Chapter 4

Genome-Wide Binding Profile of Androgen Receptor in Dihydrotestosterone-Induced LNCaP Cells

This research has been supervised by Peter B. Dervan (California Institute of Technology) and conducted in conjunction with the Wold lab (California Institute of Technology). Ali Mortazavi, Anthony Kirilusha, Ken McCue, Diane Trout, and Brian Williams have provided valuable help on performing ChIP and assisting with data work-up.

Abstract

The nuclear hormone receptor, androgen receptor, has been mapped genome-wide in LNCaP cells utilizing chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq). We observe as many as 7200 androgen receptor binding sites present in 2764 enriched, immunoprecipitated regions. We find a canonical androgen response element in the sequence data that maps to more than half of the immunoprecipitated regions. Furthermore, we define a secondary sequence motif that may be a dimer between an androgen receptor half-site and a forkhead protein. While such interactions are known in the literature, none have observed highly conserved sequence motifs. We observe fourteen other transcription factor motifs to be highly enriched within the binding regions. We characterize the majority of binding regions to be present in gene enhancer regions. Finally, we observe that fewer than 10% of the gene expression changes resulting from DHT-induction correlate with the presence of an androgen receptor binding region.

Introduction

Androgen Receptor Biology

Androgen receptor (AR), one of several nuclear hormone receptors, is a 110 kDa basic helix-loop-helix protein that binds a 5'-GGWACANNNTGTTCT-3' consensus androgen response element (ARE) as a homodimer (Figure 4.1A).[1,2] It resides outside of the nucleus until bound by a steroid, such as dihydroxytestosterone (DHT), which enables translocation into the nucleus, binding to androgen response elements, and modulation of androgen-responsive genes. AR helps to regulate the growth, differentiation, and survival of epithelial cells in the normal prostate.[3] Genotropic actions of AR are responsible for prostate cancer disease progression.[4]

The promoters of several key genes driven by androgen receptor occupancy have been studied in cell culture.[5–10] LNCaP cells have been used primarily because they are most widely available and are one of the oldest established immortalized cell lines.[11–14] The prostate specific androgen (PSA) promoter, a marker gene used to test for the presence of prostate cancer has been well mapped.[8,9] It contains a complex similar to the one drawn in Figure 4.1B. There are several ARE binding events, one in the promoter region, and a handful several kilobases upstream in the enhancer region. Additionally, other transcription factors are known to bind nearby in this promoter and synergistically cooperate in the induction of PSA, such as HIF-1α[15] and CREB.[16] As the complex forms, RNA polymerase II is recruited, and transcription begins. Chromatin immunoprecipitation has validated the presence of AR in both enhancer and promoter regions, as well as the presence of RNA polymerase II in these regions.[8,9]

Mapping the genomic AR-bound loci in LNCaP cells is vital to understand those genes directly regulated by DHT-induced AR binding events. Recent ChIP-chip (ChIP followed by microarray study of immunoprecipitated DNA) analysis has mapped AR occupancy to at most 3% of the contiguous human genome at no better than 500-bp

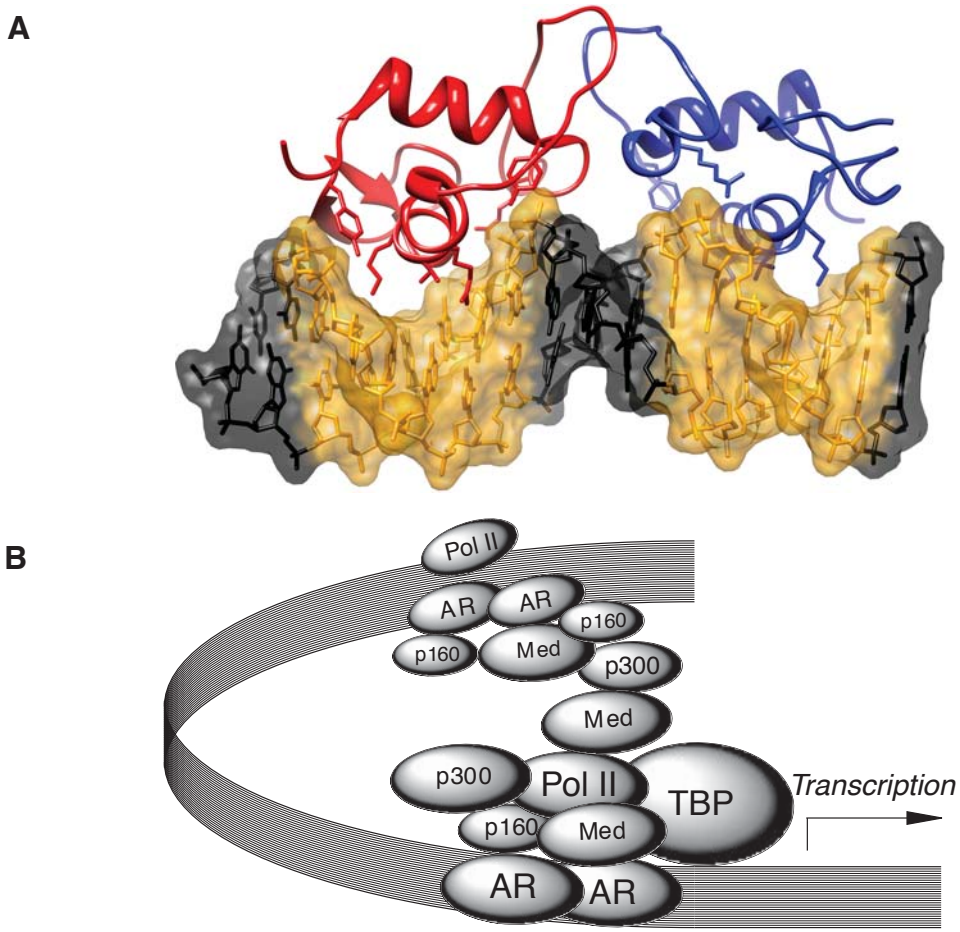


Figure 4.1. Overview of Androgen Receptor Biology. a) Crystal structure of androgen receptor DNA binding domain (PDB 1R4I). b) Example of an androgen receptor promoter / enhancer complex

resolution using either R1881 (1 nM[17,18] or 10 nM[19]) or DHT-induced (10 nM[20] or 100 nM[21]) LNCaP,[18,19,21] C4-2B,[20] or HPr-1AR cells[17] for a variety of time points. These studies have revealed potential new binding partners for androgen receptor, although none has shown a conserved motif of AR and a separate protein. Multiple examples of AR binding near other proteins (within 5–20 base pairs) exist, but a strongly conserved pattern has not emerged.

Disruption of AR binding at the AR-DNA interface may serve as a platform for future therapeutic approaches to mediating prostate cancer disease progression.

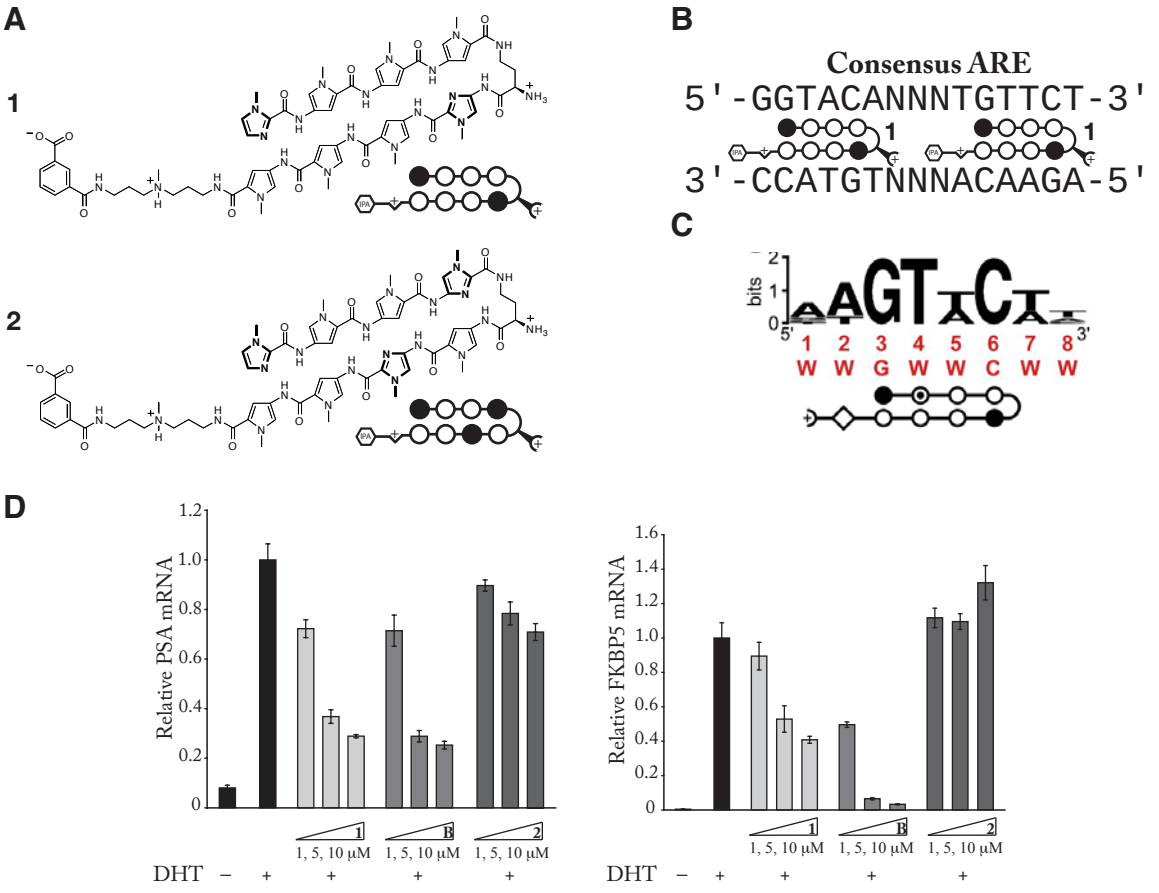


Figure 4.2. Polyamides can target and regulate Androgen Receptor (AR)-driven Gene Transcription. a) Match (1) and mismatch (2) polyamides for AR studies. b) Consensus ARE. c) CSI-derived sequence logo for a polyamide similar to 1. d) Polyamides specifically downregulate expression of PSA and FKBP5, two well characterized genes regulated by AR.

Polyamide Modulation of Androgen Receptor-Driven Gene Expression

We have discovered a polyamide (**1**) that targets the 5'-WGWWCW-3' subset of the androgen response element (ARE) consensus sequence and disrupts the AR-ARE binding event in LNCaP cell culture, resulting in downregulated PSA mRNA transcripts (Figure 4.2). Microarray mRNA transcript analysis demonstrates that polyamide **1** modulates a distinct set of genes from bicalutamide, and from an off-target mismatch polyamide (**2**) that binds to 5'-WGWCGW-3'. These findings suggest that disruption of binding at the protein-DNA interface is empirically distinguishable from protein-small molecule antagonism and that sequence specificity drives observed gene expression changes.[22] It is important to

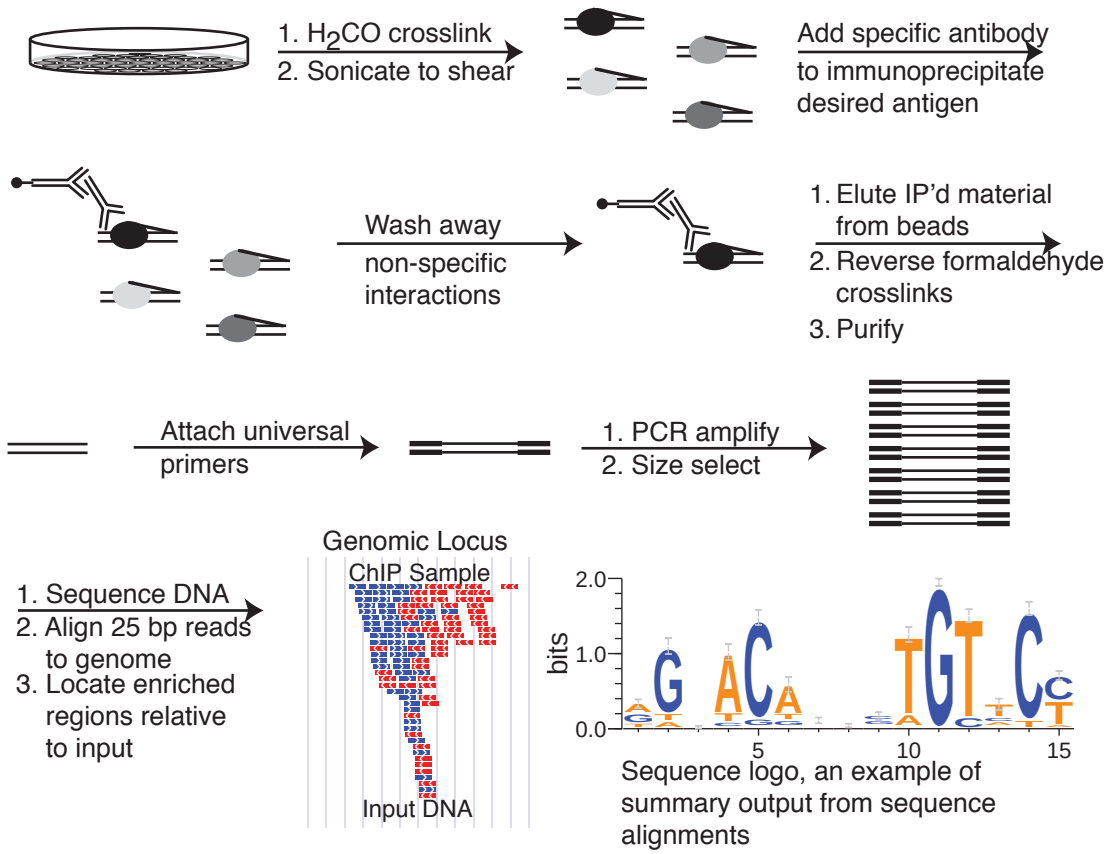


Figure 4.3. Overview of Chromatin Immunoprecipitation followed by High-throughput DNA Sequencing (ChIP-Seq)

understand whether polyamide **1** disrupts the subset of AR-ARE binding events that will be useful for slowing prostate cancer disease development.

Chromatin Immunoprecipitation followed by High Throughput Sequencing

Several new direct ultrahigh-throughput sequencing technologies enable more thorough functional genomics studies to be performed.[23,24] Chromatin immunoprecipitation (ChIP) isolates specific antigen-bound DNA fragments using an antibody bound to an immobilized support, such as magnetic bead, and has been utilized to map DNA occupancy by specific proteins.[25] ChIP-Seq is simply ChIP followed by direct ultra high-throughput DNA sequencing of the antibody-immunoprecipitated sample.[26] Genomic transcription-factor binding events have begun to be measured using ChIP-Seq (Figure 4.3).[26–30] This

technology enables antigen binding events to be resolved at 25 bp resolution, 20-fold better than previous generation ChIP-chip (chromatin immunoprecipitation followed by microarray elucidation of binding regions).[26] It requires no prior knowledge of sequence content. For genome-wide studies, it is substantially less expensive and more efficient—it only sequences material that is immunoprecipitated. It does not probe for other DNA.[24]

Overview of Experiment

This chapter focuses on providing a genome-wide binding baseline for androgen receptor occupancy in DHT-induced LNCaP cell culture. It will resolve the regions occupied by androgen receptor, the frequency of androgen receptor occurrence, binding preferences of androgen receptor, functional locations within nearby genes to binding events, and the correlations between prior microarray data and androgen receptor occupied regions. This data stands as a baseline for future experiments to create a displacement map of polyamide activity.

Results

Androgen receptor (AR)-bound DNA was immunoprecipitated from sonicated, 1 nM dihydrotestosterone (DHT)-induced LNCaP cell lysates using the rabbit polyclonal Santa Cruz Biotech antibody N-20. Real-Time, quantitative PCR (qPCR) revealed 100 (±9)-fold enrichment at the PSA ARE III locus and 48 (±6)-fold enrichment at the FKBP5 intronic locus over the average of two genomic negative loci (Figure 4.4). The immunoprecipitated fragments were then sequenced with an Illumina Genome Sequencer high throughput DNA sequencing machine. A mock sample, which was simply input DNA for the chromatin immunoprecipitation experiment, was sequenced as a control for non-random genomic shearing from the sonication. Sequenced fragments were deconvoluted in ELAND (Solexa), trimmed to 25 base pairs, and aligned to the genome using bowtie.[31] A 1 nM dihydrotestosterone (DHT)-induced sample was examined. Using ERANGE

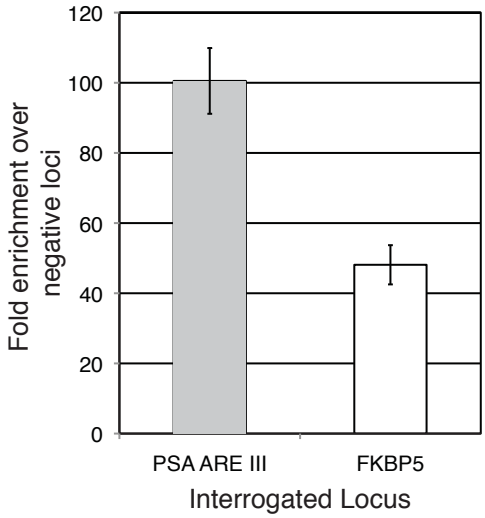


Figure 4.4. Verification of Initial ChIP data for Sequencing Submission. Enrichments are for 1 nM DHT-induced LNCaP cells and are compared relative to other loci in the human genome. An input sample calibrates the relative levels of genomic loci.

Table 4.1. Summary statistics of ChIP-Seq data. Enriched regions were called based on a four-fold enrichment relative to the input sample and a minimum of one read per million reads present in the enriched region.

	Input	ChIP sample
25 base pair reads	24.7 M	20.4 M
Fraction of reads mapped to human genome	69.8% (14.2 M)	43.4% (10.7 M)
Number of uniquely mapped loci	11.9 M	8.9 M
Number of loci mapped to multiple genomic regions	2.3 M	1.8 M
Fraction of reads mapped to enriched regions	1 %	
Number of enriched regions	2764	

3.0alpha,[26,32] 2,764 regions were found to be significantly enriched relative to the input control.

Summary Statistics of Sequenced Samples

While there is no standardized method of reporting data for ChIP-Seq, there are a few summary statistics that should be reported. Table 4.1 summarizes these statistics for both the induced, immunoprecipitated sample and the induced, input sample. There were 24.7 million 25 base pair reads generated for the ChIP'ed sample and 20.4 million 25 base pair reads generated for the input sample. Of these reads 43.4% were mapped to the human genome for the ChIP'ed sample and 69.8% were mapped in the input sample.

Once the reads were mapped to the human genome, regions were characterized as enriched if a given area of the genome was at least fourfold enriched in the ChIP-ed sample relative to the input sample. An additional requirement of one read per million reads was required for each region to be called enriched. With these parameters, roughly 1% of all reads mapped to enriched regions. There were 2,764 regions called enriched relative to the input. Each region spans on average 214 ± 75 base pairs.

Motif Searching

Because AR has been immunoprecipitated, one would expect to find the canonical ARE as a primary motif within the 2,764 enriched regions. The motif searching is computationally faster if highly-enriched events are searched first (Figure 4.5). A motif found from these regions can then be utilized to search all regions. Inevitably some regions will not contain the first motif. These left-over regions can then be submitted for motif searching, and the process can continue iteratively.

Utilizing the 593 most highly-enriched AR binding regions as a seed, motif searching was conducted through the ERANGE 3.0alpha package, using the motif-finding software MEME.[26,32,33] The 15 bp canonical ARE was observed (Figure 4.6). To determine

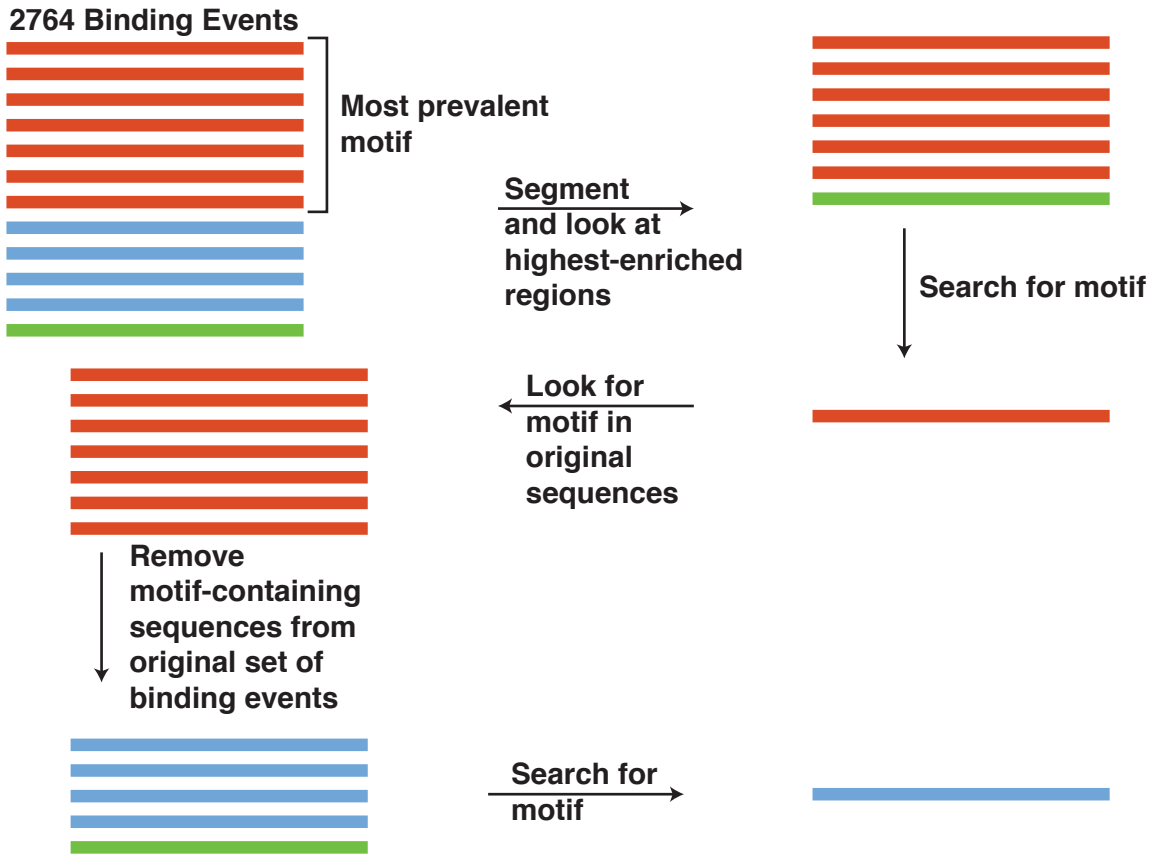


Figure 4.5. Method for finding multiple sequencing motifs using motif-finding algorithms

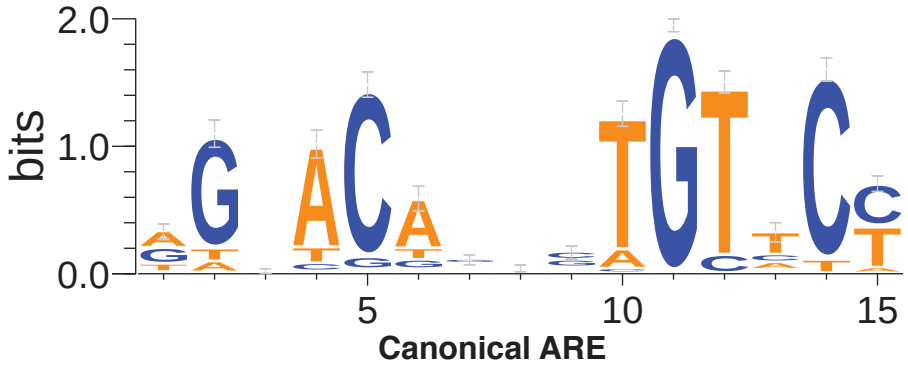
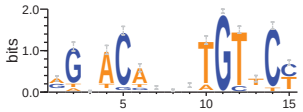


Figure 4.6. The Canonical ARE sequence was found using the 593 most strongly enriched binding regions.

Table 4.2. Prevalence of other ARE Elements in the genome and an upper estimate on the number of ARE binding sites

Motif	Matches within 2,764 Regions
	2,752
TGTTCTnnnAGAACA	206
TGTTCTnnnTGTTCT	318
AGAACAnnnTGTTCT	1,074
TGTTCT (half-site)	10,522
Estimated number of Genomic AR Binding Events (Number of half sites – 2 x Number of Canonical Motif – other AR motifs)	7,246

the prevalence of this motif in the 2,764 regions, the frequency matrix for this empirically determined motif was utilized to search the regions and yielded 1,800 regions and 2,752 canonical ARE binding sites.

The ARE binding profiles have been previously segmented to look specifically for ARE half-sites defined as 5'-AGAACA-3,' ARE head-to-head binding 5'-AGAACA$[N]_{0-8}$TGTTCT-3,' ARE tail-to-tail binding 5'-TGTTCT$[N]_{0-8}$AGAACA-3,' and a typical ARE head-to-head binding with three nucleotide spacer 5'-AGAACANNNTGTTCT-3.'[21] In this study, the ARE half-site and ARE dimers were utilized to search the immunoprecipitated regions. For ARE tail-to-tail binding, ARE direct-repeat binding, and ARE head-to-head binding (canonical ARE), a fixed three nucleotide spacer, 5'-NNN-3,' was utilized. Table 4.2 summarizes the findings from these searches.

We can utilize this data to estimate the number of genomic androgen receptor binding events. By counting the ARE half-sites, we have an upper bound on the total number of binding events. Inevitably, two half-sites will be contained within the canonical

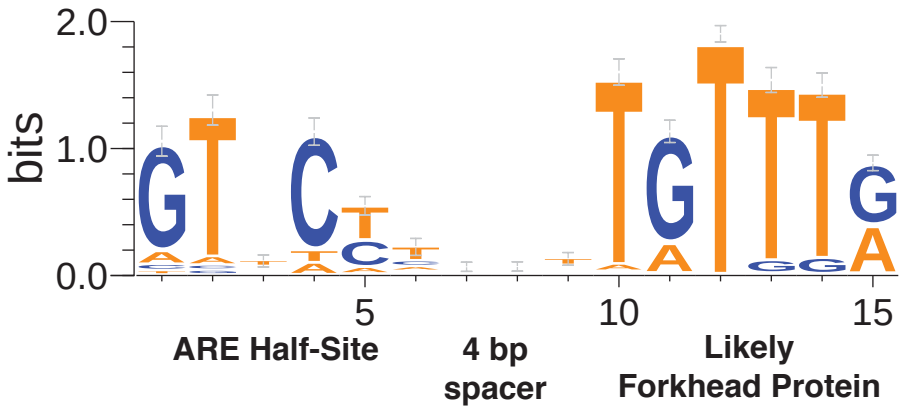


Figure 4.7. Sequence logo of a conserved motif that contains an ARE half-site and a forkhead protein binding site

ARE motif and other ARE dimer motifs. Thus, we can subtract out twice the number of ARE dimer motifs from the number of ARE half-sites. This would give 7,246 putative genomic AR binding events from 2,764 androgen receptor immunoprecipitated regions.

To discover other prominent motifs by the motif-finding software mentioned above, the 964 regions that did not contain the canonical ARE were searched for motifs. This search resulted in a motif containing an ARE half-site and what appears to be a motif for one of the forkhead proteins (Figure 4.7). The frequency matrix of this motif was used to search the entire 2,764 region sample. This motif occurs 1,622 times in 1,275 of the 2,764 regions.

There are several instances in the literature that note potential binding partners with androgen receptor. In previous ChIP-chip work, Brown and co-workers found the ARE half-site close to Oct1, GATA2, and HNF-3α (FOX-A1).[21] A recent ChIP-chip paper from the Coetzee lab noted an overrepresentation of HNF-3α sequences within androgen receptor occupied regions (each region is ~500 bp).[20] LNCaP cells are known to express HNF-3α, and thus it would be a likely candidate for the motif that is observed.[20,21,34] It is also possible that other forkhead proteins may be binding adjacent to the ARE half-site. Because of the forkhead proteins' similar binding preferences, it is not possible to differentiate the occupancy by sequence motif alone. The motif found in these regions

marks *the first instance of a well-conserved motif for the ARE half-site bound with a forkhead protein*.

The ARE-forkhead dimer motif exhibits a strong preference for a four base-pair spacer between the androgen response element and the forkhead binding region (Figure 4.7). Previously published data on AR interactions with HNF-3α showed variable distances of 0 bp to 10 bp between ARE half-site and forkhead binding consensus sequences. The PSA promoter must be bound by HNF-3α to enable transcription.[34]

An alternate approach to searching for motifs involves utilizing public consensus sequence databases such as TRANSFAC[35] or JASPAR[36] to search for overrepresented motifs within the binding regions. A publically available website (the cis-regulatory element annotation system, CEAS) enables such searching by utilizing the enriched immunoprecipitated regions as an input.[37] Eighteen distinct motifs relevant to human cell lines are listed (Figure 4.8). HNF-3α and several other forkhead proteins were most enriched after AR, glucocorticoid receptor, and progesterone receptor. These three nuclear hormone receptors have highly homologous consensus binding sequences, hence it is not surprising to observe their enrichment. Future work will seek to elucidate which of these factors is influential on modulating gene expression in conjunction with AR.

Regions of Genomic Occupancy

In addition to searching for sequence motifs of androgen receptor, we can observe nearby genes to each androgen receptor immunoprecipitated region. These may be suitable targets for androgen receptor regulation. Current algorithms search for the nearest gene within a specified radius of the antigen binding region. By examining different radii mapping with genes, we observe that the number of regions that fail to map to genes decreases rapidly when increasing from a 10 kilobase (kb) radius to a 500 kb radius (Figure 4.9). Six immunoprecipitated regions fail to map to any genes, even within megabase radii. This result is consistent with a 0.14% false discovery rate in the enriched regions.

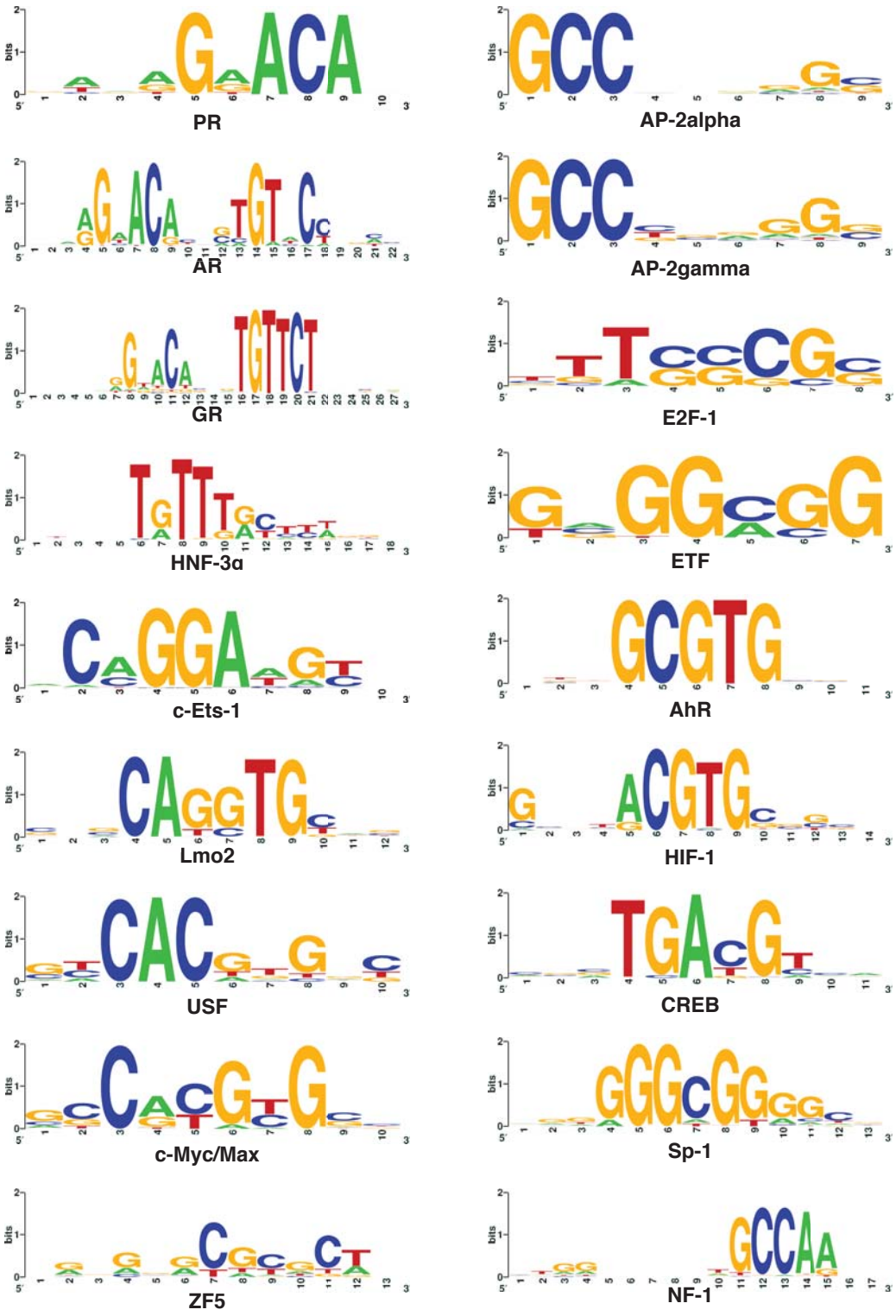


Figure 4.8. Sequence logos of known transcription factor motifs overrepresented within the Androgen Receptor Immunoprecipitated Regions

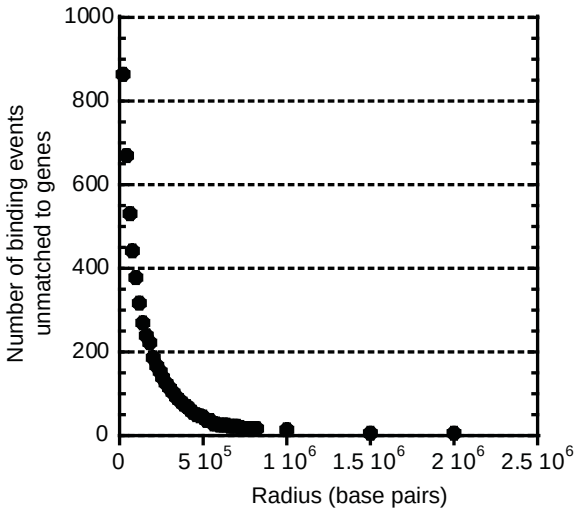


Figure 4.9. An overview of the number of androgen receptor immunoprecipitated regions that fail to map with nearby genes as a function of search radius. 1901 genes map within 20 kb of the androgen receptor binding regions.

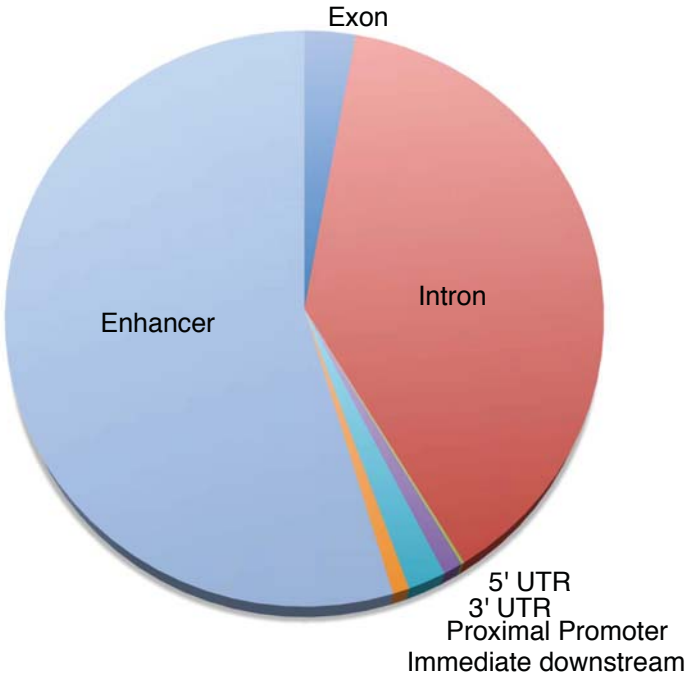


Figure 4.10. The distribution of regions of gene occupancy for androgen receptor binding regions within proximity to genes

Utilizing a 20 kb radius for mapping genes, we observe 1901 regions that map to nearby genes. These regions can be segmented into those located within enhancers (54.6%), exons (2.8%), introns (38.5%), 5'-untranslated regions (0.2%), 3'-untranslated regions (1.0%), proximal promoters (2.0%), and immediate downstream regions (0.9%) of the gene (Figure 4.10).

Correlating Microarray Transcript Analyses with ChIP-Seq Data

We can correlate androgen receptor genomic occupancy nearby genes with changes in mRNA microarray data[22] for vehicle-treated and DHT-induced conditions. Of the 1901 regions associated with genes, 356 regions overlap with 273 genes that change in expression by 1.4-fold or more with a *P* value of less than 0.01 (Figure 4.11). It is intriguing that fewer than 10% of the modulated genes correlate to androgen receptor binding. This could result from long-range (across other genes) androgen receptor transcription modulation via chromosomal looping. It could also be an artifact of androgen receptor-induced genes

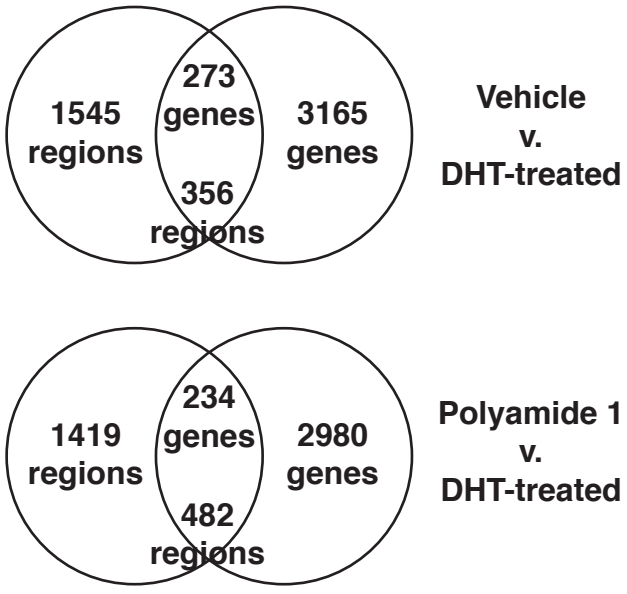


Figure 4.11. Venn diagram correlating AR binding events in proximity to genes with changes in mRNA transcript levels on DNA microarrays. A 1.4-absolute fold change and 0.01 *P* value cut-off were used for microarray values.

activating or repressing the expression of other genes.

Applying the same correlation with androgen receptor occupancy to polyamide **1**-treated microarray data, one observes fewer genes (234) intersecting with more regions (482) as compared with DHT-induced conditions above (Figure 4.11). This observation could signify that polyamides need to interfere with multiple androgen receptor binding events to achieve down- or up-regulation of genes. Future work with ChIP-Seq of polyamide **1**-treated, DHT-induced cells will help to elucidate where the polyamide is acting.

Conclusions

In this initial study of DHT-induced, genome-wide androgen receptor occupancy in LNCaP cells, we have found an upper bound to the number of genomic androgen receptor binding events, roughly 7200. We have observed the canonical ARE to be widely present in the androgen receptor immunoprecipitated regions and have found a defined sequence motif that may be explained by adjacent pairing of AR with HNF-3α. We have defined a set of 1901 regions that occur within 20 kilobases of known genes and observed that the majority of these regions are found within enhancers. By correlating mRNA microarray transcript analyses with androgen receptor immunoprecipitated regions, we observe that fewer than 10% of significantly changed genes can be correlated by androgen receptor occupancy. Herein, we have defined an initial data set of genome-wide binding of androgen receptor.

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