# Molecular Mechanisms of Androgen Action: Characterization of the Androgen Regulated Gene KLK4 and the Role of Nuclear Dynamics in Androgen Receptor-Mediated Transcription

Thesis submitted for the degree of *Philosophiae Doctor*

by

**Tove Irene Klokk**



The Gene Programme

Department of Molecular Biosciences

Faculty of Mathematics and Natural Sciences

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## ABBREVIATIONS

| | |
|---|---|
| **AF** | **Activation Function** |
| **AR** | **Androgen Receptor** |
| **ARE** | **Androgen Response Element** |
| **BPH** | **Benign Prostatic Hyperplasia** |
| **BRM** | **Brahma** |
| **CAS** | **Casodex (Bicalutamide)** |
| **CBP** | **CREB-Binding Protein** |
| **CPA** | **Cyproterone Acetate** |
| **CREB** | **cAMP Response Element Binding Protein** |
| **DBD** | **DNA Binding Domain** |
| **DHT** | **5α-dihydrotestosterone** |
| **ER** | **Estrogen Receptor** |
| **FLIP** | **Fluorescence Loss In Photobleaching** |
| **FRAP** | **Fluorescence Recovery After Photobleaching** |
| **FRET** | **Fluorescence Resonance Energy Transfer** |
| **GFP** | **Green Fluorescent Protein** |
| **GR** | **Glucocorticoid Receptor** |
| **GRIP1** | **Glucocorticoid Receptor Interacting Protein 1** |
| **HAT** | **Histone Acetyl Transferase** |
| **HDAC** | **Histone Deacetylase** |
| **HDACi** | **Histone Deacetylase Inhibitor** |
| **HRE** | **Hormone Response Element** |
| **HSP** | **Heat Shock Protein** |
| **KLK** | **Kallikrein** |
| **LBD** | **Ligand Binding Domain** |
| **LH** | **Lutenizing Hormone** |
| **LHRH** | **Lutenizing Hormone Releasing Hormone** |
| **LTR** | **Long Terminal Repeat** |
| **MMTV** | **Mouse Mammary Tumor Virus** |
| **MR** | **Mineralocorticoid Receptor** |
| **NAPDH** | **Nicotinamide Adenine Dinucleotide Phosphate** |
| **NLS** | **Nuclear Localization Signal** |
| **OHF** | **Hydroxyflutamide** |
| **PIN** | **Prostatic Intraepithelial Neoplasia** |
| **PolII** | **RNA Polymerase II** |
| **PR** | **Progesterone Receptor** |
| **PSA** | **Prostate Specific Antigen** |
| **Q-PCR** | **Quantitative Polymerase Chain Reaction** |
| **RNA FISH** | **RNA Fluorescence In Situ Hybridization** |
| **RXR** | **Retinoid X Receptor** |
| **SAHA** | **Suberoyl-Anilide Hydroxamic Acid** |
| **SDS-PAGE** | **Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis** |
| **SHBG** | **Steroid Hormone Binding Globulin** |
| **SRC** | **Steroid Receptor Coactivator** |
| **SWI/SNF** | **Switch/sucrose Non Fermentable** |
| **TAU** | **Transcription Activation Unit** |
| **TBP** | **TATA-box Binding Protein** |
| **TSA** | **Trichostatin A** |
| **TST** | **Testosterone** |
| **uPA** | **Urokinase-type Plasminogen Activator** |
| **uPAR** | **Urokinase-type Plasminogen Activator Receptor** |

## LIST OF PAPERS INCLUDED

**Paper I.** **Z. Xi, T.I. Klokk, K. Korkmaz, P. Kurys, C. Elbi, B. Risberg, H. Danielsen, M. Loda, and F. Saatcioglu (2004).** Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. *Cancer Research*, 64 (7): 2365-70.

**Paper II.** **T.I. Klokk[*], P. Kurys[*], C. Elbi, A.K. Nagaich, A. Hendarwanto, T. Slagsvold, C-Y. Chang, G.L. Hager, and F. Saatcioglu (2007).** Ligand-specific dynamics of the androgen receptor at its response element in living cells. *Mol Cell Biol.*, 27 (5): 1823-43.

**Paper III.** **T.I. Klokk, A. Kilander, Z. Xi, H. Wæhre, B. Risberg, H. Danielsen, and F. Saatcioglu (2007).** Kallikrein 4 is a proliferative factor that is overexpressed in prostate cancer. *Cancer Research, in press*.

**Paper IV.** **T.I. Klokk, P. Kurys, and F. Saatcioglu (2007).** Reduced mobility of the androgen receptor at its target sites in living cells in response to HDAC inhibition. *Manuscript.*

[*]Equal first authorship

The papers will be referred to by their roman numerals in the rest of the thesis.

## ABSTRACT

Male sex hormones (androgens) are important for the normal development of the male sexual characteristics and maintenance of the male reproductive system, including the prostate gland. Androgens are also involved in pathological conditions such as prostate cancer, which is the third leading cause of cancer-related deaths for men in western industrialized countries. Androgens mediate their action through the androgen receptor (AR), a ligand-dependent transcription factor of the nuclear receptor superfamily. Upon ligand-binding, AR translocates to the nucleus and binds specific sequences in the promoter or enhancer of androgen-responsive genes. Androgen-regulated genes have thus been of special interest for a long time for better understanding of normal prostate biology and in the search for potential biomarkers and therapeutic targets in prostate cancer. One such gene that was recently discovered is kallikrein 4 (KLK4), which is androgen regulated and specific to the prostate for expression. KLK4 belongs to the human tissue kallikrein family, consisting of 15 closely related members whose genes are tandemly located in a large cluster on chromosome 19q13.4. Interestingly, KLK4 was shown to have a different gene structure than the other members of this family, as KLK4 transcripts did not contain the putative exon 1 predicted to encode a signal peptide targeting the protein for secretion. The lack of a signal peptide resulted in an intracellular KLK4 which was predominantly expressed in the nucleus of prostate cancer cells and in the basal cells of the prostate epithelium. KLK4 was also overexpressed in malignant prostate as compared to normal prostate glands, both at the mRNA and protein level. Furthermore, we demonstrated that overexpression of KLK4 induces proliferation of the prostate cancer cell lines PC-3 and DU145. The increased rate of proliferation was at least in part due to changes in the expression of cell cycle regulatory genes. We suggest that KLK4 may have a role in prostate cell growth and is an important factor in the development and progression of prostate cancer; thus, KLK4 has potential utility as a diagnostic or prognostic marker, or therapeutic target in prostate cancer therapy.

In addition to identification and characterization of androgen target genes, it is also important to understand the molecular details of AR function to gain full insight into androgen action. To that end, we studied the interactions of AR with its target sites in

chromatin in living cells. Using fluorescence microscopy techniques, we found that there is a transient and dynamic interaction of AR with target genomic sites in the presence of agonists, which coincides with the recruitment of chromatin remodeling complexes and RNA Polymerase II, resulting in transcriptional activation. The kinetics of these interactions are ligand-dependent, as the interaction of antagonist-bound AR was dramatically faster than for agonist-bound AR. Furthermore, the interaction of a transcriptionally compromised mutant AR with target sites was faster than for wild type AR, and occurred without transcriptional activation, suggesting a correlation between transcriptional activity and residence time on the promoter. Furthermore, there were intramolecular interactions between the N- and C-termini of promoter-bound AR in its active state which were important for transcriptional activity. Finally, we elucidated how AR nuclear dynamics are changed in response to altered chromatin acetylation status. Interestingly, and in further support of a direct correlation between nuclear dynamics and transcriptional activity, we found that increased AR transcriptional activity, induced by histone deacetylase inhibitors, resulted in reduced mobility of AR at its target promoter. These data challenge the traditional static view of nuclear receptor action, and support the more recent view of transcription factor–chromatin interactions that constitute a highly dynamic system in continuous flux involving transient and rapid molecular interactions. These findings thus provide a kinetic and mechanistic basis for regulation of gene expression by androgens and anti-androgens in living cells.

# INTRODUCTION

## 1. Androgens and the Androgen Receptor

### 1.1. Androgens

The male sex hormones are known as androgens, a name derived from the Greek *andros*, man, and *gennan*, to produce. The importance of androgens was first discovered in 1849, when Arnold Bechter linked the behavorial and physiological changes of castration to a substance secreted by the testes into the bloodstream. The testicular hormone later known as testosterone was isolated in 1934, and artificially produced only one year later (Freeman et al., 2001). In the decades to follow, other androgens were also identified (Table 1).

**Table 1. Androgens commonly found in man**

| Androgen | Abbreviation | Type | Characteristics |
|---|---|---|---|
| Testosterone | TST | Steroid hormone | Produced in the testis, is the main circulating androgen |
| 5α-dihydrotestosterone | DHT | Steroid metabolite | The active metabolite of TST |
| Dehydroepiandrosterone | DHEA | Steroid hormone | Produced in the adrenal cortex |
| Androstenedione | Andro | Steroid hormone | Produced in the testis, adrenal cortex, and ovaries |
| Androstenediol | - | Steroid metabolite | Is a regulator of gonadotropin secretion |
| Androsterone | - | Steroid metabolite | Chemical by-product from break-down of other androgens |

Androgens are necessary for normal development of the penis, scrotum, testicles, and male secondary characteristics at puberty. Testosterone is the main circulating androgen, and in the developing male, the fetal testis secretes testosterone at sufficient levels to stimulate the differentiation and growth of the male reproductive organs. After birth, the serum testosterone levels decrease to a low level maintained until puberty, when the level increases to the adult range (Isaacs, 1994).

Approximately 90% of the androgens are produced by the Leydig cells in the testes, while the remainder is secreted by the adrenal cortex. The production of testosterone is regulated by negative feedback regulation by LH (Lutenizing Hormone) and the LHRH (Lutenizing Hormone Releasing Hormone) via the gonad-hypothalamus-pituitary axis (see Figure 1). The action of androgens can be blocked by anti-androgens which are described in more detail below. In the blood, testosterone is found complexed to either albumin (54%) in a low affinity fashion, or to SHBG (Steroid Hormone Binding Globulin) (44%), while only 1-2% is free. Testosterone can either enter the cell passively in its free form or by dissociation of albumin near the membrane, or it can be actively transported into the cell through a membrane receptor when bound to SHBG (Rosner et al., 1999) (see Figure 3). Once inside the cell, 90% of the testosterone is irreversibly converted to its more active metabolite DHT (5α-dihydrotestosterone) by the enzyme 5α-reductase in a sequential series of steps involving the cofactor NADPH (Levy et al., 1990). Testosterone or DHT then binds to the AR (Androgen Receptor), where DHT has five-fold higher affinity.

### 1.2. Androgen Receptor

The effects of androgens are mediated by AR, which is a ligand-dependent transcription factor that belongs to the nuclear receptor (NR) superfamily. This family of transcription factors consists of more than 150 members that are likely to have arisen from a single ancestor gene (Escriva et al., 2000) and comprise the largest family of transcription factors known. The importance of this protein family can be explained by the diversity and importance of their ligands: from sex steroids and thyroid hormones, to bile acids and vitamins (Mangelsdorf et al., 1995). Historically, these ligands were isolated in the early part of the 20$^{th}$ century based on their abilities to affect development, differentiation, metamorphosis, and homeostatis. Many of these ligands are also associated with human diseases, such as many cancers (see e.g. (Wiseman & Duffy, 2001; Singh & Kumar, 2005)). In the mid-1970s, it became evident that steroid hormones were targeted to their responsive tissues by the presence of specific high affinity receptor proteins. Due to the lipophilic character of the steroid hormones, they can pass through the lipid bilayer of the cell membrane and interact with intracellular receptors. The identification of hormone

responsive genes within these tissues then led to the identification and cloning of the steroid hormone receptors in the mid-1980s. Later studies revealed the presence of receptors for all known nuclear hormones, as well as a myriad of orphan receptors, which led to the concept of a nuclear receptor superfamily (reviewed in (Robinson-Rechavi et al., 2003)).

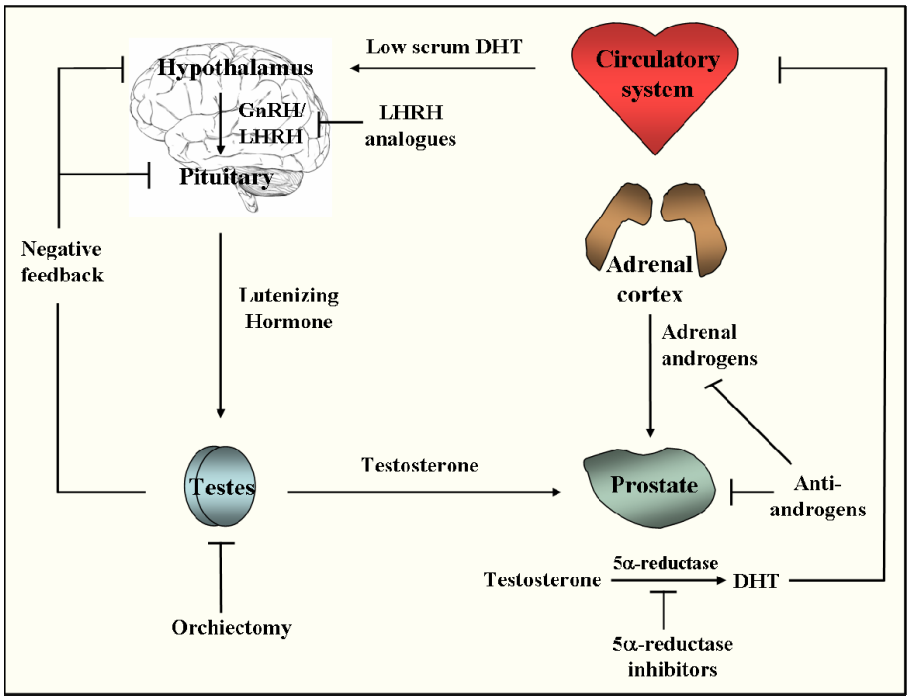


**Figure 1. The role of androgens in the gonad-hypothalamus-pituitary axis**
GnRH (Gonadotropin Releasing Hormone)/LHRH (Lutenizing-Hormone Releasing Hormone) is produced in the hypothalamus which signals the pituitary gland to produce LH (Lutenizing Hormone). LH then stimulates the Leydig cells of the testes to produce testosterone (TST), which is released into the bloodstream. In the prostate, TST is converted to DHT (5α-dihydrotestosterone) which activates the androgen receptor. TST and DHT production is regulated via a negative feedback loop to the hypothalamus. Anti-androgens can block the function of TST in the prostate and adrenal androgens produced by the adrenal cortex. Orchiectomy is medical castration for the inhibition of testosterone production.

NR family members can be classified into three groups based on their ligand binding properties: steroid hormone receptors, RXR (Retinoid X Receptor) receptors, and orphan receptors (sometimes also divided in dimeric and monomeric orphan receptors). AR belongs to the steroid hormone receptor subfamily, which also includes the progesterone receptor (PR), glucocorticoid receptor (GR), estrogen receptor (ER), and mineralocorticoid receptor (MR). The steroid hormone receptor subfamily is activated upon binding of its steroid ligand that are small lipophilic molecules, and in general bind inverted half-sites in DNA as homodimers, although other binding sites are also reported (for a review, see (Beato & Klug, 2000)). The RXR receptors bind DNA (both direct and inverted half sites) as heterodimers, usually with RXR as partner. The orphan receptors form the largest group of NRs, for which no ligands were originally identified, and these receptors bind DNA either as homodimers, or as monomers, to direct repeat of single half-sites (Mangelsdorf et al., 1995; Khorasanizadeh & Rastinejad, 2001).

### 1.2.1. AR gene and protein structure

The AR gene is localized on chromosome Xq11.2-12. It consists of eight exons, which encodes a 98 kDa protein (110 kDa on SDS-PAGE) (see Figure 2). Only one AR cDNA has been identified, so the various AR ligands probably bind the same receptor (Lubahn et al., 1988a; Lubahn et al., 1988b). The NRs have a common protein structure, with three distinct domains: a divergent N-terminal domain (NTD), a highly conserved DNA binding domain (DBD), and a moderately conserved C-terminal ligand binding domain (LBD). In AR, the NTD contains one large activation function (AF1) which is made up of two discrete regions: one required for full ligand-inducible transcriptional activity (Transcription Activation Unit 1, TAU-1) and one ligand-independent region (TAU-5) (Jenster et al., 1991; Simental et al., 1991; Jenster et al., 1995). Furthermore, the NTD contains two motifs involved in intramolecular interactions with the LBD (He et al., 2000). The LBD is made up of 12 conserved α-helical regions and two anti-parallell beta-sheets folded into a three-layered helical sandwich (Matias et al., 2000; Sack et al., 2001; Pereira de Jesus-Tran et al., 2006). In addition to being involved in ligand binding, the LBD also stabilizes homodimerization and orchestrates interaction with coregulators. The other activation function, AF-2, is also placed in the LBD. It is a ligand-dependent

transactivation function, and is also involved in interactions with co-regulators (Jenster et al., 1991; Slagsvold et al., 2000). Furthermore, the AF2 core is involved in the intramolecular interaction with two motifs of the NTD (described in more detail in paragraph 1.2.4) (Doesburg et al., 1997; Langley et al., 1998; He et al., 2000). The DBD is made up of approximately 70 amino acids, which folds into two zinc-finger motifs in which two perpendicular oriented α–helices specify DNA recognition (Freedman & Luisi, 1993). At the border of the DBD and the hinge region, connecting the DBD with the LBD, there is a nuclear localization signal (NLS) that targets the AR homodimer for translocation to the nucleus (Jenster et al., 1993; Zhou et al., 1994).

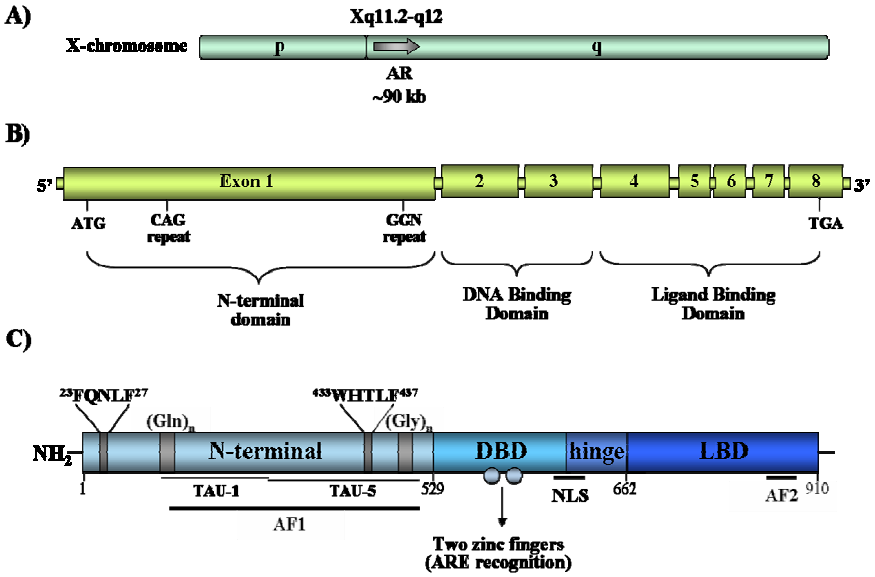


**Figure 2. Schematic presentation of the gene, mRNA, and protein structure of AR**
(A) Chromosomal location of the AR gene. (B) Exon structure of AR mRNA, with localization of the polymorphic CAG and GGN repeats, with indication of which exons encode the different domains of the AR protein. (C) Domain-structure of the AR protein: The N-terminal domain with the transactivation function AF1, divided into the two discrete regions TAU-1 and TAU-5, and the position of two motifs involved in intramolecular N/C interaction; the central DBD with two zinc-finger motifs specifying ARE (Androgen Response Element) recognition; the hinge region and the nuclear translocation signal (NLS); and the C-terminal LBD with the ligand dependent transactivation function AF2. The numbering for aminoacids in the AR protein is based on 20 polyglutamine and 16 polyglycine repeats.

The AR gene contains 2 polymorphic trinucleotide repeat segments, CAG and GGN, which encode polyglutamine and polyglycine tracts, respectively, in the N-terminal transactivation domains of AR. Both repeats, but especially the N-terminal polyglutamine repeat, have been linked to several disease states (Giovannucci et al., 1997; Kantoff et al., 1998; Krithivas et al., 1999). The polyglutamine repeat ranges from 8 to 31 repeats in normal individuals, with an average of 20 repeats (Hardy et al., 1996). *In vitro*, the length of the polyglutamine repeat is inveresely correlated with AR transcriptional activity (Chamberlain et al., 1994; Kazemi-Esfarjani et al., 1995). Longer polyglutamine repeats results in decreased AR activity and is associated with impaired spermatogenesis and infertility (Tut et al., 1997) and generally a lower risk of prostate cancer, whereas a shorter repeat length is associated with hyperactive AR and may increase prostate cancer risk (Irvine et al., 1995; Giovannucci et al., 1997). Expansion of the polyglutamine tract to more than 40 repeats causes the rare neuromuscular disorder spinal and bulbar muscular atrophy (SBMA or Kennedy's disease) (La Spada et al., 1991).

### *1.2.2. AR transcriptional activation*

In the absence of ligand, AR is found in the cytoplasm complexed with heat-shock proteins (HSP). Upon ligand-binding, AR dissociates from this complex, forms a homodimer which is phosphorylated and translocates to the nucleus where it binds to androgen response elements (AREs) in the enhancers or promoters of target genes. The AREs contain two hexanucleotide half-sites oriented as palindromes, spaced by three nucleotides (AGAACAnnnTGTTCT). However, other types of AREs also exist, such as direct repeats and elements with altered site sequence (Robins et al., 1994; Zhou et al., 1997; Geserick et al., 2005). Once bound to its response element, AR initiates gene transcription by the recruitment of chromatin modifying and remodeling complexes, coregulators and other factors of the basal transcription apparatus (Lemon & Tjian, 2000; Dilworth & Chambon, 2001; Hager, 2001; Nye et al., 2002; Orphanides & Reinberg, 2002; Shang et al., 2002; Belandia & Parker, 2003; Huang et al., 2003; Metivier et al., 2003). A schematic presentation of AR transcriptional activation is given in Figure 3.

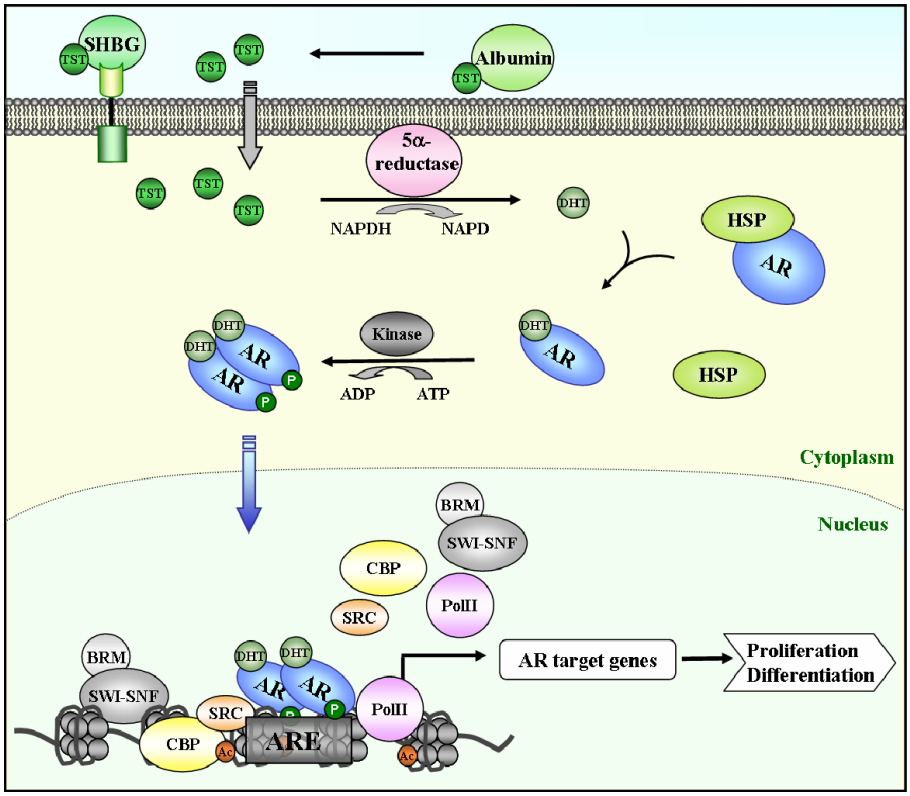


**Figure 3. AR transcriptional activation**
Testosterone (TST) dissociates from albumin close to the cell surface and diffuses into the cell, or enters the cell through a SHBG (Steroid Hormone Binding Globulin) receptor. TST is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase, and binds the androgen receptor (AR). AR dissociates from the complex with heat shock protein (HSP) and dimerizes with another ligand-bound AR. The homodimer is phosphorylated and translocates to the nucleus. Here AR binds androgen response elements (AREs) of target genes, recruits coregulators and the general transcriptional machinery resulting in transcription of AR target genes generally inducing proliferation and differentiation.

Coregulators strongly influence AR transcriptional activity, and a wide range of both coactivators and corepressors for AR have been described (for reviews, see e.g. (Heinlein & Chang, 2002; Wang et al., 2005a; Burd et al., 2006)). These augment or repress AR-mediated transcription through variable mechanisms, such as modulating ligand selectivity and DNA-binding capacity, histone modifications, or recruitment of chromatin

remodelling complexes and other factors of the general transcriptional machinery. Coregulators can be categorized based on their functional characteristics, and can be divided into two major types. Type I coregulators function primarily with AR at the target promoter, promoting DNA occupancy, chromatin remodelling or by recruitment of general transcription factors associated with the RNA Polymerase II (PolII) holocomplex. Examples of these coregulators are CBP (CREB Binding Protein)/p300 and SRC-1 (Steroid Receptor Coactivator-1), which both harbour histone acetyl transferase (HAT) activity, and also the SWI/SNF chromatin remodelling complex. The type II coregulators function mainly through modulating the appropriate folding of AR, aiding in ligand binding or facilitating AR intramolecular N/C interaction, thereby contributing to AR stability or influence its subcellular localization. This category include coregulators such as the ARA70 that stabilizes the ligand-bound receptor, and filamin that facilitates the nuclear translocation of AR (reviewed in (Heinlein & Chang, 2002)). Corepressor may in addition repress AR activity by inhibiting the recruitment of coactivators. The correct balance of coactivators and corepressors ensure the ligand and tissue-specific activity of AR, and a deregulation in the levels of these coregulators may cause inproper AR activity and therefore be involved in disease states such as prostate cancer (for review, see e.g. (Culig et al., 2004; Burd et al., 2006)).

The timing and order of events in the recruitment process during transcriptional activation induced by AR has been under scrutiny for many years. By the use of time-course based chromatin immunoprecipitation (ChIP) assays, the temporal recruitment of the AR and associated factors to AREs in chromatin have been described (Shang et al., 2002; Kang et al., 2004; Wang et al., 2005b). These studies have revealed that there are differences in the ligand-induced loading of AR, its cofactors and PolII between promoters and enhancers of the same gene, and between different genes; however, there seems to be a functional coordination between the promoter and enhancer regions through shared factors in the transcription complex (Shang et al., 2002; Wang et al., 2005b). Brown and colleagues suggest a model in which the agonist-bound AR is recruited to both the promoter and the enhancer, followed by the ordered recruitment of p160 proteins, CBP, and other factors, which results in a chromosomal loop that allows

PolII to track from the enhancer to the promoter and initiate transcription (Shang et al., 2002; Wang et al., 2005b). In contrast to what have been observed for ER (Shang et al., 2000; Metivier et al., 2003), there does not seem to be cyclical recruitment of AR and its cofactors to the promoter or enhancer (Wang et al., 2005b). Furthermore, antagonist-bound AR was shown to be recruited to the promoter of the PSA gene, but not the enhancer, followed by the formation of a corepressor complex (Shang et al., 2002).

Although the ChIP assay is a powerful tool, it has limitations in that one averages the events occurring in a population of cells and the process involves crosslinking which will obscure dynamic interactions. These limitations have recently been addressed by live cell imaging techniques, such as FRAP (Fluorescence Recovery After Photobleaching) and FLIP (Fluorescence Loss After Photobleaching), allowing the real-time imaging of molecules in single cells, thus making it possible to measure molecular dynamics at much smaller timescales compared to ChIP analysis. These techniques have been applied to the study of steroid hormone receptors such as AR, GR, ER and PR and suggest a much more dynamic interaction between the receptor and the chromatin than what was believed earlier (McNally et al., 2000; Stenoien et al., 2001a; Farla et al., 2004; Farla et al., 2005; Rayasam et al., 2005). Nuclear receptor dynamics are described in more detail in paragraph 2.5.

### *1.2.3. AR antagonists*

Given the important role of androgens in prostate cancer development, AR antagonists or anti-androgens have been developed, some of which are currently used in the treatment of prostate cancer (see Table 3). Anti-androgens antagonize AR function by binding to the LBD of AR in competition with the natural agonists TST and DHT (Denis & Griffiths, 2000; Klotz, 2000; Masiello et al., 2002). In general, the AR-antagonist complex does not activate transcription, although in some circumstances it can occur (Miyamoto et al., 1998; Fujimoto et al., 1999), but it is not clear which steps in the AR signaling pathway are influenced. For example, it has long been held that the antagonists may block nuclear import or DNA binding, based largely on biochemical and *in vitro* experiments. However, data exists supporting the opposing view (e.g. (Kemppainen JA, 1992;

Masiello et al., 2002)). It has also been suggested, as for the estrogen receptor (ER) (for a review, see (Greschik H, 2003)), that antagonist-binding gives rise to a different conformation of the LBD compared with the agonists, thereby affecting the interactions of AR with coactivators and corepressors when bound to DNA (Poujol et al., 2000; Bohl CE, 2005). Furthermore, recent reports suggest that AR antagonists actually facilitate AR-DNA association, but inhibit transcriptional activation via the recruitment of corepressors to the promoter (Shang et al., 2002). In support of this view, a recent study demonstrated that antagonist function can be blocked by the disruption of corepressor recruitment (Zhu et al., 2006). However, the molecular details of AR antagonist function are at present still not clear.

### *1.2.4. AR intramolecular N/C interaction*

Genetic and biochemical experiments have indicated that the LBD of AR interacts with its NTD upon ligand binding (Langley et al., 1995; Doesburg et al., 1997; Langley et al., 1998) similar to that observed for ER (Kraus et al., 1995). This interaction is mediated by two N-terminal motifs ($^{23}$FQNLF$^{27}$ and $^{433}$WHTLF$^{437}$) and the C-terminal AF2 (He et al., 2000; Slagsvold et al., 2000; Steketee et al., 2002; He et al., 2004), and has been shown to be important for optimal receptor activity, occuring only in the agonist-bound receptor (Doesburg et al., 1997; Schaufele et al., 2005). AR cofactors, such as the histone acetyl transferase CBP, facilitate this agonist-dependent N/C interaction (Ikonen et al., 1997), and recent studies have suggested that other AR cofactors also modulate this interaction (Shenk et al., 2001; Bai et al., 2005; Hsu et al., 2005).

The initial studies on AR N/C interactions were in large part performed with truncated versions of the receptor in mammalian or yeast two-hybrid systems, or in biochemical experiments *in vitro*. However, agonist-dependent N/C interaction was recently also demonstrated for the full length receptor in human cells by the use of FRET (Fluorescence Resonance Energy Transfer) technology (Schaufele et al., 2005). AR with one fluorophore linked to the C-terminus, and another fluorophore linked to the N-terminus was used in FRET analysis to determine the time and subcellular location of ligand-induced conformational changes. The AR antagonist hydroxyflutamide, OHF,

blocked the N/C interaction within AR, which was also closely correlated with loss of AR transcriptional activation. Mutation of the α-helical motif ($^{23}$FQNLF$^{27}$) in the NTD disrupted the N/C interaction, confirming the involvement of this motif in the interaction (Schaufele et al., 2005). Interestingly, in a recent study using the *Xenopus oocytes* as a model system, the AR N/C interaction was demonstrated to be involved in AR binding to chromatin, suggesting a novel role of this interaction in control of AR transcriptional activity (Li et al., 2006).

### *1.2.5. AR modifications*

AR undergoes several posttranslational modifications such as phosphorylation, acetylation, ubiquitination, and sumoylation (Poukka et al., 2000; Lin et al., 2002; Fu et al., 2004; Faus & Haendler, 2006). For instance, the NTD of AR is constitutively phosphorylated at Ser-94 and becomes phosphorylated at multiple additional sites in response to ligand binding (Gioeli et al., 2002). The kinases responsible for the phosphorylation of AR and the functional importance of AR phosphorylation have, however, not been established, although some studies suggest MAPKs (Mitogen Activated Protein Kinases) and Akt to play a role (Wen et al., 2000; Gioeli et al., 2006). Furthermore, a cyclin-dependent kinase 1 (CDK1) has recently been identified as an AR Ser-81 kinase. AR phosphorylation at Ser-81 increased AR protein expression and CDK inhibitors decreased not only AR Ser-81 phosphorylation, but also AR protein expression and transcriptional activity in prostate cancer cells (Chen et al., 2006). In addition, tyrosine phoshorylation induced AR activity and was elevated in hormone-refractory prostate tumors (Guo et al., 2006). The AR acetylation sites are clustered to a KXKK motif in the hinge region, and mutation of the lysine residues in this motif severely impairs AR function and delays nuclear translocation (Fu et al., 2000; Fu et al., 2002; Fu et al., 2004; Thomas et al., 2004). All steroid hormone receptors are subjected to ubiquitination, and some of the enzymes involved have been identified, although the exact sites have proven difficult to map (Faus & Haendler, 2006). A similar process to ubiquitination is sumoylation which leads to the covalent attachment of a SUMO chain onto a lysine residue in the consensus ψKxE motif (Seeler & Dejean, 2003). AR was the first steroid hormone receptor shown to be modified by SUMO, namely at K386 and

K520 (Poukka et al., 2000). The exact functional relevance of ubiquitination and sumoylation to AR function remains unclear. For an overview of post-translational modifications of steroid receptors, see (Faus & Haendler, 2006).

## 2. Nuclear Receptor Dynamics

Nuclear receptors mediate the action of their specific ligands through interaction with chromatin and the initiation of transcription of target genes. The identification of hormone responsive genes within different tissues and the subsequent molecular cloning of the steroid hormone receptors in the mid-1980s, followed by the expansion to a nuclear receptor superfamily, completed the initial characterization of the steroid hormone signaling pathway. This led to the classical model of nuclear receptor action where ligand-binding is followed by an allosteric change in receptor conformation which allows the receptor-ligand complex to translocate to the nucleus and bind high affinity sites in chromatin to regulate transcription (Yamamoto, 1985). The development of the chromatin immunoprecipitation assay (ChIP) enabled the study of NR binding to target promoters in cell culture models. These studies, together with more traditional biochemical studies on receptor-DNA interaction, built further upon the classical view of nuclear receptor action. According to this view, the nuclear receptors are stably associated with their target sites in chromatin for as long as the ligand is present, leading to the sequential recruitment of large transcriptional complexes (McKenna & O'Malley, 2002; Shang et al., 2002). The assembled protein complexes were thought to have long residence times on the DNA template, with changes in the composition of these complexes occurring on the time scale of minutes or hours. However, the ChIP technology is not sensitive enough to detect rapid protein movements, due to the need of fixation of the complete DNA/protein environment of the cell which takes time. Furthermore, the results represent the averaging of events across a cell population and cannot account for heterogenous cell responses. Therefore, there has been a need to develop new technologies to study NR-chromatin interactions in shorter time scales.

### 2.1. Hit-and-run model for nuclear receptor action

An alternative approach to study the dynamics of protein-chromatin interactions came with the advances in GFP (Green Fluorescent Protein) technology and quantitative live cell microscopy, allowing the visualization of protein dynamics in single living cells (Schaffner, 1988; Rigaud et al., 1991; McNally et al., 2000; Fletcher et al., 2002; Nagaich et al., 2004a; Nagaich et al., 2004b). Using this technology, a real time view of protein interactions with stable structures in live cells is possible. As chromosome movement is restrained in live cells (Marshall et al., 1997), it is possible by the use of photobleaching techniques, such as FRAP and FLIP, to characterize the interaction of a soluble transcription factor with the chromatin template. To specifically and visually study the interaction between NRs and their DNA response elements in chromatin, the regulatory sites must be amplified in the chromosome, creating a high density of binding sites, thus enabling the visualization of the GFP-tagged NR on its response element. This was first achieved with the establishment of a cell line with 200 copies of the steroid hormone receptor inducible MMTV (Mouse Mammary Tumor Virus) promoter stably integrated into the chromosome of a murine mammary adenocarcinoma cell line (McNally et al., 2000). The LTR (Long Terminal Repeat) of MMTV contains HREs to which steroid hormone receptors can bind specifically (see Figure 4), and the GFP-tagged receptor binding to a regulatory element can thus be observed by microscopy (McNally et al., 2000). For the use of such arrays, it is important to establish that the genes within the array behave similarly to normal, single copy sequences. For the MMTV array, the hormonal response of the MMTV promoters within the array have been rigorously characterized (Fragoso et al., 1998; Kramer et al., 1999). The position and extent of nucleosome remodeling in the amplified array was compared to that observed in low-copy and single-copy MMTV in chromatin which showed that the chromatin reorganization event summed over the individual promoter copies in the array is indistinguishable from the event averaged over many cells with single gene copies. Furthermore, the kinetics of receptor induced transcription observed in the array cells is also identical to that originally described in low copy cells (Archer et al., 1994; Smith et al., 1997).

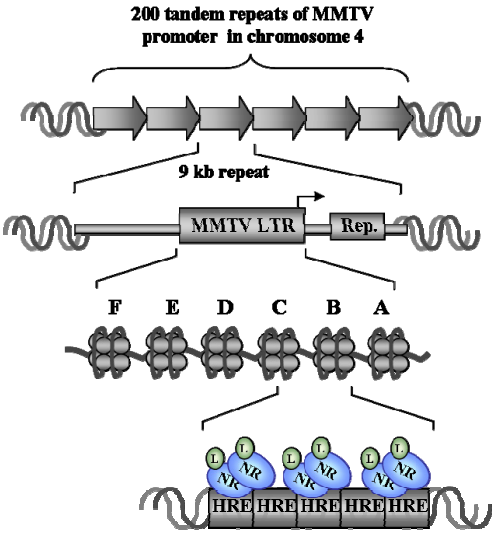


**Figure 4. Structure and organization of the MMTV array**
200 copies of a 9 kb repeat of the MMTV (Mouse Mammary Tumor Virus) promoter integrate into the host chromosome, creating an MMTV array. The MMTV Long Terminal Repeat (LTR) is characterized by a series of positioned nucleosomes (A-F) and liganded nuclear receptors (NR) can bind to hormone response elements (HREs) in the nucleosome B-C region, driving the transcription of a reporter gene (Rep).

Direct measurements of the residence time of GR on the MMTV promoter using FRAP and FLIP analysis demonstrated a very rapid and dynamic interaction between GR and chromatin, with the receptor only present at the template for a period of 10-20 seconds at a time (McNally et al., 2000). These unexpected results were in disagreement with the traditional view of a long-term and stable transcription initiation complex. However, similar high mobility of other transcription related factors has been demonstrated in the same (Becker et al., 2002; Rayasam et al., 2005) or similar systems (Stenoien et al., 2001a; Dundr et al., 2002; Agresti et al., 2005; Bosisio et al., 2006). This has led to the proposal of an alternative model for nuclear receptor action, called the hit-and-run model (see Figure 5). According to this model, the receptor transiently interacts with the promoter, recruits other factors, and is itself dynamically displaced from the promoter (for reviews, see e.g (Hager et al., 2004)). These confounding results and the resulting new model for NR action have given new insights into protein-movement in the nucleus

and their dynamic equilibrium with multiple targets in the nuclear compartment (Phair & Misteli, 2000; Misteli, 2001; Phair et al., 2004).

The “static” versus “dynamic” view on the development of transcriptional complexes on regulated promoters can be integrated in a model that provides a possible resolution of these two apparently opposing views (Hager et al., 2006; Metivier et al., 2006). It has been suggested that the initiating factor, e.g. a NR, exists in the nucleoplasm in different complexes with its coregulators. These complexes search for their binding sites by three-dimensional scanning of the genome, and then interact randomly and dynamically with response elements in target promoters (Phair et al., 2004). Most of these interactions are not productive, as the promoter must be in the appropriate state for the complex to initiate transcription. As chromatin, and also the cofactors themselves, are being modified, and other factors are being recruited, the stability of the complex is enhanced and may initiate transcription. ChIP analysis at varying times during this process would trap the complexes at a specific stage of promoter development giving the impression of a statically bound complex, although the actual dynamics of site occupancy are rapid (see illustration of this “return to template” model in (Hager et al., 2006)). The rapid cycling of factors on and off its template allows promoters to be activated very rapidly upon stimulation, an obvious advantage for efficient promoter function.

In addition to this dynamic cycling of factors on and off its regulatory element, detailed studies using ChIP analysis of ER responsive promoters have revealed a periodic cycling of ER and cofactors on the promoter over periods in the range of 15-45 minutes (Shang et al., 2000; Burakov et al., 2002; Metivier et al., 2003; Reid et al., 2003). Using an ultrafast UV crosslinking assay, a similar periodic binding and displacement of GR from its chromatin template was also observed in an *in vitro* system (Nagaich et al., 2004b). The underlying mechanisms and the significance of this cyclical behavior are at present unknown. It has been suggested that proteasome-mediated degradation of the receptor and phosphorylation of Pol II are important factors in this process (Reid et al., 2003).

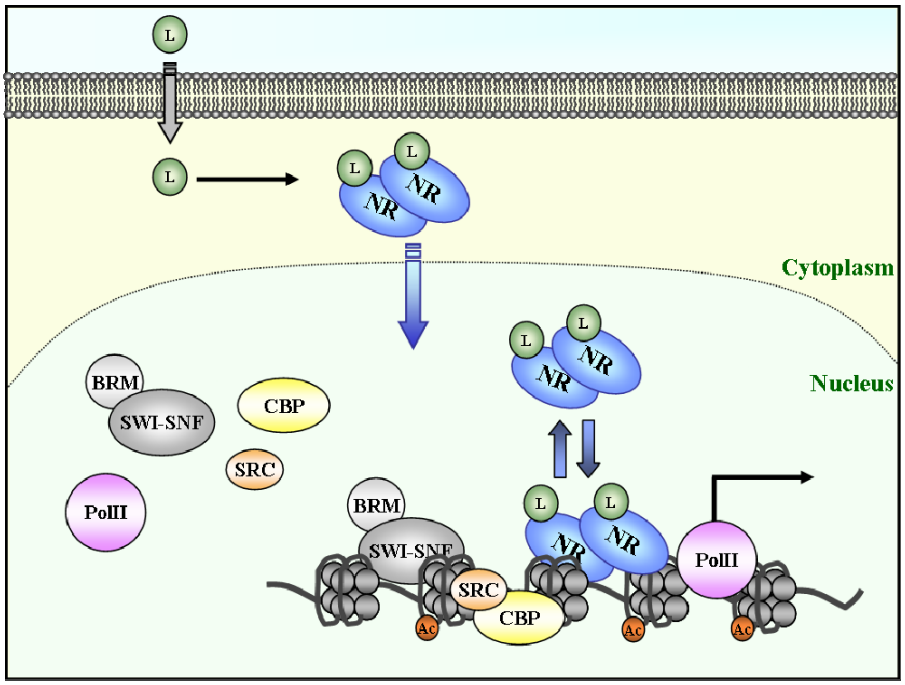


**Figure 5. Hit-and-Run model for nuclear receptor action**
Upon ligand-binding, the nuclear receptor (NR) is translocated to the nucleus where it is associated with target sites in the chromatin. Factors of the transcriptional machinery are recruited, including chromatin remodeling complexes (SWI/SNF), histone acetyl transferases (CBP), coactivators (SRC) and RNA Polymerase II (PolII). The chromatin is remodeled, allowing for more proteins to bind and a fruitful transcriptional initiation complex is established. NR is dynamically displaced (symbolized by the arrows) and shuttles between the chromatin-bound and free nucleoplasmic state.

### 2.2. Chromatin remodeling and chaperone dependency

In addition to highly dynamic protein-chromatin interactions, emerging evidence suggests that energy-dependent processes contribute significantly to the rapid movement of proteins in live cells, and to the rapid exchange of sequence-specific DNA-binding proteins with regulatory elements. This was demonstrated by a strong ATP-dependence on factor movement, as ATP-depletion inhibited protein movement in the nucleus, as well as site specific interactions with a template (Elbi et al., 2004; Stavreva et al., 2004;

Agresti et al., 2005). Two separate energy-dependent mechanisms have been implicated in transcription factor mobility: chromatin remodeling and chaperone dependency.

ATP-dependent chromatin remodeling complexes play essential roles in the regulation of transcription, DNA repair, cell cycle and development (Wallberg et al., 2000; Narlikar et al., 2002; Peterson, 2002; Gregory & Shiekhattar, 2004). According to the ATPase that forms the main component of the complex, they can be classified into three major types: SWI/SNF, ISWI, and Mi-2. The mammalian SWI/SNF complex is probably the best described, and was first identified in yeast (Peterson et al., 1994). It contains one of two ATPases, BRG1 or BRM, and several BRG1-associated factors. Even though BRG1 and BRM are highly homologous ATPases, they can play roles in very different cellular pathways through selective association with certain coregulatory proteins (Hsiao et al., 2003; Kadam & Emerson, 2003; Salma et al., 2004). Although BRG1 has been shown to be the preferred ATPase for GR-induced (Fryer & Archer, 1998) and PR-induced (Mymryk & Archer, 1995) chromatin remodeling, a strong dependence for BRM as the core ATPase for AR activity has been demonstrated (Marshall et al., 2003). SWI/SNF is recruited to the AR transcription site via the histone acetyl transferases (HATs) CBP and p300. Although histone acetylation enhances the recruitment of SWI/SNF, it is not required for SWI/SNF-induced chromatin remodeling. However, both SWI/SNF remodeling activity and CBP/p300 HAT activities are required for hormone dependent activation. Hence, there is not only direct recruitment by NR's, but also cofactor-cofactor, and cofactor-histone interactions occurring at the active transcription site (Huang et al., 2003).

Receptor mobility is also dependent on the presence of chaperone proteins. Unliganded GR, PR and AR usually reside in the cytoplasm in complex with several chaperones, such as certain heat shock proteins (HSPs). It is thought that chaperones are important for the insertion of the steroid ligand into the hydrophobic environment of the receptor LBD (Pratt et al., 1996). Other functions of molecular chaperones were demonstrated by Elbi et al., using digitonin to permeabilize cells such that much of the free cytoplasmic protein of the cells is released (Elbi et al., 2004). Due to their size, GFP-labeled receptors (GR

and PR) were retained in the cytoplasm enabling the study of their motility by FRAP analysis. Under conditions where the cells were permeabilized, the steroid receptors were totally immobile, but this loss was in large part recovered when a cocktail of seven chaperone proteins was added to the cells (Elbi et al., 2004). This process was also completely ATP-dependent. These results suggest a more general role of chaperones in NR action: chaperones are not only required for ligand assimilation but also the movement of receptor within the nucleus.

### 2.3. Histone acetylation

In addition to chromatin remodeling complexes, enzymes that catalyze posttranslation modifications of histones also regulate the accessibility of promoters to the transcription and replication machinery (Berger, 2002). Changes in the multiple modifications of the N-terminal tails of histones can control chromatin packaging and create binding-sites for chromatin-associated proteins (Jenuwein & Allis, 2001; Fischle et al., 2003). Several different covalent modifications of histones have been identified: acetylation (of lysine residues), methylation (of lysine or arginine residues), phosphorylation (of serine residues) and ubiquitination (of lysine residues) (for review see (Berger, 2002)). Histone modification and ATP-dependent chromatin remodeling are functionally connected for gene regulation, although it is unclear whether there exists an actual mechanistic interrelationship between them. Promoters are usually envisioned to be in either a non-accessible off-state, or in a more accessible on-state allowing gene transcription. However, it now seems that genes pass through a continuum of activity states, and the evolution of these states can be quite complex (reviewed in (Hager et al., 2006)).

Histone acetylation is one of the most well studied histone modifications. In general, histone acetylation induces transcription by converting chromatin from a low-acetylated, ‘closed’ form, to an acetylated, ‘open’, more accessable form (Verdone et al., 2005). The key observation to support this view was that several promoter-associated coactivators possessed HAT activity, suggesting that HAT activity was important for transcriptional activation (Kuo & Allis, 1998). Several enzymes with HAT activity have been identified,

many of which are components of large multisubunit complexes, recruited to promoters by interaction with DNA-bound activator proteins.

HAT activity is required for optimal AR activity. CBP, a well described histone acetyltransferase, was identified as a coactivator for AR, and the overexpression of CBP was also able to rescue the activity of transcriptionally compromised AR mutants (Fronsdal et al., 1998). Furthermore, AR agonists and antagonists exhibit differences in their ability to promote recruitment of HAT complexes to promoters, indicating that receptor-binding to chromatin is followed by histone modifications (Kang et al., 2004). In support of this, it was demonstrated by ChIP analysis that both CBP and the related p300 were recruited to the promoter and enhancer of PSA gene by agonist-bound AR (Shang et al., 2002; Wang et al., 2005b). However, in the presence of the antagonist bicalutamide, CBP was not recruited, confirming the important role of HAT activity in AR transcriptional activation (Shang et al., 2002). Other AR coactivators possessing HAT actvtivity include SRC-1 and SRC-3 (also called AIB1, pCIP, and TRAM1) that interacts with CBP (Liao et al., 2002), and PCAF (p300/CBP associated factor), also involved in interaction with p300/CBP and in the acetylation of non-histone targets such as various transcription factors and also AR itself (Fu et al., 2000).

### *2.3.1. HDAC inhibitors*

Enzymes called Histone Deacetylases (HDACs) function in opposition to HATs by deacetylating histone tails. In general, HDACs create a “closed”, non-accessible form of chromatin, inhibiting transcription of many genes, and are commonly associated with transcriptional repression (reviewed in (Marks et al., 2003)). At present, there are eleven identified HDACs in humans which can be divided into four classes based on sequence homology to yeast HDACs: class I (HDAC 1, 2, 3, and 8), class II (HDAC 6 and 10), class III (HDAC 4, 5, 7, and 9), and class IV (HDAC 11). The global chromatin acetylation status is dependent upon the correct equilibrium between HAT and HDAC activity. Genetic abnormalities in HAT/HDAC genes may cause an inbalance in chromatin acetylation status resulting in repression of, e.g. cell cycle control genes, or overexpression of oncogenes, which may promote tumorigenesis and cancer. If the

inbalance in histone acetylation is a result of inhibited HAT activity or increased HDAC activity, HDAC inhibitors (HDACis) may restore this balance and thereby block tumor cell proliferation. A variety of agents, both natural and synthetic, with HDACi activity have been discovered, and can be divided into five main classes: short-chain fatty acids, hydroxamic acids, electrophilic ketones, cyclic tetrapeptides, and amino benzamides (see Table 2). Genes silenced in cancer cells, such as many tumor suppressor genes, seem to be especially sensitive to HDACis. Interestingly, HDACis are growth suppressive and apoptotic only in transformed cells. They act very selectively, and alter the transcription of fewer than 2% of expressed genes. Many HDACis have therefore been explored for potential anti-cancer activity, and some of these are in clinical trials for cancer treatment (reviewed in (Monneret, 2005; Gallinari et al., 2007)).

Although the general effect of HDACis is to increase acetylated chromatin and the resulting activation of several genes, there are several examples where HDACs appear to be required for gene activation, and HDACis then actually repress gene transcription (Lallemand et al., 1996; Siavoshian et al., 2000; Laribee & Klemsz, 2001; Ferguson et al., 2003; Qiu et al., 2006). The effect of HDACis may also be dependent on the promoter and transcription factor context. This is exemplified by the MMTV promoter, at which the GR activity is inhibited upon treatment with the HDAC inhibitor TSA, in contrast to AR which is activated (List et al., 1999a; List et al., 1999b).

### *2.3.2. HDAC inhibitors in prostate cancer*

A number of HDACis have proved to have antiproliferative effects in cultured human prostate cancer cells and in mouse xenograft models. The mechanisms by which these inhibitors exhibit their antiproliferative effect vary widely among the inhibitors. The hydroxamic acid pyroxamide caused growth inhibition through cell cycle arrest in prostate cancer cells, and inhibited the growth of the CWR22 prostate cancer xenografts (Butler et al., 2001), as did also its analogue SAHA (Butler et al., 2000), both with relatively low toxicity.

**Table 2. Natural and synthetic HDAC inhibitors and their properties**

| HDAC inhibitor | Type | Activity | Clinical trials | References |
|---|---|---|---|---|
| AN-9 (pivaloyloxymethyl butyrate) | Short-chain fatty acid | H, C, A | Phase I/II | (Zimra et al., 1997; Reid et al., 2004) |
| CI-994 | Synthetic benzamide derivate | H, C, A | Phase I | (LoRusso et al., 1996; Loprevite et al., 2005) |
| Depsipeptide (FK228/FR901228) | Natural (bacterial) cyclic tetrapeptide | H, C, A | Phase II | (Furumai et al., 2002; Piekarz et al., 2006) |
| LAQ-824 | Synthetic hydroxamic acid derivate | H, C, A | Phase I | (Catley et al., 2003; Kato et al., 2007) |
| MS-275 | Synthetic pyridyl carbamate derivative | H, C, A | Phase II | (Lee et al., 2001; Gojo et al., 2006) |
| Na-Butyrate | Short-chain fatty acid | H, C, A | Phase I/II | (Prasad, 1980; Newmark et al., 1994) |
| Na-Phenylbutyrate | Short-chain fatty acid | H, C, A | Phase I | (Gore et al., 2002; Camacho et al., 2007) |
| PXD101 | Synthetic hydroxamic acid derivate | H, C, A | Phase I | (Plumb et al., 2003; Qian et al., 2006) |
| Pyroxamide | Hydroxamic acid | H, C, A | - | (Butler et al., 2001) |
| Suberoylanilide hydroxamic acid (SAHA) | Synthetic hydroxamic acid | H, C, A | Phase II | (Vrana et al., 1999; Ruefli et al., 2001; Duvic et al., 2007) |
| Trapoxin | Natural (fungal) cyclic tetrapeptide | H, C | - | (Kijima et al., 1993) |
| Tributyrin | Short-chain fatty acid | H, C, A | Phase I | (Chen & Breitman, 1994; Conley et al., 1998) |
| Trichostatin A (TSA) | Natural (fungal) hyroxamic acid | H, C | - | (Yoshida et al., 1987; Yoshida et al., 1995) |
| Valproic acid | Short-chain fatty acid | H, C, A | Phase I/II | (Gottlicher et al., 2001; Kuendgen et al., 2005) |

H-inhibits purified HDAC
C-inhibits growth of transformed cells
A-inhibits *in vivo* tumor growth in animal models

Valproic acid, another hydroxamic acid, inhibited prostate cancer cell growth, *in vitro* and *in vivo*, by inducing apoptosis (Angelucci et al., 2006; Xia et al., 2006). In a similar manner, sodium butyrate and TSA synergize with 1,25-(OH)-vitamin D3 to inhibit the growth of LNCaP, PC-3 and DU145 by inducing apoptosis (Rashid et al., 2001). The short chain fatty acid phenylbutyrate inhibited the invasive properties of prostate cancer cells (Dyer et al., 2002) and inhibited prostate cancer cell and xenograft proliferation through cell cycle arrest and induction of apoptosis (Melchior et al., 1999). The cyclic tetrapeptide depsipeptide (FK228) inhibited prostate cancer cell growth *in vitro* and *in vivo*, through the effect on the expression of angiogenesis factors (Sasakawa et al., 2003a; Sasakawa et al., 2003b). Possibly the most promising current HDACi is SAHA, which at doses without detectable toxicity, reduced tumor growth by 97% in mice transplanted with CWR22 human prostate tumors (Butler et al., 2000). It is also the most advanced HDACi in clinical trials, with meaningful clinical responses in patients with different types of cancer (Gallinari et al., 2007). However, there is at present no HDACi in clinical trials for prostate cancer.

## 3. Androgens in Prostate Cancer

Even before the discovery of testosterone, it was very well known that there was a strong dependency between the testes and the prostate. As early as 1895, reports showed the inverse correlation between prostate size and castration in elderly men. After the isolation of testosterone in 1934, Huggins and Hodges demonstrated that androgens, secreted from the testes, are important for the development and growth of prostate cancer (Huggins, 1941). It is now clear that androgens have a critical role in the development and maintenance of the male reproductive system and have roles in physiological and pathological conditions, including the normal prostate and prostate cancer (reviewed in (So et al., 2003; Karayi & Markham, 2004)).

### 3.1. Androgens in prostate biology

The prostate is an exocrine gland of the male mammalian reproductive system. Its main function is to store and secrete a clear, slightly basic fluid that constitutes up to one-third

of the volume of semen. Some of the proteins contained in the prostate secretion helps liquefy the semen. However, the specific function of the prostate gland is still unknown, and it is in fact the largest organ of the human body of unknown specific function (Isaacs, 1994). In the fetus, testosterone stimulates budding of the prostate epithelium from the urogenital sinus and signals the differentiation and growth of the prostate gland. If sufficient levels of testosterone are not present, the prostate gland does not develop. The prostate remains small (1-2 grams) until puberty when it grows to its adult size of approximately 20 grams. This period of exponential growth between the age of 10-20 years is the same period when serum testosterone levels are rising from the initial low levels to the high levels seen in the adult male (Isaacs, 1994). When the adult size of the prostate is reached, there is normally no more net growth of the gland.

The normal adult prostate gland shows a high degree of cellular organization, and is composed of a glandular epithelial and a fibromuscular stroma compartment. The epithelial compartment is made up of two major morphologically distinct cell types: the luminal and basal cells. Luminal cells tend to be differentiated and androgen dependent, with a relatively low proliferative capacity and high apoptotic index, while the basal cells generally appear undifferentiated and androgen independent, with high proliferative capacity and low apoptotic index, attributes characteristic of stem cells. The prostate epithelium has also a third cell type, the neuroendocrine cells, which are scattered at low percentage throughout the gland. In addition, a transiently proliferating/amplifying cell population, serving as an intermediate between the undifferentiated stem cells of the basal layer and the highly differentiated exocrine, and also neuroendocrine, cells of the lumen has been proposed (Isaacs & Coffey, 1989).

### 3.2. Prostate carcinogenesis

Androgen levels increase in puberty, resulting in a net growth of the prostate until it reaches its maximum adult size around the age of 20. After this age, the prostate normally ceases its continuous net growth, and androgens regulate the total number of prostatic cells by stimulating the rate of proliferation and at the same time inhibiting cell death/apoptosis (Isaacs, 1994). An inbalance in this regulation, either by increased

proliferation or inhibited apoptosis, may give rise to an abnormal growth of the prostate, eventually leading to prostate cancer.

Carcinoma of the prostate is the most frequently diagnosed non-cutaneous malignancy in men. It accounts for one third of all cancers diagnosed and it is the third leading cause of cancer-related death in men in western industrialized countries (Jemal et al., 2007). Prostate cancer is predominantly a disease of elderly men, with a steeply increasing incidence in the 7$^{th}$ decade of life. The recently observed rise in incidence of prostate cancer may, therefore, partly be explained by an ageing population. However, the age-adjusted incidence has also increased, hence other factors such as genetic disposition, life style and diet are probably also important factors (Parkin et al., 2001). There is a striking difference in prostate cancer risk between ethnic groups, with a more than 10-fold higher incidence of prostate cancer in Western industrialized countries compared to East Asian countries (Quinn & Babb, 2002b; Quinn & Babb, 2002a). Furthermore, in the United States, the risk of prostate cancer is approximately 60% higher in African-American than in European-American men and the comparative mortality rate is more than twice as high (Powell, 2007). However, immigrant studies have demonstrated that genetic disposition can only account for some of this difference, suggesting that other factors, such as life style and diet, are important (reviewed in (Jankevicius et al., 2002)). In contrast to the increasing incidence of prostate cancer, the mortality rate has declined since the early 1990s, possibly due to the use of PSA (Prostate Specific Antigen) screening leading to earlier diagnosis and treatment (see also paragraph 4.3). However, it is still debatable if the decline in mortality rate is actually a consequence of PSA screening (Constantinou & Feneley, 2006). Another reason could be a mis-certification of cause of death in a large group of men in the 1980s-1990s (Feuer et al., 1999).

During the progression of prostate cancer (see Figure 6), the prostate retains some of its glandular structure and is therefore classified as adenocarcinoma. The first detectable morphological change in the development of prostate cancer is considered to be prostatic intraepithelial neoplasia (PIN). PIN may occur in men in their twenties (Isaacs, 1994), and can be detected histologically by thickening of the epithelial layer, and also loss of

distinct basal and secretory layers. The progression of the disease is slow, and clinically detectable prostate cancer does not typically arise until the sixth decade. The carcinoma is firstly confined to the prostate, but about one third of prostate tumors become locally invasive, spreading beyond the tissue capsule, and finally developes into metastatic disease (Isaacs, 1994). The most frequent metastatic sites for prostate carcinomas are bone, liver and lung, and the metastases usually appear undifferentiated. Most prostate cancer tumors regress upon initial androgen depletion therapy; however, the tumors in most cases recur in an androgen independent state for which there is no efficient therapy at present. The molecular mechanisms of transition from androgen-dependence to androgen-independence remain poorly understood, although it appears that AR signaling remains important throughout the course of the disease (Balk, 2002; Chen et al., 2004). For the last decade, there has been a major research focus on the molecular mechanisms of this transition which is crucial for the development of effective therapies (for reviews, see (Feldman & Feldman, 2001; Navarro et al., 2002; Agoulnik & Weigel, 2006)).

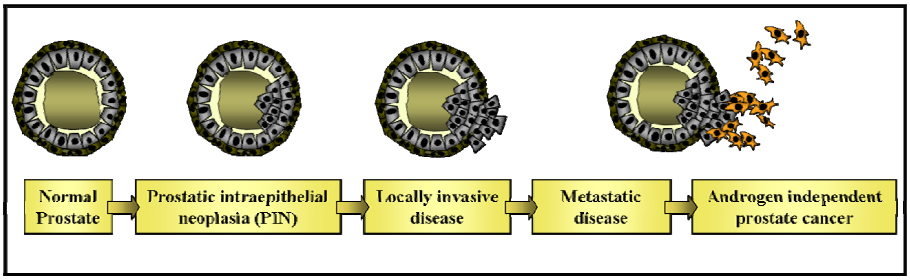


**Figure 6. Prostate cancer progression**
The epithelium of the prostate gland is composed of luminal cells (grey) oriented towards the lumen of the gland, basal cells (brown) and neuroendocrine cells scattered throughout the gland. In prostatic intraepithelial neoplasia (PIN), the cells start to proliferate towards the lumen of the gland, until the tissue capsule breaks and the tumor becomes locally invasive. The tumor then progresses to a metastatic state spreading to distant organs, and then finally to an androgen-independent state.

### 3.3. Anti-androgens in prostate cancer treatment

The initial treatment of prostate cancer is usually radical prostatectomy or radiation to remove or destroy the cancerous cells that are still confined within the prostate capsule. However, many patients are not cured by this treatment and their cancer recurs, or the patient may not have been diagnosed until after the cancer has spread beyond the tissue capsule (Pirtskhalaishvili et al., 2001). The first systemic therapy for advanced prostate cancer emerged in 1941 with the discovery that surgical or medical castration, leading to a reduction in the levels of circulating androgens, caused regression of prostate tumors (Huggins, 1941). This induced reduction in androgen-levels is called androgen ablation therapy, which still is the only successful treatment for advanced prostate cancer. There are several types of androgen ablation therapy: surgical castration, medical castration using LHRH analogues, anti-androgen monotherapy, and maximum androgen blockade (MAB) which is a combination of castration and anti-androgen administration. Surgical and medical castration lowers the levels of free testosterone in the circulation; however, some testosterone (~5%) still remains since androgens are also produced by the adrenal cortex, which is not affected by the treatment, and anti-androgens are then used to block the function of the remaining androgens (Pirtskhalaishvili et al., 2001; Anderson, 2003). Recently, monotherapy with anti-androgen alone have proved to be an attractive alternative to castration as it results in less severe side-effects (reviewed in (Anderson, 2003)). Table 3 lists anti-androgens that were previously or are currently used in the treatment of advanced prostate cancer. The non-steroid antagonist bicalutamide is at present probably the most favorable anti-androgen used in prostate cancer therapy (Anderson, 2003; Miyamoto et al., 2004).

**Table 3. Anti-androgens previously and/or currently used in prostate cancer treatment**

| Anti-androgen | Trade name | Type |
|---|---|---|
| Cyproterone Acetate (CPA) | Androcur, Climen, Diane 35, Ginette 35 | Synthetic steroid, partial antagonist |
| Bicalutamide | Casodex | Non-steroid, pure antagonist |
| Flutamide | Eulexin | Non-steroid, pure antagonist |
| Nilutamide | Nilandron | Non-steroid, pure antagonist |

### 3.4. Prostate cancer models

*In vitro* cell culture is one of the most commonly used models in cancer research. For the study of prostate cancer, the androgen-sensitive cell line LNCaP is the most widely used model system (Horoszewicz et al., 1980). This cell line is cultured from a lymph node metastasis of a white Caucasian man. LNCaP cells express AR, but with a T877A mutation in the LBD which renders it more sensitive to a wider range of steroid ligands than wild type AR (Veldscholte et al., 1990; Veldscholte et al., 1992; Tan et al., 1997). Some androgen non-responsive prostate cancer cell lines, such as PC-3 (bone metastasis) (Kaighn et al., 1979) and DU145 (brain metastasis) (Stone et al., 1978) are also widely used. Other cell lines are also available, but many of these have proved to be either derivatives of the three mentioned cell lines, or of other non-prostatic cell lines, or are not freely available (van Bokhoven et al., 2003). The available cell lines also do not span the range of prostate cancer phenotypes. Primary cultures, of both malignant and normal epithelial prostate cells, are therefore also necessary. Technical improvements over the last decades have made the use of primary cultures more widespread, and there are now several primary cultures of human prostatic cells, representing the different stages of prostate cancer, but some hurdles remain for their routine use (for review, see (Peehl, 2005)). Xenografts derived from human prostate cancer cell-lines, is another means of obtaining *in vivo* models for human prostate cancer. At present, various xenograft models representing the various stages of clinical prostate cancer, and also in some cases cell lines established from these, are available (reviewed in (van Weerden & Romijn, 2000)).

Animal models, mainly mouse and rat, are widely used tools in cancer research. Despite the obvious anatomical differences between the mouse and human prostate, several mouse models have been developed which recapitulate many features of human prostate cancer (reviewed in (Abate-Shen & Shen, 2002)). The most commonly used model is the TRAMP (transgenic adenocarcinoma mouse prostate) mice, expressing SV40 viral oncogenes specifically in the prostate driven by the rat probasin promoter (Greenberg et al., 1995). The TRAMP mice develop high-grade PIN and/or prostate cancer within 12 weeks of birth, and also ultimately develop metastases by 30 weeks. Androgen depletion results in decreased tumor incidence, as well as the subsequent appearance of androgen-

independent disease (Gingrich et al., 1997). Thus, the TRAMP mice recapitulate many aspects of human prostate cancer and have given significant insights into the molecular mechanisms of prostate cancer development and progression. Other transgenic and knock-out mouse models have also been developed (for a review, see (Abate-Shen & Shen, 2002)). Especially useful knock-out models for prostate cancer have been the NKX3.1 (Bhatia-Gaur et al., 1999) and PTEN (Di Cristofano et al., 1998) models. Human NKX3.1 is localized to chromosomal region 8p21, a region which undergoes loss-of-heterozygosity (LOH) in ~80% of prostate cancers (He et al., 1997). However, there are discrepancies in the literature about how or if NKX3.1 expression is changed during prostate cancer progression (Bowen et al., 2000; Xu et al., 2000; Ornstein et al., 2001; Korkmaz et al., 2004b; Bethel et al., 2006); thus, it is under debate if the NKX3.1 gene is actually lost in the Chr8p21 deletion. PTEN maps to chromosomal region 10q23, a region that also undergoes LOH at advanced stages in many cancers, including prostate cancer (Di Cristofano & Pandolfi, 2000). The cooperativity between loss of NKX3.1 and PTEN has also been studied, and was shown to be restricted to the prostate, and importantly, the knock-out mice displayed carcinoma lesions that resemble early stages of human prostate cancer (Kim et al., 2002).

Under physiological conditions, cancer cells reside histologically as three-dimensional organoids, and the host microenvironment is known to be pivotal to malignant progression of the cancer cells (Chung et al., 2005). The established *in vitro* models do not fully recapitulate the prostate tumor environment, and further insight into cancer biology and therapy requires new and improved research models. Recently, several 3D co-culture models for the study of prostate cancer growth have been developed (reviewed in (Wang et al., 2005c)). Formation of human prostate tissue from embryonic stem cells which shows species–conserved signaling mechanisms, was also recently achieved (Taylor et al., 2006). These model systems may prove useful for studies of human prostate development and maturation and may give insights into mechanisms of prostate carcinogenesis.

### 3.5. Prostate cancer biomarkers

As the treatment options for advanced prostate cancer are limited, early detection of the disease is essential. A biomarker that allows for the detection of prostate cancer at an early stage is therefore of significant importance, and much effort has been invested in the search for prostate specific molecules that might serve as cancer biomarkers or as therapeutic targets. AR regulated genes have been of special interest, which led to the discovery of PSA (Prostate Specific Antigen), a widely used biomarker for prostate cancer (see paragraph 4.3). Although increased levels of PSA are correlated with risk of prostate cancer, PSA has its limitations both for the detection and grading of prostate cancer due to high rates of false positive and negatives, and therefore other more specific markers are needed for the improved diagnosis and monitoring of disease progression. In this regard, other AR target genes have been of interest and the advances in microarray technology over the last decade have accelerated the insight into AR-mediated gene expression programs (see e.g. (DePrimo et al., 2002; Nelson et al., 2002; Nantermet et al., 2005; Asirvatham et al., 2006)). The majority of large-scale expression studies have been performed in LNCaP cells, and gene expression profiling studies have revealed that 1.5% to 4.3% of the LNCaP transcriptome is either directly or indirectly regulated by androgens (for a review, see (Dehm & Tindall, 2006)). How these changes translate to the protein levels in most cases remains unclear, and is a subject of future research.

## 4. Human Tissue Kallikreins

The human tissue kallikreins (KLKs) is a family of proteins primarily expressed in the glandular epithelia of many organs, also the prostate. Their transcription is in many cases regulated by sex steroid hormones, which are involved in the development of several endocrine-related tumors. The most well studied member of this family is KLK3 or PSA, which is, as already described, a commonly used marker for prostate cancer. Other members of this family are also under scrutiny as potential biomarkers for prostate cancer and other endocrine-related cancers (reviewed in e.g. (Borgono & Diamandis, 2004)).

### 4.1. The human tissue kallikrein locus

The human KLKs form a family of 15 closely related serine proteases encoded by conserved genes tandemly located in a large gene cluster (320 kb) on chromosome 19q13.4 (Figure 7). The first three members of the family, KLK1 (tissue kallikrein), KLK2, and KLK3 (PSA) were long thought to be the only members of the family. However, during the last decade the availability of human genome sequences and extensive screening of the KLK locus has revealed the presence of 12 additional KLK genes (Riegman et al., 1992; Gan et al., 2000; Clements et al., 2001). This gene cluster represents the largest cluster of contiguous protease genes in the human genome (Puente et al., 2003; Yousef et al., 2003). The human KLK locus has its rodent counterpart with a cluster of 28 functional genes in mouse (Evans et al., 1987; Olsson & Lundwall, 2002) and 10 functional genes in rat (Southard-Smith et al., 1994), and tissue KLKs have to date been identified in six mammalian orders. The significance of the different numbers of KLK genes in the different organisms is currently not known. Alternative splicing is prevalent within the human KLK locus, a trait not observed with rodent genes, and alternative splice variants have been described for all but one (KLK14) of the KLK genes (reviewed in (Kurlender et al., 2005)).

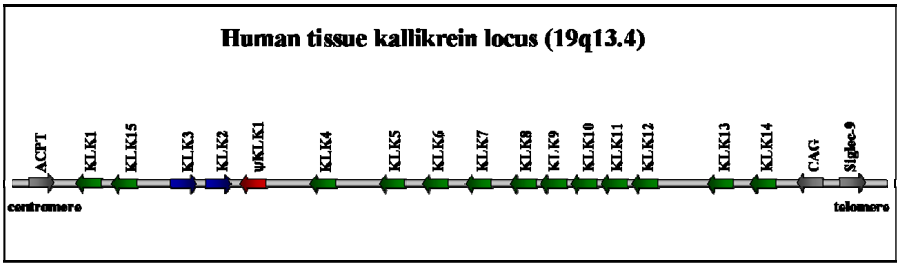


**Figure 7. The human tissue kallikrein gene locus**
Position and orientation of the kallikrein genes KLK1-KLK15 in the KLK gene locus at Chr19q13.4, with genes with telomeric to centromeric orientation in green, centromeric to telomeric orientation in blue, and pseusogenes in red. Non-kallikrein genes are in grey.

### 4.2. Kallikreins as cancer biomarkers

With the complete description of the human KLK locus, the main research effort is now centered around the elucidation of potential biological functions of the KLKs. The KLK genes encode putative serine proteases, with a conserved catalytic triad made up of histidine (H), aspartic acid (D) and serine (S), giving trysin- or chymotrypsin-like specificity. *In vitro* studies have shown that some KLKs can auto-activate while others can activate each other, suggesting that the KLKs may be part of an enzymatic cascade (Yousef & Diamandis, 2002; Borgono & Diamandis, 2004). Serine proteases play key roles in diverse physiological processes and vary widely with respect to substrate specificity (Rawlings & Barrett, 1993). The KLKs are expressed in a wide range of tissues, suggesting a functional role in diverse physiological and pathophysiological processes, including skin desquamation and other skin diseases, tooth development and enamel defects, Alzheimer's disease, and Parkinson's disease, in addition to several cancers. Hence, many members of the KLK family have shown potential as diagnostic or prognostic markers, especially in hormone dependent cancers such as prostate, breast, testicular and ovarian cancer (for reviews, see (Diamandis & Yousef, 2002; Borgono & Diamandis, 2004; Clements et al., 2004; Paliouras et al., 2007)). It has been suggested that KLKs might promote or inhibit cancer cell growth, angiogenesis, invasion and metastasis by activation of growth factors and other proteases, release of angiogenic or anti-angiogenic factors, and degradation of the extracellular matrix (ECM) (reviewed in (Borgono & Diamandis, 2004)). As KLKs possibly promote tumor growth through their proteolytic activity, the design of KLK inhibitors that may have potential in anticancer therapies is under development. For instance, highly specific serpins to KLK2 have been designed which displayed unique reactivity to KLK2 (Cloutier et al., 2004). Further research is required to reveal the functional roles of KLKs in various tissues and determine whether they have clinical utility as biomarkers for disease states, and possibly also as therapeutic targets.

### 4.3. Prostate Specific Antigen (PSA)

PSA is a widely used clinical tumor marker for detection and monitoring of prostate cancer progression (Stamey et al., 1987; Partin et al., 2002; Stephan et al., 2002). PSA is

produced at very high concentrations by the prostate gland, and it is secreted into the seminal plasma at a concentration from 0.5 to 5 mg/mL under normal physiological conditions. PSA degrades the seminal vesicle proteins semenogelin I and II and aids in the liquefaction of the semen, an event that is integral to sperm motility (Ban et al., 1984; Lilja, 1985; Lilja et al., 1989). Prostate cancer and physical trauma to the prostate, resulting in the perturbation of the prostate gland, can result in significant rise in the PSA concentration of the blood. Thus, elevated PSA levels are used as a marker for prostate gland abnormalities (Stephan et al., 2002; Lilja, 2003).

The advantages of using PSA as a prostate cancer marker is that it is secreted and enters the circulatory system, allowing easy detection of PSA in patient's serum samples. In addition, virtually all primary prostate tumors maintain PSA expression. However, there are some problematic issues concerning the use of PSA as a marker for prostate cancer. Due to PSA expression in benign prostatic hyperplasia (BPH), it is difficult to discriminate between BPH and prostate cancer, resulting in a high level of false positives and unnecessary biopsies (Barak et al., 1989; Drago et al., 1989). Another problem is that PSA can fail as a marker for residual disease since not all metastases maintain PSA expression (Sissons et al., 1992; Daher & Beaini, 1998; Constantinou & Feneley, 2006). Thus, the necessity to find additional markers for prostate cancer still remains.

In addition to PSA, another gene of this family that is androgen regulated and highly enriched to prostate for expression is KLK2, which may also have utility as a prostate cancer marker in conjunction with PSA (Rittenhouse et al., 1998; Stenman, 1999; Stephan et al., 2005). KLK2 is of particular interest in the discrimination of benign and malignant disease when the PSA levels are low, and between locally advanced and organ-confined prostate cancer (Haese et al., 2005). Yet another member of the family, KLK4, has more recently been identified as prostate-specific and androgen regulated, and is described in more detail below.

### 4.4. Kallikrein 4 (KLK4)

KLK4 was first cloned in 1999 by different approaches and is also known as prostase, KLK4-L1, PRSS17 and ARM-1 (Nelson et al., 1999; Stephenson et al., 1999; Yousef et al., 1999; Korkmaz et al., 2001). The KLK4 gene has the typical kallikrein gene structure, with five exons and four introns (Nelson et al., 1999; Stephenson et al., 1999) (see Figure 8A). Initial computer analysis of the gene predicted a transcript encoded by all five exons, which would be translated into a pro-KLK4 of 254 amino acids (aa), with a 26-aa signal peptide that would result in an active protein of 224 aa after cleavage of the pro-piece. However, extensive screening of cDNA libraries and RACE analysis did not permit the cloning of a 5'-extension with the putative first exon (Korkmaz et al., 2001). By the use of reverse transcriptase-PCR of mRNA from the prostate cancer cell line LNCaP and the androgen-dependent prostate cancer xenograft CWR22, the vast majority of KLK4 mRNA was found to have only four coding exons (Korkmaz et al., 2001). This transcript would thus give rise to a protein lacking the signal peptide that normally targets the protein for secretion, and was therefore the first member of the kallikrein family that was predicted to be intracellularly localized.

The KLK4 gene gives rise to 8 different mRNA forms through alternative splicing and/or alternative transcription start sites and expected to give rise to at least 7 different protein moieties (reviewed in (Kurlender et al., 2005)). It is at present not clear which of these transcripts are most relevant to prostate cancer. There have been some reports suggesting to prove the secretion of KLK4, however with questionable validity. Based on a KLK4-specific immunoassays, it was claimed that KLK4 is secreted into biological fluids (Obiezu et al., 2002; Obiezu et al., 2005). However, the specificity of the antibodies used in these studies was not verified, it was for instance not demonstrated that the antibodies detect endogenous KLK4 in prostate cancer cells. Furthermore, sample numbers are not high enough for proper statistical analysis. Another study reports the secretion of KLK4 based on the presence of KLK4-specific antibodies in prostate cancer patient sera (Day et al., 2002), which is only indicative of the presence of secreted KLK4. Importantly, they were also not able to detect endogenous KLK4 in LNCaP cells, questioning the specificity of the antibody used. A more recent report analyzed the compartmentalized

expression of endogenous KLK4 in prostate cancer cell lines and prostate tissue, and found that the full-length KLK4 transcript and the exon-1 deleted transcript are expressed in prostate cancer, resulting in a cytoplasmic and a nuclear form of the KLK4 protein, respectively (Dong et al., 2005), confirming the presence of a nuclear KLK4 as proposed (Korkmaz et al., 2001). Although these data suggest that KLK4 may be expressed in two major isoforms (see Figure 8), more extensive analysis is required for the determination of which forms of KLK4 are expressed, and their relative importance, in prostate cancer.

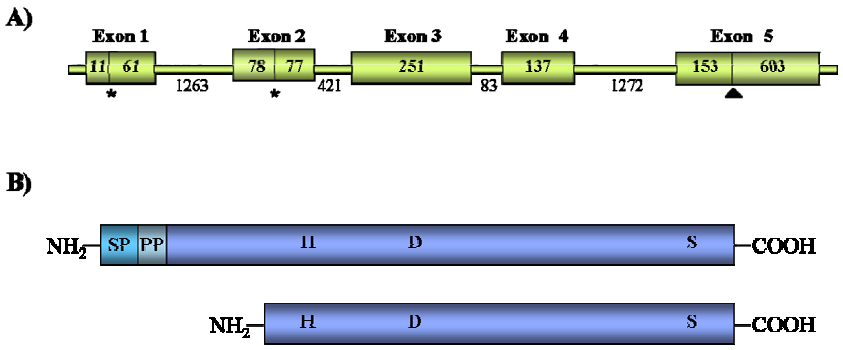


**Figure 8. Structure of the KLK4 gene and two KLK4 protein isoforms**
(A) Exon/intron organization of the KLK4 gene. Two translational start sites (*), and the stop codon is indicated (arrow). (B) Structure of the two KLK4 protein isoforms detected in prostate cancer, encoded by the full-length KLK4 transcript (upper) and the exon 1-deleted transcript (lower). The signal peptide (SP) and pro-piece (PP) of the secreted KLK4 is given. The positions of the three amino acids of the conserved catalytic triad (H, D, S) are indicated.

The biological function of human KLK4 is at present unknown. Strong evidence suggests that the murine and porcine KLK4 is involved in the regulation of enamel matrix protein processing and further function in defining structure and composition of enamel (Hu et al., 2002; Simmer & Hu, 2002; Nagano et al., 2003). Recent studies reported that mutations of KLK4 results in enamel defect (Stephanopoulos et al., 2005; Hart, 2006). Human KLK4 is highly prostate enriched, and is androgen regulated (Nelson et al., 1999; Korkmaz et al., 2001), which suggests that it may function in prostate or seminal plasma

similar to KLK2 and PSA. The full-length KLK4 transcript, encoding a secreted protein, has been used in several studies to express recombinant versions of KLK4 in order to examine its substrate specificity. Takayama and coworkers showed that a recombinant, chimeric form of KLK4 (ch-KLK4) in which the pro-piece of KLK4 was replaced by that of PSA to create an activation site susceptible to trypsin-type proteases, had a trypsin-type substrate specificity (Takayama et al., 2001). In addition, ch-KLK4 also readily activated both pro-PSA and single chain urokinase-type plasminogen activator (scuPA, pro-uPA), and completely degraded prostatic acid phosphatase (PAP), indicating that KLK4 may have a role in the physiological processing of seminal plasma proteins, as well as in the pathogenesis of prostate cancer through its activation of pro-uPA (Takayama et al., 2001). In a recent report, it was demonstrated that the three-domain receptor of uPA, uPAR, is also a target for KLK4, cleaved in the D1-D2 linker sequence and, to a lesser extent, in its D3 juxtamembrane domain (Beaufort et al., 2006). These data suggest a role of KLK4 in modulation of the tumor-associated uPA/uPAR-system activity by either activating pro-uPA or cleaving the cell surface-associated uPA receptor. Furthermore, recombinant KLK4 was reported to cleave extracellular matrix proteins, suggesting a role of KLK4 in tissue remodeling (Obiezu, 2006). However, there is at present not sufficient substrate and enzymatic evidence to support the notion that KLK4 has a functional role in seminal liquefaction.

In addition to these *in vitro* studies with recombinant protein, there have been a few studies trying to reveal the biological function of endogenous KLK4 in prostate cancer cells. Veveris-Lowe et al. showed that cytoplasmic KLK4, as well as PSA, increases cell migration when ectopically expressed in the prostate cancer cell line PC-3. This was associated with loss of E-cadherin and an increase of vimentin, suggesting an involvement of cytoplasmic KLK4 in the epithelial-mesenchymal transition, a crucial event in the progression of cancer to an invasive phenotype (Veveris-Lowe et al., 2005). Recently, a role of secreted KLK4 in prostate cancer metastasis to bone was also suggested, based on the dependency of KLK4-expression in the interaction between prostate cancer cells and osteoblasts in bone metastasis (Gao et al., 2007). These results suggest that the secreted form of KLK4 may have a role in prostate cancer development

and progression, although more studies are needed in order to elucidate its actual presence in biological fluids, and exact role in prostate cancer biology. It is at present no report on the biological function of nuclear KLK4. For a complete understanding of the role of KLK4 in prostate cancer development and progression, it is important to elucidate the relevance of the different forms of KLK4 expressed. The functional properties of the encoded protein(s) may then be elucidated in greater detail.

## AIMS OF THE STUDY

As detailed in the introduction, androgens are involved in important physiological and pathological processes, such as normal prostate biology and prostate cancer; however, the molecular mechanisms of androgen action remain largely unclear. The major aim of this study was thus to examine in greater detail the molecular mechanisms underlying androgen action in the cell. To this end, there were two main focus areas:

1. Characterization of the androgen target gene KLK4
2. Nuclear dynamics of AR-mediated transcriptional activation

KLK4 has been identified as a prostate specific and androgen regulated gene, with potentially important functions in prostate cancer. Previous work suggested that KLK4 may have a different gene structure than the other members of the kallikrein family; thus we set out to map the 5' end of the KLK4 transcript in detail. The cellular localization of the encoded protein, as well as its androgen regulation in prostate cancer cells was studied. To elucidate the potential role of KLK4 in prostate carcinogenesis, we examined the expression levels of KLK4 in benign compared to malignant human prostate glands. Furthermore, the functional properties of KLK4 in prostate cancer cells were elucidated using adenovirus-mediated overexpression and siRNA technology.

AR is a ligand-dependent transcription factor and the main mediator of androgen action in the cell. Thus, a detailed understanding of the mechanisms by which AR regulates transcription is essential for elucidating androgen action. We therefore investigated AR-mediated transcription in detail, with a special focus on its interaction with chromatin in response to androgens and anti-androgens. To this end, we developed a system enabling the visualization of GFP-tagged AR when it is bound to its response element in living cells. We then used advanced fluorescence microcopy techniques to elucidate the nature of AR interactions with target sites, and correlated these interaction kinetics with the recruitment of factors of the transcriptional apparatus and the initiation of transcription. Finally, we investigated how HDAC inhibitors affect AR transcriptional activity and chromatin interaction dynamics in living cells.

## SUMMARY OF PAPERS

**Paper I. Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer**

Kallikrein 4 (KLK4) is a member of the human tissue kallikrein family, consisting of 15 closely related serine proteases. It was demonstrated by systematic PCR analysis that KLK4 has a gene structure differing from the rest of the family members. The putative exon one, encoding a signal peptide targeting the protein for secretion, is not part of the main KLK4 transcript, and KLK4 is thus an intracellular protein. Immunostaining of ectopically expressed KLK4 in COS-7 cells and endogenous KLK4 in LNCaP cells demonstrated that KLK4 is predominantly localized in the nucleus, which was further confirmed by biochemical fractionation experiments. KLK4 is strongly androgen-regulated in LNCaP cells, and is not expressed in the androgen-insensitive cell lines PC-3 and DU145, suggesting that KLK4 expression is correlated with the presence of functional AR. Furthermore, we showed that KLK4 mRNA is overexpressed in prostate cancer compared to normal prostate by *in situ* hybridization of prostate tissue microarrays, being expressed predominantly in the nucleus of basal cells of the prostate epithelium. This is the first report of nuclear localization of a member of the kallikrein family, suggesting that it may have unique functions compared to the other members of the family. Importantly, its androgen regulation and overexpression in prostate cancer suggest that it might have important roles in prostate carcinogenesis.

**Paper II. Ligand-specific dynamics of the androgen receptor at its response element in living cells**

Cell lines with tandem repeats of the MMTV promoter stably integrated into its genome have previously been used to demonstrate rapid interactions between steroid hormone receptors and chromatin in live cells. As the hormone response elements of the MMTV LTR function also as AREs, we adopted this system and established MMTV array containing cell lines stably expressing GFP-fusions of AR and a transcriptionally impaired mutant (AR-E897A). FRAP analysis in combination with other methods was used to study the dynamics of AR-chromatin interactions in live cells in response to a wide set of AR ligands. A rapid interaction of AR with target genomic sites in living cells in the presence

of agonists was demonstrated, which coincided with the recruitment of PolII and the SWI/SNF chromatin remodeling complex, resulting in transcriptional activation. The interaction of antagonist-bound or mutant AR with its target site was kinetically different: it was dramatically faster, and occurred without the recruitment of SWI/SNF or PolII, and without any transcriptional activation. ATP- and SWI/SNF-dependent displacement of AR from the MMTV chromatin was also demonstrated *in vitro*. Furthermore, FRET analysis of wild type and mutant AR, when associated with its target sites, showed that intramolecular interactions between the N- and C-termini of AR play a key functional role in AR transcriptional activation. These data provide a kinetic and mechanistic basis for regulation of gene expression by androgens and anti-androgens in living cells.

**Paper III. Kallikrein 4 is a proliferative factor that is overexpressed in prostate cancer**

As demonstrated in Paper I, KLK4 is a unique member of the human tissue kallikrein family. Here we further elucidate the functional properties of KLK4 in prostate cancer cells, and its expression in normal prostate and prostate cancer specimens. Firstly, we examined the expression of KLK4 at the protein level in prostate tissue microarrays by immunohistochemistry. Consistent with its mRNA expression, KLK4 is significantly overexpressed in malignant prostate carcinomas as compared to benign prostate glands. Furthermore, KLK4 is predominantly expressed in the nucleus of basal cells of the prostate epithelium. An adenovirus-mediated expression system for KLK4 was generated and used to conditionally express KLK4 in the prostate cancer cell lines PC-3 and DU145. The expression of KLK4 in these cell lines dramatically induced proliferation as demonstrated both by colony formation and proliferation assays. The increased proliferation was at least in part through significant alterations in cell cycle regulatory gene expression as demonstrated by cell cycle specific oligonucleotide array analysis. Consistent with these data, siRNA-mediated knockdown of endogenous KLK4 in LNCaP prostate cancer cells inhibited cell growth. These data identify KLK4 as the first member of the kallikrein family with proliferative properties mediated through the alteration of cell cycle regulatory gene expression, and indicate that KLK4 may have important roles in prostate cancer development and progression.

**Paper IV. Reduced mobility of the androgen receptor at its target sites in living cells in response to histone deactylase inhibiton**

As reviewed in the introduction, histone acetylation is an important factor in gene regulation. Compounds that affect histone acetylation, such as inhibitors of histone deacetylases (HDACis), can thus be used to regulate gene expression. The mechanisms by which histone acetylation regulates transcription is unclear, hence we elucidated the effect of altered chromatin acetylation on the dynamic interactions between AR and chromatin as described in paper III. To this end, HDACis were used to induce AR transcriptional activity, and its corresponding nuclear mobility was examined by FRAP analysis. Furthermore, the mobility of a transcriptionally impaired AR mutant (AR-E897A), and also GR, was analyzed in a similar manner. We demonstrated that AR and AR-E897A mobility is strongly reduced in response to HDAC inhibitors TSA and SAHA, which correlated with increased transcriptional activity. However, TSA and SAHA did not affect mobility of antagonist bound AR and AR-E897A with no change in transcriptional activity. Importantly, the same inhibitors did not increase the transcriptional activity of agonist-bound GR, and its mobility on the target promoter was not affected. These data suggest that histone acetylation is involved in the dynamic interaction between steroid receptors and target sites in chromatin through alteration of receptor transcriptional activation.

## RESULTS AND DISCUSSION

**Kallikrein 4 is an intracellular protein that is overexpressed in prostate cancer**

Previous work from our laboratory (Korkmaz et al., 2001) suggested that KLK4 has a different gene structure than the other family members, with only four coding exons. The KLK4 main transcript was proposed to lack the putative exon 1, thus encoding a protein without a signal peptide, possibly resulting in an intracellular protein. This would suggest a different function of KLK4 compared to the other members of the kallikrein family, hence it was of significant importance to explore the nature of the KLK4 transcripts.

We therefore set out to analyze the 5' end of the KLK4 transcript in greater detail. Through a detailed PCR analysis using a set of different 5'-primers, it became evident that a transcript without the putative exon 1 was the physiological relevant form of KLK4 mRNA. A transcript containing all five exons was also present, although at a 1000-fold less abundancy than the exon 1-deleted transcript. A transcript lacking the putative exon 1 would encode a protein not targeted for secretion, and we therefore decided to examine the cellular localization of KLK4. Ectopic expression of tagged KLK4 in COS-7 cells showed predominantly nuclear localization of the protein. A KLK4-specific antibody was raised, and used to stain endogenous KLK4 in LNCaP cells, confirming the nuclear localization of the protein. Biochemical fractionation experiments furthermore confirmed the localization of KLK4 to the nucleus (Figure 9).

The androgen regulation of KLK4 had been demonstrated at the mRNA level (Nelson et al., 1999; Yousef et al., 1999; Korkmaz et al., 2001). Having a KLK4-specific antibody, Western analysis was used to demonstrate androgen regulation also at the protein level, with a 20-fold increase in KLK4 after 48 hours of androgen treatment of LNCaP cells. No KLK4 was detected in the androgen-insensitive prostate cancer cell lines DU145 and PC-3, suggesting that KLK4 expression is correlated with the presence of functional AR. The fold-regulation of KLK4-expression upon androgen treatment has varied widely among different studies (Nelson et al., 1999; Stephenson et al., 1999; Yousef et al., 1999; Korkmaz et al., 2001; Dong et al., 2005), possibly due to differences in cell culture

conditions and antibody-specificity, and clearly also discrepancies in which KLK4 isoform has been studied.

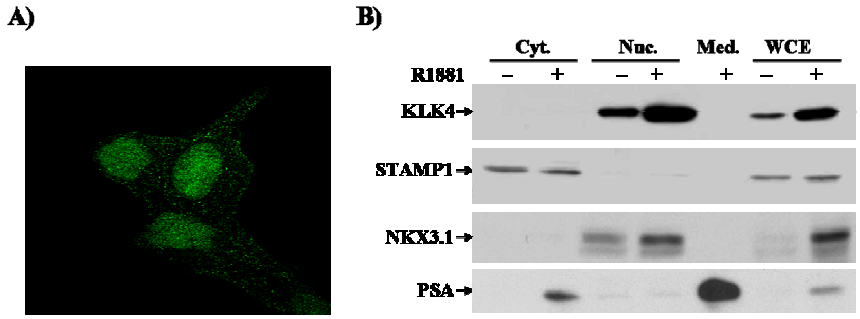


**Figure 9. Nuclear localization of KLK4**
(A) LNCaP cells were treated with R1881 for 48 hours and subjected to immunofluorescence analysis with a KLK4-specific antibody, or (B) nuclear (nucl.), cytoplasmic (cyt.), whole cell (WCE) and secreted (Med.) protein extracts were made and subjected to Western analysis using specific antibodies for KLK4, STAMP1, NKX3.1 and PSA.

The size of the protein detected by Western blot was around 45 kDa, in contrast to the calculated size of about 28 kDa. This suggests that KLK4 may be post-translationally modified. However, despite thorough analysis no glycosylation modifications of KLK4 could be detected, suggesting that KLK4 may be modified in other ways such as by ubiquitination or sumoylation. Further analysis is thus needed to determine the nature of KLK4 post-translational modifications.

The prostate-specific, and androgen regulated expression of KLK4 suggest a potential involvement in prostate cancer. Hence, we used a KLK4-specific riboprobe to study KLK4 mRNA expression in prostate tissue microarrays, containing normal and tumor glands from human prostates. The analysis showed that KLK4 was significantly overexpressed in prostate carcinoma compared to benign prostate glands. Furthermore, KLK4 was predominantly expressed in the nucleus of the basal cells of the prostate epithelium. Together these findings confirm the presence of an intracellular form of KLK4, with potential function in prostate carcinogenesis.

The presence of an intracellular form of KLK4 has later also been confirmed by others (Dong et al., 2005). There are some discrepancies in the exact N-terminal sequence of the nuclear form of KLK4 which should be elucidated in future studies. Furthermore, Dong et al. did not detect any androgen regulation of nuclear KLK4 in LNCaP cells (Dong et al., 2005), in contrast to what has been repeatedly observed in our laboratory. The exchange of cells and antibodies between the laboratories might aid in elucidating the reason for this obvious discrepancy. In line with our observations, Dong et al. also did not detect any glycosylation of nuclear KLK4, proposing that the discrepancy between calculated and detected size of the protein is due to other modifications.

Although the full-length KLK4 transcript was found to be much less abundant than the exon 1–deleted transcript, it cannot be ruled out that also the full-length transcript encodes a protein with important physiological roles. As discussed in the introduction, there have been some reports on the secretion of KLK4, however with questionable validity (Day et al., 2002; Obiezu et al., 2002; Obiezu et al., 2005). More recently, work by Dong et al, which confirmed the presence of a nuclear form of KLK4, also detected a cytoplasmic KLK4 in prostate cancer cell lines and prostate cancer tissue (Dong et al., 2005). Furthermore, secreted KLK4 was also detected in seminal fluid from a prostate cancer patient, not present in a healthy individual. Later work from the same laboratory propose cytoplasmic KLK4 to have roles in epithelial-mesenchyme transition processes, indicating that it may be involved in the progression of prostate cancer through the promotion of tumor cell migration (Veveris-Lowe et al., 2005). Furthermore, a recent report from Gao et al. suggests that secreted KLK4 potentially is involved in the cellular interaction between prostate cancer cells and osteoblasts, thus proposing a role of KLK4 in prostate cancer bone metastasis (Gao et al., 2007). Taken together with the data presented in this thesis, it can be suggested that KLK4 is expressed in both a nuclear and secreted form. Both forms may have important function in prostate carcinogenesis, and their properties and relative importance in prostate cancer needs to be explored individually.

The abundancy of the exon-1 deleted KLK4 transcript, its overexpression in prostate cancer and its strong androgen regulation suggest a potentially important physiological role of nuclear KLK4 in prostate carcinogenesis. Thus we set out to elucidate its expression in prostate specimens, and its functional role in prostate cancer cells in greater detail. Firstly, we analyzed KLK4 protein expression in prostate tissue microarrays by immunohistochemical staining. The analysis confirmed the results at the mRNA level, with significant overexpression of KLK4 in malignant prostate carcinoma compared to benign prostate glands. This is also in line with what has been observed in another study (Veveris-Lowe et al., 2005), however in contrast to what was found by others (Obiezu et al., 2005). Both these studies examined the cytoplasmic and secreted form of KLK4, probably giving rise to the discrepancies. One study reports the expression of nuclear KLK4 in human prostate specimens, with stronger signal in cancer cells than in benign glands (Dong et al., 2005), however without any statistical analysis. This is therefore the first report of significant overexpression of nuclear KLK4 in prostate cancer. The immunohistochemical staining further confirmed the nuclear localization of KLK4, with expression predominantly in the basal cells of the prostate epithelium.

**KLK4 is a proliferative factor in prostate cancer cells**

To study the functional properties of KLK4 in prostate cancer cells, we developed an adenoviral-mediated expression system. The system was designed to express a His-tagged KLK4 under a doxycycline-inducible promoter (Figure 10A). The proliferative effect of KLK4-expression in the prostate cancer cell lines PC-3 and DU145 was evaluated by colony formation and proliferation assays, and the results clearly demonstrated a proliferative effect of KLK4 in both cell lines (Figure 10B).

Increased proliferation of cancer cells can be mediated through several mechanisms, such as inhibition of apoptosis or induction of cell cycle progression. To evaluate if the latter was the case in our system, cell cycle specific oligoarrays were probed with biotin-labeled cRNA from KLK4-expressing PC-3 cells, and compared to non-expressing cells. The analysis revealed that the proliferative effect of KLK4 was at least in part through the alteration in cell cycle regulatory gene expression. Several genes involved in

progression of the cell cycle, such as E2F1 and cyclin B1, as well as the proliferative markers PCNA and Ki-67, were upregulated in response to overexpression of KLK4. Furthermore, several inhibitors of the cell cycle, such as the CDK (cyclin dependent kinase) inhibitors p15, p16 and p21, were downregulated in the same cells. CDK inhibitors act as tumor-suppressor genes, and their down-regulation or loss is commonly seen in prostate cancer (reviewed in (Fernandez et al., 2002)). These data suggest that KLK4 induces proliferation of prostate cancer cells through the alteration of cell cycle regulatory gene expression. As KLK4 is not a transcription factor, intermediate factors must be involved and the identification of these will give important information about the mechanisms of action of KLK4.

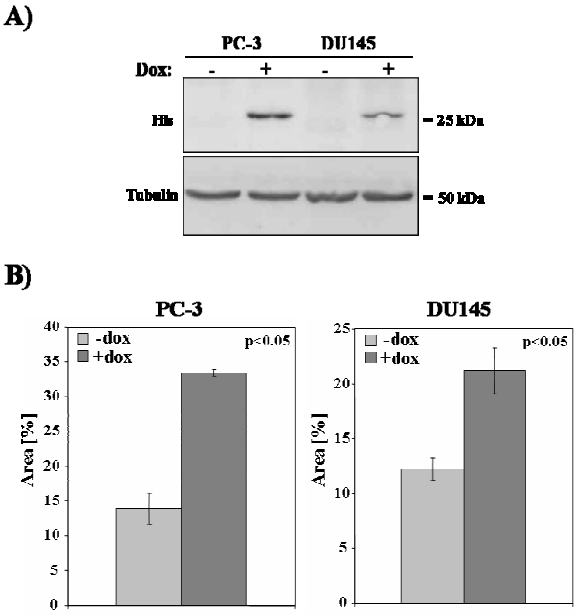


**Figure 10. Overexpression of KLK4 induces proliferation of prostate cancer cells**
(A) PC-3 and DU145 cells were infected with KLK4-expressing adenovirus, and the expression of His-tagged KLK4 upon addition of doxycycline was verified by Western analysis using a His-specific antibody. (B) Quantification of colony formation by PC-3 and DU145 cells expressing (+dox) or not expressing (-dox) KLK4. $p<0.05$ as assessed by Student's t-test.

The size of the adenovirus-expressed KLK4 was about 26 kDa, which is significantly smaller than the size of endogenous KLK4 in LNCaP cells (see Paper I), questioning if the physiological properties of KLK4 are maintained in this system. In addition, the overexpression of proteins may cause non-specific side-effects on cellular properties. Hence, in order to elucidate if the observed proliferative effect of KLK4 also applied to endogenous KLK4, siRNA was used to specifically knock down the expression of KLK4 in LNCaP cells. The specific knock-down of KLK4 was confirmed both at the mRNA and protein level, and resulted in the inhibition of growth of the LNCaP cells. These results confirm the proliferative effect of KLK4 in prostate cancer cells, suggesting that the smaller size KLK4 expressed in PC-3 and DU145 cells has maintained its functional properties.

This is the first report on important biological functions of the nuclear form of KLK4. The data presented suggest a role of KLK4 in prostate cancer cell proliferation, proposing that KLK4 may be involved in the progression of prostate cancer. The specific mechanisms by which KLK4 mediates this effect remain to be elucidated

**Ligand-specific dynamics of the androgen receptor**

As detailed in the introduction, the traditional way of viewing nuclear receptor action has recently been challenged by a new dynamic model, in which the receptor transiently interacts with its target genomic sites and are dynamically displaced at the timescale of seconds (see Figure 5). The dynamics of AR interaction with target genomic sites have not previously been studied, thus we set out to analysie the kinetics of AR interaction with chromatin in respose to angonists and antagonists using advanced fluorescence microcopy techniques.

Cell lines with integrated tandem repeats of the MMTV promoter, containing 800-1000 binding sites for AR, stably expressing GFP-tagged AR and the mutant AR-E897A were generated. These cell lines have maintained their response to AR ligands with respect to nuclear translocation and transactivation potential. The high density of AR binding sites within the tandem MMTV repeat enabled the visualization of the GFP-tagged receptor

when bound to its response element, and FRAP analysis was used to analyze the dynamics of the interaction between the receptor and its promoter in living cells in response to agonists, partial antagonists and antagonists. The FRAP recovery curves (Figure 11A) clearly demonstrate a rapid and dynamic interaction between AR and its target promoter, with times for half-maximum recovery in the scale of seconds. The recovery of AR is strongly ligand-dependent, with significantly delayed recovery in response to agonist as compared to antagonist. The delayed recovery corresponds to increased AR transcriptional activity, as demonstrated by RNA FISH analysis (Figure 11B). Furthermore, the recovery of the transcriptionally impaired mutant AR-E897A was significantly faster than wild type AR, proposing a correlation between AR transcriptional activity and recovery kinetics at the promoter.

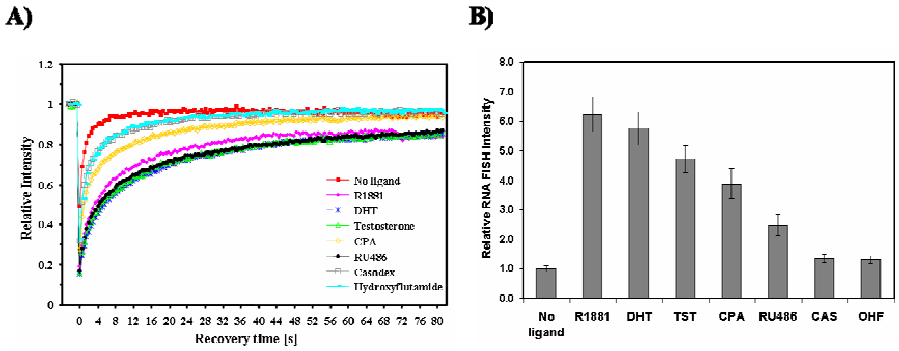


**Figure 11. Ligand-specific dynamics of AR**
GFP-AR expressing cells were treated with different ligands as indicated, and GFP-AR interaction with the MMTV array was analyzed by FRAP analysis (A), or the cells were subjected to RNA FISH analysis for quantification of transcriptional activity (B). Error bars represent standard error.

Rapid recovery of agonist bound-AR as demonstrated by FRAP analysis was first described by Farla et al. (Farla et al., 2004), and the recovery kinetics was later shown to be ligand-dependent with faster recovery of antagonist-bound AR than agonist-bound AR (Farla et al., 2005; Marcelli et al., 2006), in line with our observations. However, these studies were performed on AR in the general nucleoplasmic space, and not on a target

promoter. This is therefore the first time to demonstrate the transient and rapid interaction between AR and its response element in living cells.

Delayed recovery of agonist-bound receptor as compared to unliganded receptor has also been demonstrated for the steroid hormone receptors PR (Rayasam et al., 2005) and ER (Stenoien et al., 2001b). As observed for AR, the recovery of PR was faster in the presence of antagonist (ZK98299) than in the presence of agonist (R5020) (Rayasam et al., 2005). In agreement with our observations, the partial antagonist RU486 resulted in delayed recovery of PR compared to the pure antagonist ZK98299, suggesting that the mechanism of RU486 induced antagonism is different from other antagonists. In contrast to what was observed for AR and PR, ER mobility was dramatically reduced in the presence of antagonist ICI 182,780 compared to agonist E2 (Stenoien et al., 2001b), suggesting that antagonist function differs among the steroid hormone receptors.

The delayed recovery of agonist-bound AR coincided with the recruitment of Pol II and the chromatin remodeling complex SWI/SNF, which was not observed for antagonist-bound AR or the mutant AR-E897A. Furthermore, in an *in vitro* chromatin remodeling assay, ATP- and SWI/SNF-dependent displacement of AR from the chromatin template was seen, demonstrating the importance of chromatin remodeling in the dynamic interaction between AR and chromatin. The involvement SWI/SNF-induced chromatin remodeling in transcriptional activation has previously been described for AR, as well as other nuclear receptors (Fryer & Archer, 1998; Dilworth & Chambon, 2001; Fletcher et al., 2002; Belandia & Parker, 2003; Marshall et al., 2003; Rayasam et al., 2005).

As described in the introduction, an intramolecular interaction between the N- and C-termini of AR is important for optimal receptor activity, however, this interaction has not been demonstrated for AR in its active form when associated with its target genomic site. We therefore used FRET analysis of dual-labeled AR when bound to the MMTV array to examine the importance of this intramolecular interaction. The possible role of intermolecular interaction between two neighboring molecules was also evaluated. A set of AR and AR-E897A fusion proteins were generated, and their expected response to

androgen was confirmed by luciferase reporter assays. Acceptor photobleaching was then used for FRET analysis, and the results confirm the presence of intramolecular interactions between the N-and C-termini of wild type AR when associated with its target promoter (Figure 12). FRET was significantly reduced for the transcriptionally impaired AR-E897A, suggesting that the intramolecular interaction is important for optimal receptor activity. Some FRET was also observed when co-transfecting single-labeled AR (CFP-AR + AR-YFP), suggesting that there are some intermolecular interactions between neighboring molecules. However, these are similar for wild type and mutant AR, suggesting that they are not as important for AR transcriptional activity as the intramolecular interactions.

Together these data give significant new information about mechanisms of actions of AR, and about antagonist function. The results support the hit-and-run model of nuclear receptor action, suggesting that the model describes a general feature of at least steroid hormone receptors, and possibly also of other DNA-interacting proteins. This dynamic nature of protein-chromatin interaction allows for very rapid response to changes in the cellular environment, giving a new dimension to transcriptional regulation.

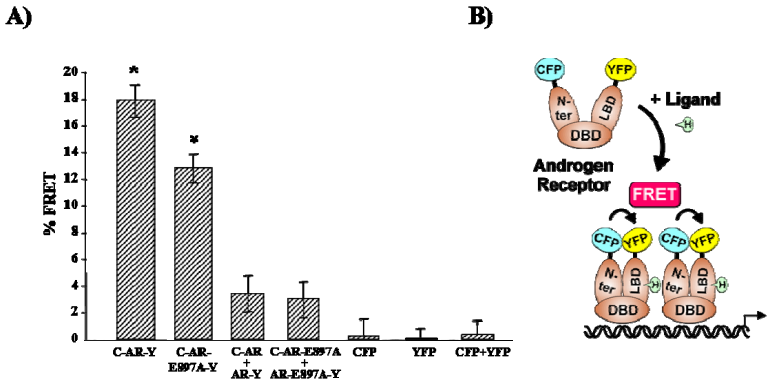


**Figure 12. Intramolecular interactions of AR at its target promoter**
(A) MMTV array-containing cells were transfected with different AR-fusion constructs as indicated, treated with R1881 for 48 hours, and subjected to FRET analysis. (B) Schematic presentation of the intramolecular interaction between the N-and C-termini of agonist-bound AR Error bars represent standard error, * means statistically difference ($p<0.05$) as assessed by Student's t-test.

**Reduced mobility of the androgen receptor in response to HDAC inhibitors**

As discussed in the introduction, histone acetylation is an important factor in gene regulation; however, the underlying mechanisms are unclear. Thus we wanted to elucidate the effect of altered chromatin acetylation on the dynamic interactions of AR with its target sites in living cells. HDACis are compounds that change the acetylation status of chromatin by inhibiting the deacetylation of histone tails, and it has been shown that AR transcriptional activity is induced by HDACis (List et al., 1999b; Shang et al., 2002; Korkmaz et al., 2004a). Hence, we decided to use the HDACis TSA and SAHA in our system to study the effect of chromatin acetylation on AR interactions with chromatin, and the possible correlation between transcriptional activity and receptor dynamics.

TSA treatment resulted in increasing acetylation of histone 3 (H3) in our cell lines in a time-dependent fashion, concomitant with an increase in AR transcriptional activity and delayed FRAP recovery curves (Figure 13). However, AR recovery dynamics was not affected by treatment with the same compound when associated with antagonist (OHF), corresponding with no change in transcriptional activity. The same effect was also seen for the HDAC inhibitor SAHA. Furthermore, TSA induced the transcriptional activity of the mutant AR-E897A, followed by significantly delayed FRAP recovery at the MMTV array. These data suggest that increased histone acetylation results in delayed recovery and increased residence times of AR at the promoter through the induction of transcriptional activity.

HDAC inhibitors can have various effects on different receptors, and in different promoter contexts. In contrast to for AR, HDAC inhibitor TSA has actually been shown to have an inhibitory effect on GR activity on the MMTV promoter (List et al., 1999a). We therefore used a previously described (McNally et al., 2000) cell line with integrated MMTV array stably expressing GFP-GR to study the effect of HDAC inhibitors on GR nuclear mobility. TSA and SAHA had the same effect on chromatin acetylation status in this cell line as in the AR cell lines; however, GR transcriptional activity was not significantly altered in response to the inhibitors and FRAP analysis demonstrated no

change in GR residence time on the promoter. These data support a correlation between increased transcriptional activity and reduced nuclear mobility. Furthermore, the data suggest that histone acetylation does not always induce transcription, but is dependent on promoter and transcription factor context. A more detailed study into the factors involved in the dynamic exchange of the receptor between its chromatin-bound state and the free nucleoplasmic state will give further information about the mode of action of nuclear receptors. It will be of special interest to examine which factors are involved in AR-mediated transcription in comparison to GR-mediated transcription, thereby identifying factors with receptor-specific roles. Potential candidates might be HDACs, which have shown to be exhibit both activation and repression properties as co-regulators for nuclear receptor action (Gallinari et al., 2007). One example is HDAC1, which has a repressive role in AR transactivation (Shang et al., 2002), while it has been identified as a coactivator for GR (Qiu et al., 2006).

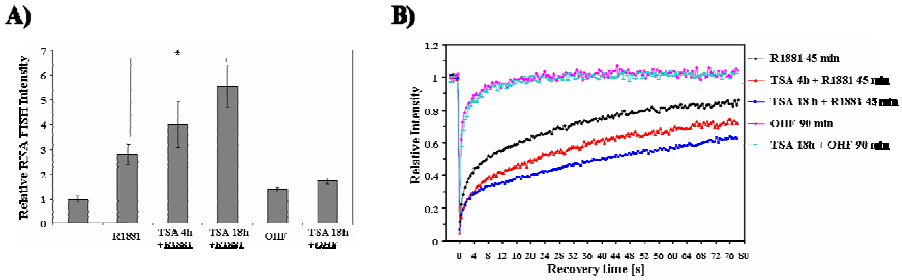


**Figure 13. Increased transcriptional activity and reduced mobility of AR in response to HDAC inhibitor TSA**
GFP-AR expressing cells were treated with TSA and R1881 or OHF for different time periods, and subjected to RNA FISH (A) and FRAP (B) analysis. Error bars represent standard error, and * indicates statistically difference ($p<0.05$) as assessed by Student's t-test.

Together these data gives further insight into the molecular details of the dynamic interaction between AR and its target sites. Furthermore, important differences between AR- and GR-dependent transcriptional activation have been identified, being potentially important in the search for novel diagnostic and therapeutic targets in prostate cancer.

The proposed correlation between transcriptional activity and AR mobility on the promoter needs to be elucidated in greater detail, but could potentially be used as a tool in high-throughput screening for novel anti-androgens for the use in prostate cancer therapy. Detailed analysis of the dynamic interaction between nuclear receptors, and also other transcription factors, and chromatin are needed in order to fully understand the molecular details of transcriptional regulation.

## FUTURE PERSPECTIVES

In this thesis, we identified KLK4 as a unique, intracellular member of the KLK family. We found that KLK4 is overexpressed in prostate cancer and induces proliferation of prostate cancer cells, suggesting that it may have important roles in prostate carcinogenesis. The mechanism by which KLK4 achieves this effect is largely unknown and will be of importance to explore in greater detail. A comprehensive study of KLK4 expression in prostate tissue specimens from large patient cohorts, representing the various stages of prostate cancer, would give clues as to how KLK4 expression varies during prostate cancer progression. The expression of KLK4 in matched normal and cancer samples from the same prostate gland would also be of interest to study to elucidate the local expression pattern of the protein. These data would give valuable information about the potential in using KLK4 as a diagnostic or prognostic marker for prostate cancer.

One important aspect of KLK4 expression is its regulation by androgens; however, the fold induction of KLK4 by androgens has varied between different studies. The reason for this discrepancy should be explored. The KLK4 promoter should be analyzed for potential androgen response elements, and the functionality of these determined. To this end, and for other analysis concerning androgen regulation, it would be of great value to check the response of KLK4 expression in other androgen regulated cell lines, such as LAPC-4 (Klein et al., 1997). There is also inconsistency in the literature regarding the size of KLK4. Possible post-translational modifications of KLK4, such as glycolysation and sumoylation, and the physiological relevance of these, if any, are also important aspects to explore. To understand the potential role of KLK4 in prostate cancer, the specific mechanisms underlying the proliferative effect of KLK4 are of importance to elucidate. This can possibly be achieved through the identification of KLK4 interaction partners, and direct up- or downstream targets, both for ectopically expressed and endogenous KLK4. This can for example be achieved by yeast two hybrid analysis and co-immunoprecipitation studies. Furthermore, examination of the effect of KLK4 expression in *in vivo* model systems, either by overexpression or knock-down/knock-out studies, would give valuable insight into KLK4 function. Given its intracellular location,

KLK4 likely has different functional roles than the other members of the KLK family. Possible nuclear variants of the other KLKs may also exist and needs to be explored in detail. The presence of the other KLK4 isoforms should also be explored, and the possible functional roles of these should be determined. Taken together, these studies would give important new information about KLK4 function and its possible role in prostate carcinogenesis.

In this thesis we also explore the molecular mechanisms of AR action in living cells, using advanced fluorescence microscopy techniques. The data presented demonstrate a very dynamic interaction between AR and its target promoter, supporting the hit-and-run model of nuclear receptor action. The traditional static view on nuclear receptor-chromatin interactions and the idea of a stable transcriptional complex staying bound to the template for as long as transcription occurs, is challenged by this model. Although a highly dynamic behavior has now been described for many nuclear proteins in the general nucleoplasmic space, it is essential that similar studies, as we present here for AR, for other transcription factors on their regulatory elements are performed. These studies would then decide if the observed behavior also applies to a wider range of DNA-interacting proteins, or is specific for just a subset of transcription factors.

The long-term loading profile of AR and its cofactors onto the MMTV promoter would be of interest to elucidate, by ChiP analysis, in order to determine if AR exhibits the same cyclical loading profile as observed for ER. The possible functional relevance of this could then be explored. The role of chromatin acetylation on receptor dynamics should also be elucidated in greater detail. HDAC inhibitors are important tools for such studies, and inhibitors working by various mechanisms could be used. Furthermore, the overexpression and/or knock down of specific HATs and HDACs might prove useful in exploring the molecular details underlying transcriptional activation by steroid hormone receptors. The comparison of factors recruited to the promoter in the presence of AR compared to GR, as well as the acetylation status of the promoter after treatment with HDAC inhibitors, may give clues to why these two receptors respond differently to these inhibitors. One approach to this could be to determine which HDACs are involved in AR-

versus GR-mediated transcription, possibly identifying factors that have unique roles in the two systems. It would also be of great interest to study the dynamics of AR on a natural promoter. In order to relate the ligand-dependent dynamics of AR to its role in prostate carcinogenesis, this should preferentially be evaluated in a prostate cancer cell line. This can be achieved by the recombinant incorporation of a serially amplified AR-responsive promoter (e.g. the PSA promoter) into the chromosome of a prostate cancer cell line. Such a system would give further information about the physiological relevance of the highly dynamic interaction between AR and chromatin. A detailed understanding of how AR interacts with response elements of target genes will give significant insight into the regulation of AR-mediated transcription, an important aspect of normal physiology as well as in the development and progression of prostate cancer.

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