Chapter 3
Negative Androgen-Response Elements in Androgen Target Genes

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Abstract Genes that are positively regulated by androgen receptor (AR) contain the positive androgen-response element (pARE) in their proximal or/and distal promoter regions. Similarly, genes that are negatively regulated by AR contain the negative androgen-response element (nARE). In this chapter, we review our work on identification and characterization of nARE. We identified seven nAREs in the negative androgen-response region in the TGF-β1 promoter. Each nARE is composed of 15 nucleotides and mediates androgen-dependent inhibition of transcription. The 5′ portions of nAREs are highly conserved and resemble core half-sites in pAREs. The nAREs interacted weakly with AR DNA-binding domain (AR DBD) in gel shift assays. Mutations on the conserved nucleotides in the nARE abolished its interaction with the AR DBD and abolished its inhibition of androgen-driven transcription, suggesting that nARE/AR interaction is essential for nARE-mediated inhibition of transcription. Taken together, these findings indicate that the AR negatively regulates the expression of its target genes via a negative androgen-response region composed of multiple nAREs.

Keywords Prostate • Prostate cancer • Androgen • Androgen receptor • Negative androgen-response element

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3.1 Introduction

In order to understand androgen receptor (AR) functions in cell survival, differentiation, and proliferation in the prostate gland, as well as in early events leading to prostate cancer, DNA microarray, DNA chip, and SAGE analyses have identified large pools of genes whose expression is affected by androgens [1–5]. Chromatin immunoprecipitation together with microarray analysis (ChIP-chip) characterized the genomic organization of AR-regulated genes [6, 7]. These studies reported a relatively high level of correlation between AR-binding and gene expression and provided the basis for further studies on how AR regulates the target gene expression at the whole genomic scale.

Many features of the androgen-induced expression program appear to be related to activation of the cell's capacity to produce seminal fluid of the prostate gland. Some AR-target genes encode transcriptional regulatory and signaling proteins, which may have important roles in programming the normal prostate cells in response to the androgen. Many genes were modulated similarly in all of prostate cell lines, suggesting conservation of a specific androgen-responsive program, perhaps a vestige of the prostatic epithelial phenotype [1]. Some AR target genes show altered expression in prostate cancer, implying that these genes may be modulated abnormally in cancer cells because of molecular genetic alterations acquired with transformation.

Stimulation of gene expression is only one aspect of the AR signaling. The androgen-repressed genes also appear to play important roles in regulating cell growth, differentiation, survival, and migration, as well as the androgen signaling axis itself [8–10]. The expression of androgen-repressed genes upon ablation therapy is thought to contribute to prostate cancer regression [11]. Despite the importance of repressed genes, most studies have focused on the transcriptionally activated targets. Additionally, researches on gene repression in the context of AR signaling have dealt with mechanisms that antagonize the positive action of AR on unregulated target genes [12, 13]. Relatively little attention has been paid to bona fide AR-mediated gene repression.

Diverse mechanisms of transcriptional repression have been proposed for eukaryotic genes [14]. Most studies of transcriptional repression by nuclear hormone receptors have focused on the nonsteroid, class II receptors such as the thyroid and retinoid hormone receptors. In the absence of ligand, these receptors bind constitutively to their respective response elements to mediate strong active repression, a mechanism that entails the formation of a corepressor complex containing of corepressors (e.g., NCoR, SMRT) and histone modifiers (e.g., HDACs) [15]. Active repression via the recruitment of corepressor complexes has also been observed for the steroidal, class I receptors such as AR [16]. This has mainly been examined in the setting of antagonist-mediated repression. Other studies on negatively regulated (agonist-mediated) AR target genes provoke a mechanism of repression through physical interference with other transcription factors at their cognate promoter elements [8, 9, 14, 17]. One such example was illustrated by the finding of a sequence element (XBE) in the prostate specific antigen (*PSA*) gene [18]. The XBE interacted

with both the AR and the p65 subunit of nuclear factor (NF)-κB. Cross-modulation of the AR and NF-κB p65 mediated inhibition of AR-mediated *PSA* promoter transactivation.

Authors have described regulation of expression of a few AR target genes via negative androgen-response elements (nAREs) [19–21]. The negative ARE was proposed to mediate repression via its direct interaction with AR. But, the nature of the negative ARE and the mechanism by which the negative ARE represses transcription remain elusive.

3.2 The Negative Androgen-Response Promoter Element Strongly Inhibits Androgen-Driven Transcription

In order to map negative androgen-response promoter element (nARPE), we made a luciferase reporter (pGL3-4xARE-E4-Luc), which includes four tandem positive androgen-response elements (pAREs) upstream E4 core promoter with a pGL3-basic backbone [22] (Fig. 3.1a). When the pGL3-4xARE-E4-luc reporter was co-transfected with expression plasmid for AR into PC3MM2 cells in the absence or presence of androgen (R1881), AR activated the reporter about 36-fold higher in the presence of androgen than in the absence androgen (Fig. 3.1c). To validate the reporter system, we cloned DNA fragments of various lengths (50, 150, and 250 bp) into pGL3-4xARE-E4-luc reporter between 4xARE and E4 and found that no significant difference between the pGL3-4xARE-E4-luc and the reporter with nonspecific DNA fragments was observed (Fig. 3.1c). Thus, the reporter pGL3-4xARE-E4-luc provided a tool to observe inhibition effect of the nARE, when inserted it into the reporter between 4xARE and E4 (Fig. 3.1b).

Using this reporter system, we identified four nARPEs in TGF-β1 (−673 to −423), CDK2 (−931 to −731), PDEF (−268 to −50), and p21 (−1925 to −1691) promoters (Fig. 3.2), and they dramatically inhibited the androgen-driven luciferase expression when inserted into pGL3-4xARE-E4-luc between 4xARE and E4 [22, 23] (Fig. 3.1c). All identified nARPEs spanned DNA sequences that were 200–250 bp long and mediated strong (16 to 40-fold) inhibition of androgen-driven transcription in close proximity of pAREs. To our surprise, when we used deletion constructors that inserted shorter DNA fragments of these nARPEs into pGL3-4xARE-E4-luc between 4xARE and E4, we could not figure out a short sequence that responses for negative regulation of AR-driven transcriptional activity. Instead, we observed gradual decrease in the activity of nARPEs when truncated from 3′ or 5′ ends. These observations suggest that these nARPEs contain multiple *cis*-elements that function synergistically.

When put four nARPEs in their natural or reverse orientation, all of them showed dramatic inhibition effect on AR-driven transcriptional activities. In contrast, when put four nARPEs about 3 kb upstream the transcription start site, all of them lost inhibition effect on AR-driven transcriptional activities. Identified nARPEs also inhibited transcription activated by ER or Gal4-p53 using pGL3-5Gal4-E4-Luc or

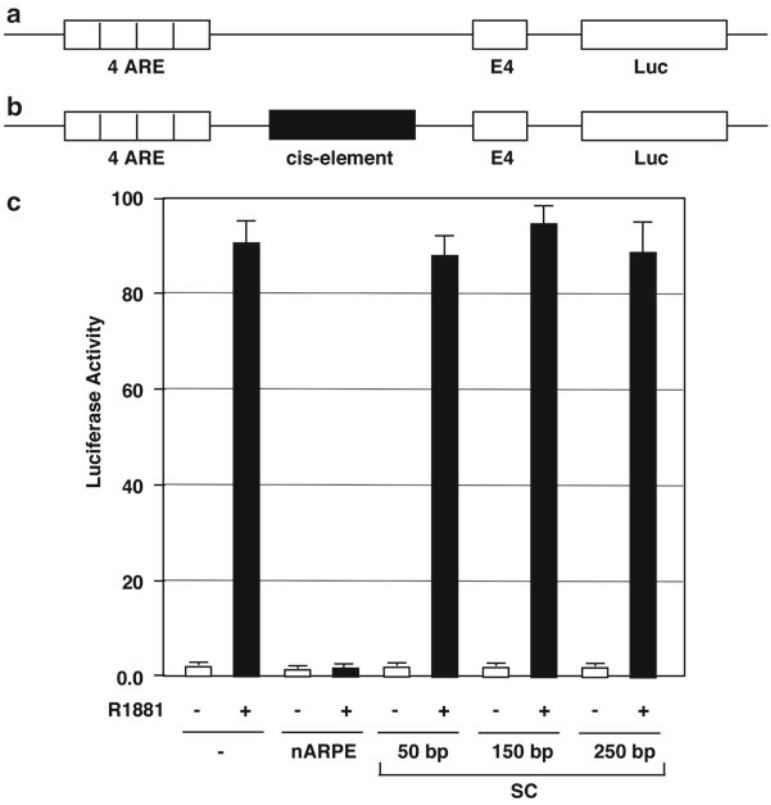

Fig. 3.1 A luciferase reporter to assay the negative androgen-response element. (**a**) Diagram of the luciferase reporter construct. (**b**) Diagrams of the luciferase reporter construct with a *cis*-element insertion. (**c**) Luciferase assay. PC3MM2 cells were transfected with 5 fmol of pcDNA-AR and 25 fmol of the luciferase reporter constructs that contain no insertion, a negative androgen-response promoter element (nARPE), or scramble control (SC) DNA fragments derived from human PDEF promoter (−3018 to −2775). Transfected cells were allowed to grow in the absence or presence of 10 nM R1881 and harvested for the luciferase assay. The values represent the mean ±SD ($n=3$)

pGL3-2ERE-E4-Luc as the reporter [22]. Enhancer can regulate transcription on both orientations and far away from transcription start site. Thus, identified nARPEs function with a mechanism distinct from the enhancer.

Previous studies suggested that the *TGF-β1* gene was negatively regulated in the prostate gland and positively regulated in prostate cancer cells by androgens [23, 24]. The *TGF-β1* promoter contains multiple positive AREs and a negative ARPE. The positive AREs interact with AR in vitro and in vivo, and both positive AREs and negative ARPE are functional in the synthetic promoters as well as in the integrated *TGF-β1* promoter [23]. These findings indicate that regulation of *TGF-β1* expression in response to the androgen is complicated and could be neutral, positive, or negative

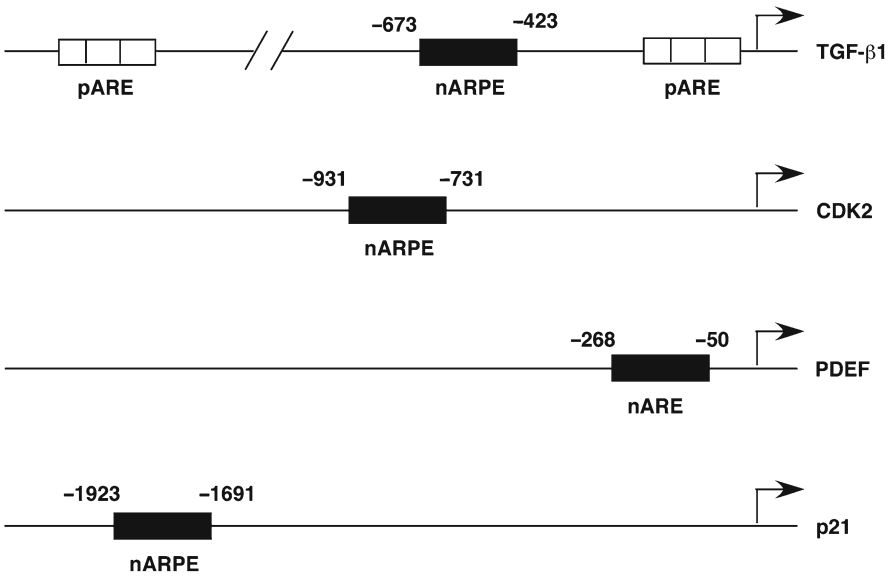

Fig. 3.2 Diagram shows the negative androgen-response promoter elements in TGB-β1, CDK2, PDEF, and p21 promoters. *pARE* positive androgen-response promoter element, *nARPE* negative androgen-response promoter element

depending on cell types or cell environments. The functional roles of the other three identified nARPEs in the regulation of their corresponding promoters are currently unknown. Two different kinds of *cis*-elements, Ets and hormonal responsive element (HRE), were identified in the maspin promoter [21]. The Ets element is active in regulating maspin expression in normal prostate epithelial cells but inactive in tumor cells. The HRE site is a negative element that is active in both cell types. This negative DNA sequence can repress a heterologous promoter recognized by the androgen receptor. Watt el al. used an enhancer trap approach with randomly cleaved overlapping DNA fragments from an approximately 55-kb P1 cosmid insert encompassing the 5′ half and upstream sequences of the PSMA gene (FOLH1) to isolate an enhancer that strongly activates the FOLH1 core promoter region [20]. The enhancer (PSME) is located in the third intron about 12 kb downstream from the start site of transcription and is characterized by a 72-bp direct repeat within a 331-bp core region. PSME-enhanced expression is repressed in the presence of androgen, mimicking the repression of the endogenous FOLH1 gene. Noss et al. sequenced and analyzed the ability of 5.5 kb of PSMA promoter/leader region to promote transcription [19]. In stably transfected LNCaP cells, the FOLH1 promoter/leader region produced a 21% downregulation in response to androgens, while addition of the enhancer resulted in a 45% downregulation. These results demonstrate significant upregulation of transcription by the PSMA promoter/enhancer, with specificity for the LNCaP prostate cell line, and downregulation of transcription in response to physiological levels of androgen.

3.3 The Negative Androgen-Response Element (nARE) Functions Synergistically to Inhibit Androgen-Driven Transcription

Genes that are positively regulated by AR contain the pAREs in their proximal or/and distal promoter regions. The pARE is composed of two short 6-bp sequences known as core half-sites. The primary sequence of the core half-sites is largely limited to two distinct motifs, AGAACA and TGTTCT. The two half-sites can be organized into either spaced palindromic or direct repeats [25]. Active AR homodimer recognizes and binds pARE and subsequently recruits cofactors to regulate transcription of its target genes [26, 27]. In general, one or two pAREs with high affinity for AR-binding localize at the proximal promoter region. On the other hand, the distal androgen-response enhancer region contains multiple low affinity pAREs. In contrast, the promoter structure of genes negatively regulated by AR is still illusive.

Our recent work identified four nARPEs from four androgen-regulated genes, which strongly inhibited androgen-driven transcription [22, 23]. By deletion analysis and sequence alignment, a nARE (5′ CAGACCCTCTTCTCC 3′) was identified from the nARPE in the *TGF-β1* gene [22]. A single copy of the nARE slightly inhibited the androgen-driven transcription, while multiplication of this nARE dramatically enhanced the inhibitory activity (Fig. 3.3).

After identifying one nARE, we built a construct containing a single copy of the identified nARE and a short DNA sequence derived from the negative androgen-response region [22]. The resulting constructs were transiently transfected into PC3 cells with pcDNA-AR, and the transfected cells were grown in the presence or absence of the androgen (R1881). The construct without any sequence insertion between nARE and E4 promoter elements was used as the control. This analysis and further deletion identified six other nAREs in the TGF-β1 nARPE (Fig. 3.4a). Three copies of each identified nARE exhibited strong inhibition of androgen-driven transcription (Fig. 3.4b). One nARE (nARE4) in particular inhibited the basal level of transcription. Six of seven identified nAREs inhibited androgen-driven transcription at a level comparable with that of TGF-β1 nARPE (Fig. 3.4b). These results reveal that the TGF-β1 nARPE contains seven functional nAREs, that together strongly inhibit androgen-driven transcription.

3.4 The Negative Androgen-Response Element Functions Through the AR Signaling

Sequence alignment of the seven identified nAREs and the previously identified XBE [18] revealed a highly conserved 5′ half end containing the three most conserved nucleotides (G3, C5, and C7) [22] (Fig. 3.5a). The nucleotides C14 and C15 were also conserved in some nAREs. We found that the 5′ parts of nAREs are similar

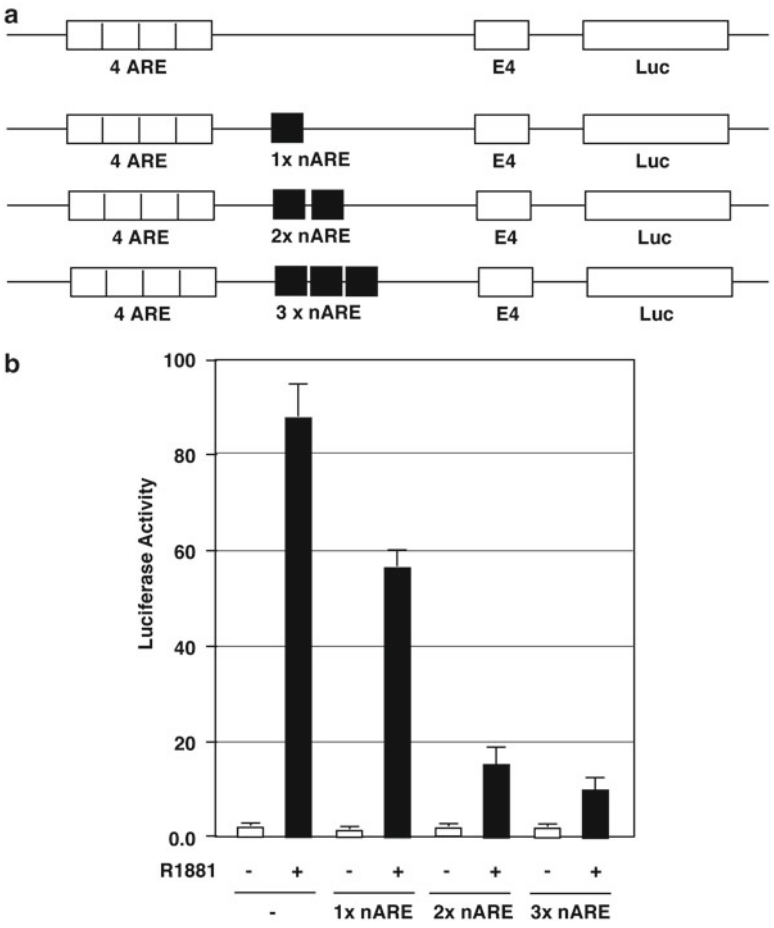

Fig. 3.3 nAREs function synergistically. (**a**) Diagrams of the luciferase reporter constructs without or with one, two, or three copies of nARE insertion. (**b**) Luciferase assay results show the synergism between nAREs. PC3MM2 cells were transfected with 25 fmol of the luciferase reporter constructs and 5 fmol of pcDNA-AR. Transfected cells were allowed to grow in the absence or presence of 10 nM R1881 and harvested for the luciferase assay. The values represent the mean ± SD ($n = 3$)

to the 5′ core half-sites in the consensus pARE. More importantly, the nucleotides involved in AR interaction with pARE (G3 and C5) are conserved in nAREs. We generated the mutations (G3A/C5T) in nARE7 and subcloned two copies of the mutated nARE7 into the pGL3-4xARE-E4-luc reporter. This mutation completely abolished nARE7-mediated transcriptional inhibition [22].

We labeled probes containing two copies of wild-type and mutant nARE7 with ^{32}P and incubated the probes with the recombinant AR DBD [22]. This AR DBD protein (100 and 200 ng) interacted with the nARE7. The shifted protein–DNA complex was completely abolished by cold nARE7 and pARE probes but not

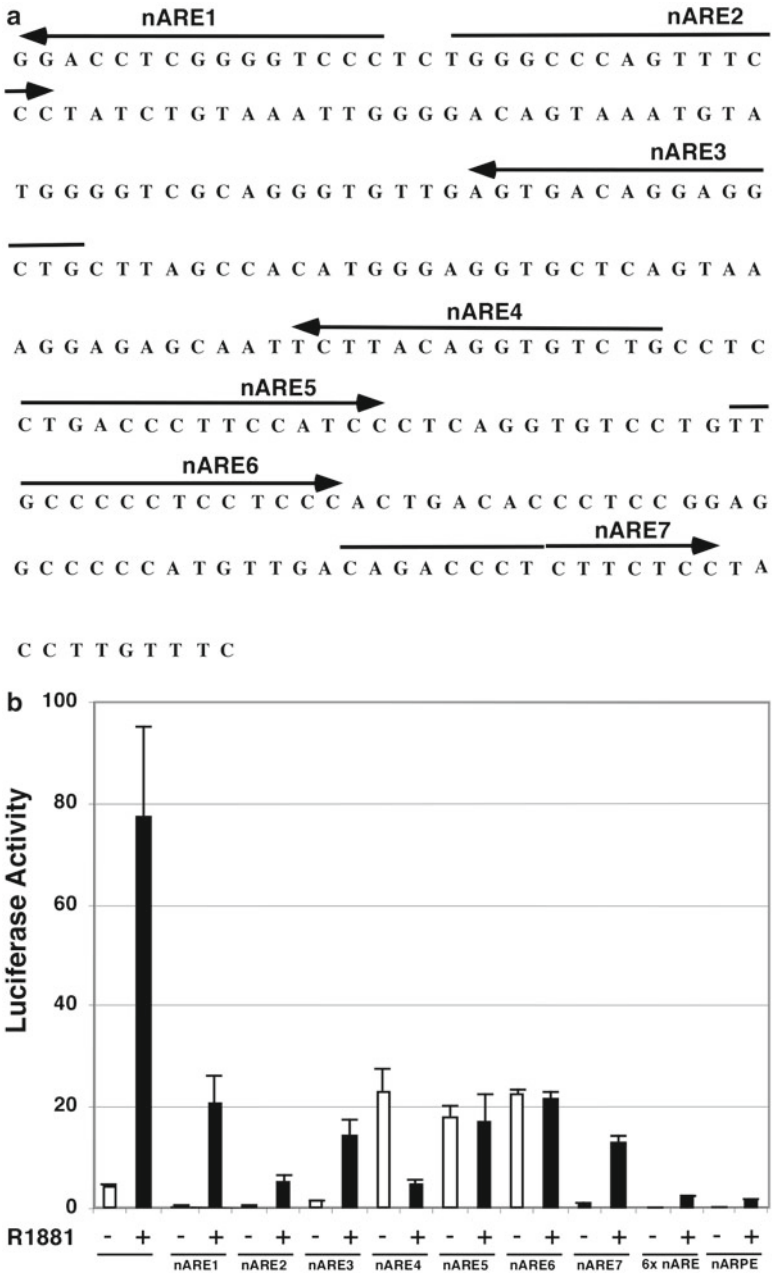

Fig. 3.4 The TGF-β1 negative androgen-response promoter element contains seven functional nAREs. (**a**) Identified 7 nAREs in the TGF-β1 negative androgen-response promoter element. Sequences and directions of nAREs are indicated by underlines and *arrows*, respectively. (**b**) Luciferase assay results show repression activity of 7 nAREs on androgen-driven transcription. PC3MM2 cells were transfected with 25 fmol of the luciferase reporter constructs and 5 fmol of pcDNA-AR. Transfected cells were allowed to grow in the absence or presence of 10 nM R1881 and harvested for the luciferase assay. The values represent the mean ± SD ($n = 3$)

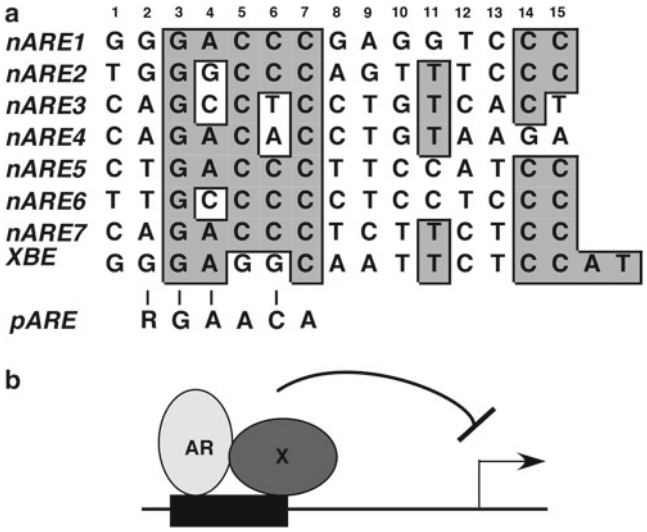

Fig. 3.5 nAREs resemble core half-sites in pARE (**a**) Sequence alignment of identified seven nAREs and XBE with pARE consensus. The conserved nucleotides are in *shadow*. (**b**) A model for AR function through nARE. *AR* androgen receptor, *X* unidentified transcriptional factor that interacts with AR and the 3′ part of nARE

affected by the mutant pARE (pAREmt) probe, which did not interact with the AR DBD [23, 28]. Mutations on the nucleotides critical for AR/DNA interaction (G3 and C5) abolished the AR DBD/nARE7 interaction. In contrast, low amounts of the AR DBD protein (2.5 and 5 ng) generated much strong shifted binds with pARE probes. These findings suggested that the AR weakly interacts with nAREs and that the AR binds to the nARE, which was consistent with the fact that the nARE contains only a consensus ARE half-site motif. The AR-nARE interaction is also correlated with the function of nAREs. Since gel shift experiments were carried out with the purified AR DBD protein and not a cell extract, it is possible that other proteins may also interact with the nARE and contribute to AR repression of gene expression through the nARE (Fig. 3.5b).

He et al. cloned 860 bp (−765 approximately +95) of masipin promoter sequence from the human genomic DNA and studied the promoter activity [29]. The 860 bp sequence and a series of deletions from 5′ and 3′ ends were inserted into the upstream of luciferase reporter gene, respectively. Results from dual luciferase reporter assay and electrophoretic mobility shift assay indicated that there was a negative androgen-responsive element (5′-GTACTCTGATCTCC-3′) in the region of −277 to −262. It appeared that AR interacted with this nARE. But it is unclear how AR regulates maspin gene expression through this nARE. The nAREs we identified do not resemble those found in the maspin gene but are similar to the 5′ part of the XBE in the *PSA* gene [18]. The XBE interacted with both the AR and the p65 subunit of nuclear factor (NF)-κB [18]. We performed a cotransfection assay and found that

NF-κB p65 did not affect the inhibitory activity of the *TGF-β1* nARPE. However, based on these results, we cannot rule out the competition of other transcriptional factors with the AR in interaction with identified seven nAREs in the *TGF-β1* promoter in transcriptional repression (Fig. 3.5b).

In reports of previous AR-binding analysis of the mammalian genome, authors described the enrichment of many other DNA-binding motifs in AR-binding sites [6, 7]. They found that a consensus AR half-site motif and potential binding sequences for AP-1, ARA, ZNF42n NHF-4a, and epidermal growth factor were linked together. Because the 5′ halves of the nAREs identified in our study are highly conserved and similar to a consensus pARE half-site motif and the 3′ halves are diversified, other transcription factors may participate in nARE function by interacting with the 3′ half of the nARE (Fig. 3.5b). Gel shift experiment was carried out with recombinant and the purified AR DBD protein and showed a weak interaction between nARE and AR DBD. We cannot exclude the possibility that the other transcription factors together with AR synergistically interact with the nAREs to contribute to AR repression of gene expression. Further studies are necessary to identify factors that can bind to the 3′ parts of nAREs to participate in nARE-mediated transcriptional repression.

3.5 Future Directions

The synthetic promoters and transient transfection assay are used for characterization and analysis of the nARE. Multiple copies of the construct in a single transfected cell and the poorly chromatinized nonintegrated promoters may render the corresponding transcription factors limiting with respect to the exogenous construct. Thus, it is necessary to further investigate whether the identified nAREs function in the integrated (single copy) chromosomal environment and in their native gene environments.

Distinct histone amino-terminal modifications generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptionally active or transcriptionally silent chromatin states [30–32]. With a few exceptions, trimethylations of H3K9, H3K27, and H4K20 are associated with transcriptional repression, whereas methylations of H3K4, H3K36, and H3K79 are associated with transcriptional activation [33, 34]. It is more likely that AR and the other transcription factor (TF) interact with the nARE to recruit corepressors to modify histone tails for suppression of transcription. It is important to investigate whether AR interaction with the nARE recruits corepressors and alters histone modifications (e.g., acetylation, methylation) in the region surrounding the nARE.

It is unknown how AREs function in the regulation of AR-target genes in animals. To fill this gap is to investigate roles of positive and negative AREs in the regulation of *TGF-β1* gene expression and in the development and functions of mouse prostate gland. The positive and negative AREs in the mouse *TGF-β1* promoter can be mutated and the mutated promoters knocked in the *TGF-β1* locus in

the mouse. The consequences of these knock-in mutations on expression of the *TGF-β1* gene and on the development and functions of mouse prostate gland can be investigated.

Expression of maspin is under the influence of both a positive Ets and a negative HRE element. Loss of maspin expression during tumor progression apparently results from both the absence of transactivation through the Ets element and the presence of transcription repression through the negative HRE element recognized by androgen receptor. The PSME activates transcription from its own and heterologous promoters in prostate cell lines; enhancement is greatest in the PSMA-expressing cell line LNCaP (>250-fold). The PSME shows essentially no activity in five non-prostate cell lines. The data demonstrate that both cell-type specificity and androgen regulation are intrinsic properties of the nARPEs. TGF-β1 is a multifunctional cytokine that influences homeostatic processes of various tissues. TGF-β1 expression is inhibited by androgens in the prostate gland, whereas its expression is enhanced by androgens in highly metastatic prostate cancer cells. How androgen signaling positively or negatively regulate TGF-β1 expression in response to various signals or under different environmental conditions is unknown.

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