H., and Roeder, R.G. (1996). Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc. Natl. Acad. Sci. USA *93*, 8329–8333.

Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. *14*, 121–141.

Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. (1996). p300 is a component of an estrogen receptor coactivator complex. Proc. Natl. Acad. Sci. USA *93*, 11540–11545.

He, B., Kemppainen, J.A., Voegel, J.J., Gronemeyer, H., and Wilson, E.M. (1999). Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. J. Biol. Chem. *274*, 37219–37225.

Heinlein, C.A., and Chang, C. (2002). Androgen receptor (AR) coregulators: an overview. Endocr. Rev. *23*, 175–200.

Horike, S., Cai, S., Miyano, M., Cheng, J.F., and Kohwi-Shigematsu, T. (2005). Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. Nat. Genet. *37*, 31–40.

Hu, X., and Lazar, M.A. (2000). Transcriptional repression by nuclear hormone receptors. Trends Endocrinol. Metab. *11*, 6–10.

Jenster, G. (1999). The role of the androgen receptor in the development and progression of prostate cancer. Semin. Oncol. *26*, 407–421.

Jia, L., Choong, C.S., Ricciardelli, C., Kim, J., Tilley, W.D., and Coetzee, G.A. (2004). Androgen receptor signaling: mechanism of interleukin-6 inhibition. Cancer Res. *64*, 2619–2626.

Johnson, K.D., Christensen, H.M., Zhao, B., and Bresnick, E.H. (2001). Distinct mechanisms control RNA polymerase II recruitment to a tissue-specific locus control region and a downstream promoter. Mol. Cell *8*, 465–471.

Kang, Z., Janne, O.A., and Palvimo, J.J. (2004). Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. Mol. Endocrinol. *18*, 2633–2648.

Kuras, L., and Struhl, K. (1999). Binding of TBP to promoters in vivo is stimulated by activators and requires pol II holoenzyme. Nature *399*, 609–613.

Labrador, M., and Corces, V.G. (2002). Setting the boundaries of chromatin domains and nuclear organization. Cell *111*, 151–154.

Lewis, B.A., and Reinberg, D. (2003). The mediator coactivator complex: functional and physical roles in transcriptional regulation. J. Cell Sci. *116*, 3667–3675.

Louie, M.C., Yang, H.Q., Ma, A.H., Xu, W., Zou, J.X., Kung, H.J., and Chen, H.W. (2003). Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc. Natl. Acad. Sci. USA *100*, 2226–2230.

Malik, S., and Roeder, R.G. (2000). Transcriptional regulation through mediator-like coactivators in yeast and metazoan cells. Trends Biochem. Sci. *25*, 277–283.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835–839.

McKenna, N.J., and O'Malley, B.W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. Cell *108*, 465–474.

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.

Moras, D., and Gronemeyer, H. (1998). The nuclear receptor ligand-binding domain: structure and function. Curr. Opin. Cell Biol. *10*, 384–391.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953–959.

Orphanides, G., and Reinberg, D. (2002). A unified theory of gene expression. Cell *108*, 439–451.

Quigley, C.A., De Bellis, A., Marschke, K.B., el-Awady, M.K., Wilson, E.M., and French, F.S. (1995). Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr. Rev. *16*, 271–321.

Recillas-Targa, F., Bell, A.C., and Felsenfeld, G. (1999). Positional enhancer-blocking activity of the chicken β-globin insulator in transiently transfected cells. Proc. Natl. Acad. Sci. USA *96*, 14354–14359.

Riegman, P.H., Vlietstra, R.J., van der Korput, J.A., Brinkmann, A.O., and Trapman, J. (1991). The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol. Endocrinol. *5*, 1921–1930.

Shang, Y., and Brown, M. (2002). Molecular determinants for the tissue specificity of SERMs. Science *295*, 2465–2468.

Shang, Y., Hu, X., DiRenzo, J., Lazar, M.A., and Brown, M. (2000). Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell *103*, 843–852.

Shang, Y., Myers, M., and Brown, M. (2002). Formation of the androgen receptor transcription complex. Mol. Cell *9*, 601–610.

Shao, W., Keeton, E.K., McDonnell, D.P., and Brown, M. (2004). Coactivator AIB1 links estrogen receptor transcriptional activity and stability. Proc. Natl. Acad. Sci. USA *101*, 11599–11604.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes Dev. *12*, 599–606.

Tsai, M.J., and O'Malley, B.W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. *63*, 451–486.

Wang, H., Huang, Z.Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B.D., Briggs, S.D., Allis, C.D., Wong, J., Tempst, P., and Zhang, Y. (2001). Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. Science *293*, 853–857.

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.

West, A.G., Gaszner, M., and Felsenfeld, G. (2002). Insulators: many functions, many mechanisms. Genes Dev. *16*, 271–288.

Wolf, D.A., Herzinger, T., Hermeking, H., Blaschke, D., and Horz, W. (1993). Transcriptional and posttranscriptional regulation of human androgen receptor expression by androgen. Mol. Endocrinol. *7*, 924–936.

Xu, J., Qiu, Y., DeMayo, F.J., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1998). Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science *279*, 1922–1925.