Chapter 1: Introduction

1.1 Historical perspectives in androgenic research

Androgens are a class of steroid hormones responsible for the differentiation of male genitalia along with the maintenance of male secondary sexual characteristics and reproductive function (Kaufman et al. 2005). The role of androgens in male physiology has been considered since antiquity, with the utilisation of castrati as singers in the ancient Persian court and eunuchs as servants in imperial Chinese courts as early as the 8th Century B.C. In 1786 John Hunter transplanted testes into capons to observe possible masculinising effects, and by 1849 Adolph Berthold proposed internal secretion based upon testicular transplantation experiments. In 1889, Brown-Séquard, at age 74, reported dramatic rejuvenating effects after self-administration of testicular extracts of dogs and guinea pigs, albeit unlikely that this aqueous abstract contained any biologically active androgens. In 1935 Ernest Laqueur and his team first isolated testosterone (T) from bull testes, which is now known to be the major testicular androgen in male circulation (Kaufman et al. 2005).

1.2 Production and metabolism of androgens and the male phenotype

1.2.1 Embryonic and foetal development

Androgens are essential for the development of the male phenotype. Embryos develop by default as female until expression of the *Sry* gene from the Y chromosome triggers the differentiation of genital ridge cells into Sertoli cells (Berta et al. 1990; Jager et al. 1990; Koopman et al. 1991). Sertoli cells then orchestrate the development of the testis, by triggering increased cell proliferation in this region and causing mesonephric cell immigration to the testis. These mesonephric cells differentiate into Leydig, endothelial and peritubular myoid cells depending on cellular interactions within the forming testis. Peritubular myoid cells and endothelial cells are essential for the development, structural integrity and vasculature of the testis, whereas foetal Leydig cells are principally responsible for the synthesis and secretion of T, the crucial male sex hormone. T is converted by the enzyme 5α-

reductase in genital tubercle mesenchyme to 5α-dihydrotestosterone (DHT), which allows differentiation of the urogenital sinus into the epididymis, prostate, vas deferens and seminal vesicles, and for the formation of external male genitalia (reviewed in Wilhelm et al, 2006). Individuals with germ line deficiency in 5α-reductase activity show incomplete development of the external genitalia and prostate, but normal differentiation of the epididymis, vas deferens and seminal vesicles, indicating that the importance of the conversion of T to DHT in development od the male reproductive tract is tissue specific (Andersson et al. 1991).

1.2.2 Androgen production in the adult: the hypothalamic-pituitary-gonadal axis

The majority of androgens in adult males are produced by the coordinated actions of the hypothalamic-pituitary-gonadal (HPG) axis (Fig 1.1). The hypothalamus secretes pulses of gonadotrophin releasing hormone (GnRH) which act on the pituitary, leading to pulsatile secretions of luteinising hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream. In the testes FSH binds to its receptor on the Sertoli cell surface leading to the regulation of spermatogenesis (Walker et al. 2005). LH binds to receptors on the surface of Leydig cells, activating adenylate cyclase and thereby causes increased production of intracellular cyclic adenosine monophosphate (cAMP). This rapid increase in cAMP results in the transport of cholesterol from the cell surface to the inner mitochondrial membrane with the involvement of steroidogenic acute regulatory protein (de Kretser et al. 2001).

Chronic stimulation of the Leydig cells by LH is required for the optimal expression of the enzymes involved in T biosynthesis. A complex set of events involving these steroidogenic enzymes catalyse the conversion of cholesterol to multiple steroids, including T, DHT, 17β-estradiol (E2) and progesterone (P) (Fig 1.2)(de Kretser et al. 2001). In addition to their main function which is the formation and secretion of testosterone, Leydig cells also produce E2 in small amounts formed from T (de Kretser et al. 2001). The Leydig cells produce 90-95% of

the testosterone found in male circulation, with the remainder produced peripherally from the adrenal androgen precursors (Labrie et al. 1983).

1.2.3 Peripheral synthesis of androgens from precursors in the male

While in the rat, rabbit and dog brain small amounts of dehydroepiandrosterone (DHEA) are synthesised which act as neurosteroids (Mathur et al. 1993), primates are the only mammals whose adrenal glands synthesise androgens (DHEA) from cholesterol with pregnenolone and 17α-hydroxypregnenolone as intermediates (Fig 1.2) (Rainey et al. 2002). In primates, micromolar amounts of the structural androgen DHEA are secreted into the bloodstream where it may be converted by steroid sulphatases to its more prevalent hydrophilic sulphonated form DHEA(S). While some studies indicate that DHEA(S) is unable to readily cross lipophilic membranes and is therefore biologically inert (Hammer et al. 2005), others have reported evidence of conversion of DHEA(S) to more active steroids within synovial and granulosa cells (Bonser et al. 2000; Weidler et al. 2005). Nevertheless, several studies have highlighted that DHEA is capable of entering cells and being converted to more active sex steroids T and DHT (reviewed in Miller 2002). These androgens are all capable of binding to the intracellular mediator of genomic androgenic action, the androgen receptor (AR); DHT with the highest affinity (Kd: 0.77 nM)(Duff et al. 2005) with T binding with approximately 7 times lower affinity followed by DHEA binding with approximately 13,000 times lower affinity than DHT (Fig 1.2, green arrows)(Fang et al. 2003). Additionally, another structural androgen and testosterone precursor, androstenedione (ASD), is found in male serum in low nanomolar amounts and binds to the AR with approximately 570 fold lower affinity than DHT (Horton et al. 1966; Fang et al. 2003). The testes and adrenal glands are responsible in approximately equal parts for the secretion of 85% of the ASD found in serum, with the remainder produced in peripheral tissues from T or DHEA (Horton et al. 1967; Baird et al. 1969). Multiple enzymes are responsible for the conversion of the androgenic precursors DHEA and ASD to T or DHT and the efficiency of conversion of the androgenic precursors will depend on the expression of these enzymes in the specific peripheral tissue (Fig 1.2). The

relative contributions to overall androgenic activity of DHEA and ASD binding directly to the AR in comparison to conversion in the peripheral tissue to the more active androgens T or DHT is still unclear but the contribution of adrenal androgens to androgenic activity has been estimated to be as high as 50% of overall androgenic activity in the prostate (Labrie et al. 1986).

1.2.4 Activation and inactivation of androgens within target tissues

The inactivation of androgens by enzymatic activity in tissues is also an important control of androgenic effects at a local level. The first step in the metabolism of T within target tissues involves the irreversible reduction of a small amount of T to E2 or DHT in the prostate, skin and liver. DHT can then be converted to 3α,17βdiol (3α-diol) and androsterone and E2 into estrone (Fig 1.2) (de Kretser et al. 2001). However, the androgenic metabolites 3α-diol retains some androgenic/steroidogenic activity in tissues, and can also be converted back to DHT (Fig 1.2) (Belanger et al. 2003). The irreversible conjugation of 3α-diol, androsterone and estrone with the polar cofactor uridine 5'-diphospho- (UDP) glucuronic acid creates steric hindrance for binding to steroid receptors and thereby abolishes or inactivates the steroidogenic activity of these compounds in tissues. This process is known as glucuronidation and involves the activity of a specific UDP-glucuronyltransferase (Fig 1.2, grey arrows). Glucuronidation also solubilises these hormones and allows them to circulate in the bloodstream and be excreted as urinary metabolites (Belanger et al. 1991; Roy 1992). In addition, T itself can be inactivated directly in the liver via reduction and oxidation, followed by glucuronidation.

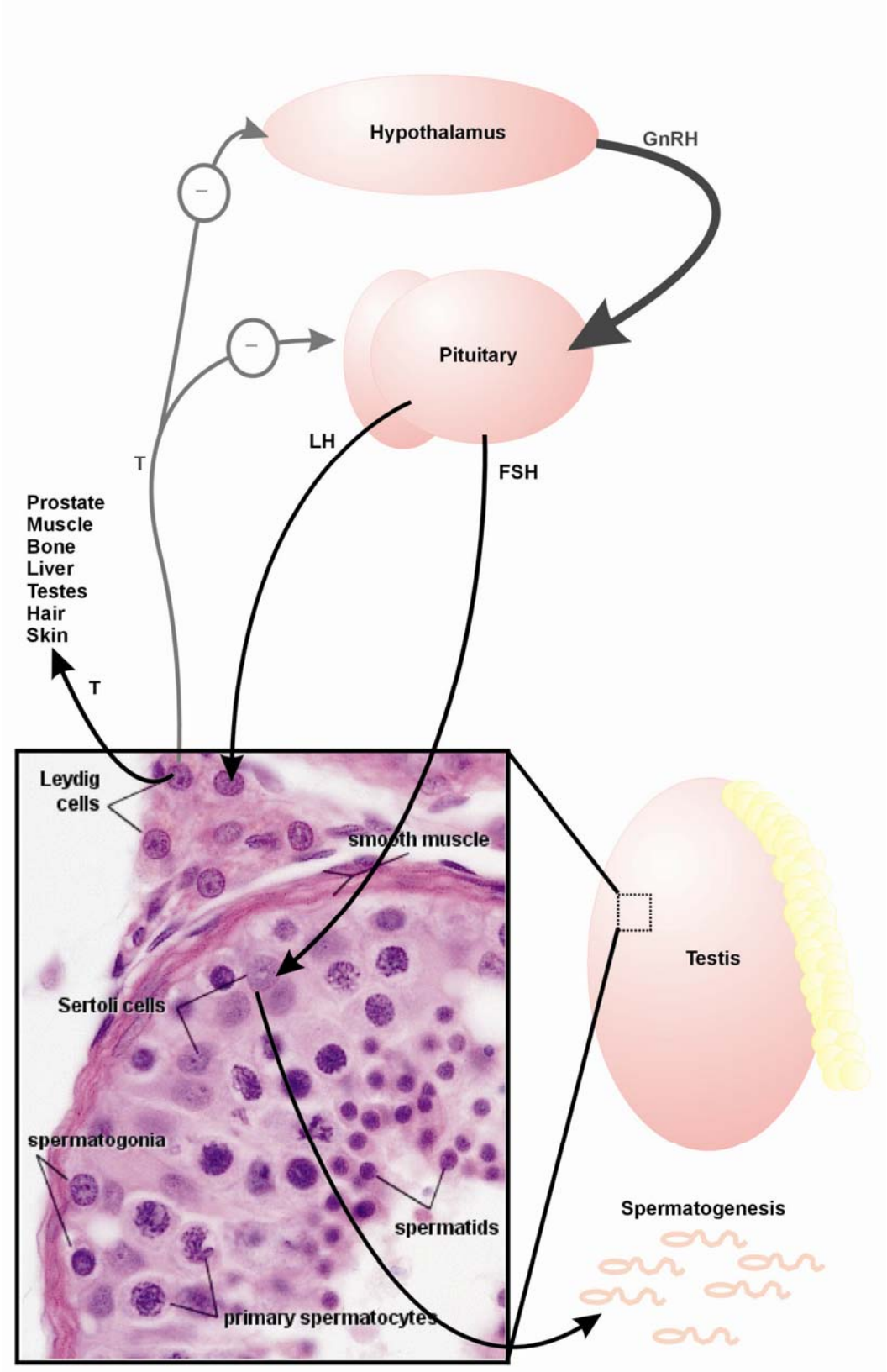
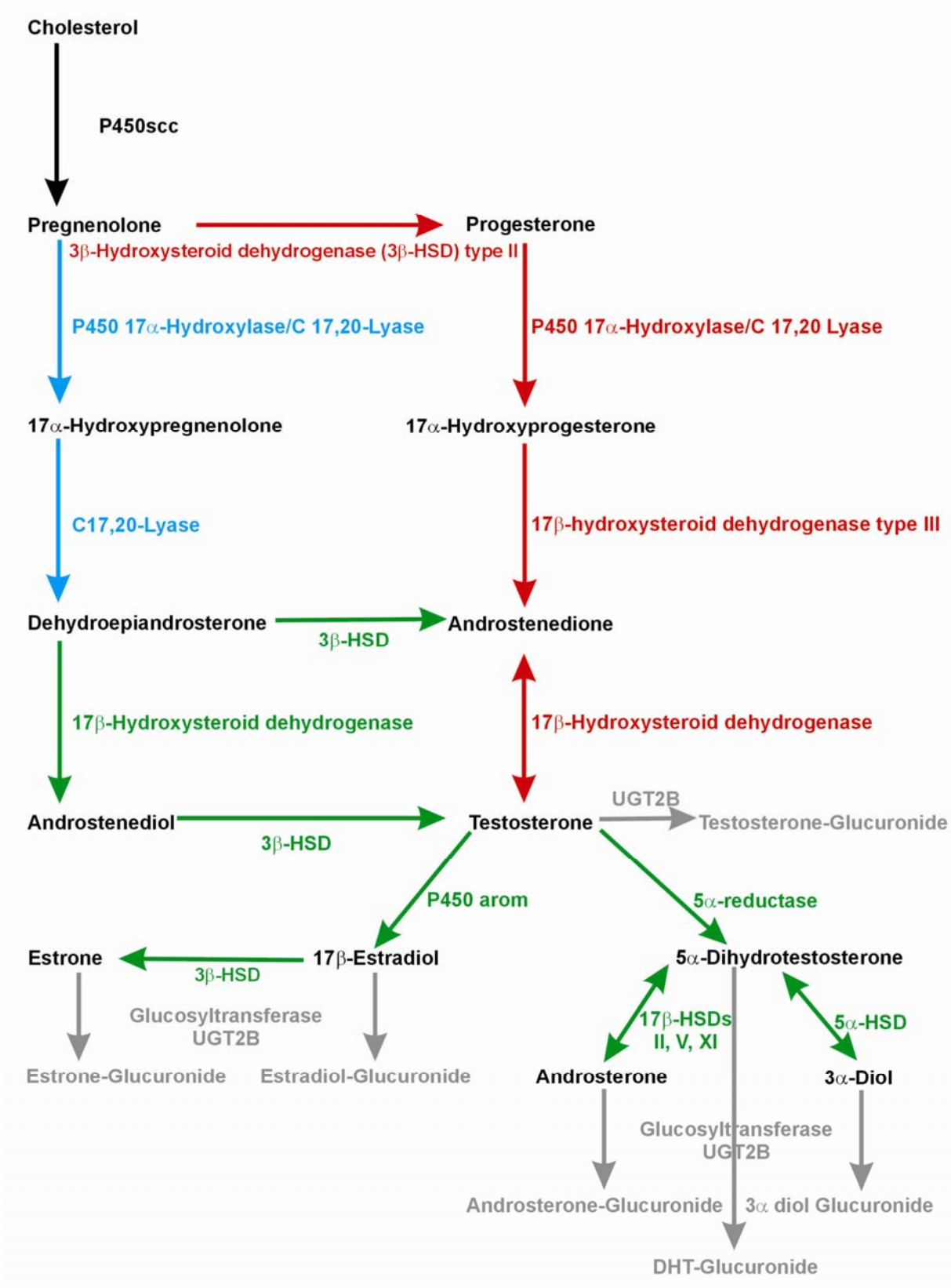


Fig 1.1 The Hypothalamic pituitary gonadal (HPG) axis.
The hypothalamus is responsible for pulsatile secretions of Gonodotropin releasing hormone (GnRH) which act on the pituitary, resulting in pulsatile release into the bloodstream of lutenising hormone (LH) and follicular stimulating hormone (FSH). These act on the testis to cause the initiation of steroid biosynthesis and spermatogenesis, respectively. Testosterone (T), the major male androgen is released into circulation where it acts on androgen target tissues. Unbound or free testosterone (FT) in circulation provides feedback on the pituitary and hypothalamus to inhibit the secretion of LH and FSH.

Fig 1.2: Steroid biosynthesis and metabolism pathways in the male.
The synthesis of all steroid hormones begins with cholesterol. The majority of T synthesis in males occurs in Leydig cells of the testis utilising the pathway depicted above by the red arrows. In the testis, cytochrome P450, cholesterol side chain cleavage (P450scc, P450 11A1) converts C_{27} cholesterol to the C_{21} steroid pregnenolone by cleaving the cholesterol side chain. Progesterone is then oxidised and stereochemically rearranged to form progesterone by the action of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase type II (3βHSD2). Progesterone is metabolised by the cytochrome P450 17α hydroxylase/C17-20 lyase via hydroxylation at carbon 17 followed by the cleavage of two carbon side chains and the reduction of the 17-ketone by 17β-hydroxysteroid dehydrogenase type 3 to form the precursor of T, C17 steroid androstenedione (ASD). Dehydroepiandrosterone (DHEA) is synthesized and secreted by the adrenal cortex and metabolised to T in the pathway depicted by the blue arrows. The conversion of DHEA or DHEA(S) to T, DHT and E2 is dependent on the expression and function of the following enzymes within peripheral tissues in the pathway depicted by the green arrows: 3β-Hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerases (3β-HSD type 1 and 2), Aldo-keto reductase family 1, member C3 (AKR1C3, aliases: 17β-Hydroxysteroid dehydrogenase type 5, 3-α hydroxysteroid dehydrogenase, type 2) (de Kretser et al. 2001). DHEA more commonly circulates in the bloodstream in its more soluble sulphonated form DHEA(S) which is catalysed by steroid sulphatase in target tissues into DHEA (Martel et al. 1994; Fujikawa et al. 1997). Testosterone is further metabolised in target tissues into the more active metabolite DHT via the actions of 5α-reductase or formed into the estrogen 17β-Estradiol (E2) via conversion of its A-ring to a phenolic structure by the cytochrome P450 aromatase ($P450_{arom}$). DHT can be reversibly converted in peripheral tissues to androsterone or 3α, 17βdiol (3α diol) and E2 into estrone. Active androgens or estrogens are irreversibly inactivated when they are conjugated with glucuronide involving the actions of a specific glucosyltransferase in the pathways represented by the grey arrows. T and androstenediol are also capable of being glucuronidated and therefore targeted for excretion (Belanger et al. 2003).


Cholesterol
P450scc
Pregnenolone
Progesterone
3β-Hydroxysteroid dehydrogenase (3β-HSD) type II
P450 17α-Hydroxylase/C 17,20-Lyase
P450 17α-Hydroxylase/C 17,20 Lyase
17α-Hydroxypregnenolone
17α-Hydroxyprogesterone
C17,20-Lyase
17β-hydroxysteroid dehydrogenase type III
Dehydroepiandrosterone
3β-HSD
Androstenedione
17β-Hydroxysteroid dehydrogenase
17β-Hydroxysteroid dehydrogenase
UGT2B
Androstenediol
3β-HSD
Testosterone
Testosterone-Glucuronide
P450 arom
5α-reductase
Estrone
3β-HSD
17β-Estradiol
5α-Dihydrotestosterone
Glucosyltransferase
UGT2B
17β-HSDs
II, V, XI
5α-HSD
Estrone-Glucuronide
Estradiol-Glucuronide
Androsterone
3α-Diol
Glucosyltransferase
UGT2B
Androsterone-Glucuronide
3α diol Glucuronide
DHT-Glucuronide

1.2.5 Androgens in male pubertal development and in the adult

Androgens are essential for the development and maintenance of male secondary sex characteristics during puberty. A dramatic increase in T production at this time results in voice deepening, facial and body hair growth and penile lengthening (Rogol 2002). After puberty, the precise roles of T are less well defined, but many cross sectional and longitudinal studies have now shown that serum T levels decline at a rate of about 0.9 - 1-3% per year (Gray et al. 1991; Harman et al. 2001; Feldman et al. 2002; Liu et al. 2007). Factors contributing to this decline include decreasing Leydig cell function with age (Zirkin et al. 2000; Anand-Ivell et al. 2006), decreases in the pulse amplitude and number of pulses of LH or GnRH (Mulligan et al. 1999), and finally decreasing supply of androgenic precursors within the testis (Takahashi et al. 1983). There is also evidence that testicular function declines with age independently of the HPG axis (Anand-Ivell et al. 2006).

The consequences of the decrease in serum T as men age are as yet unclear, due to: i) the multifactorial nature of many of the symptoms of T deficit, ii) that many of the symptoms may actually be a consequence of aging rather than T decline, and iii) that T decline may result as a consequence of the symptom rather than a cause of the symptom. However, males with borderline low levels of serum T have reported a number of vague symptoms such as lack of energy, decreased libido, loss of motivation, cantankerousness, sleepiness after lunch, and an inability to concentrate (Kelleher et al. 2004).

1.2.6 Diagnosis of androgen deficiency in the adult: measurement of serum T

In addition to the decline that has been observed with aging, it has been estimated that as many as 1 in 200 men in the Australian population produce low serum levels of gonadal steroid hormones (hypogonadism) which manifests as abnormally low levels of T. There are two types of hypogonadism, primary (hypergonadotrophic hypogonadism) resulting from

defects in testicular biosynthesis of T which results in elevated gonadotrophin levels but low levels of serum T. An example of primary hypogonadism includes individuals with Klinefelter syndrome, a condition in males resulting from extra X chromosome. This condition typically leads to small testicles and decreased serum androgens but high LH and FSH levels (Bojesen et al. 2007). In contrast, secondary (hypogonadotrophic hypogonadism) resulting from defects within the hypothalamic-pituitary-gonadal axis and manifests as abnormally low gonadotrophins and serum T. An example of secondary hypogonadism is Kallmann syndrome, where affected male individuals have intact testes but decreased sex hormone production due to various genetic defects which result in aberrant neuronal migration in the hypothalamus during development (Kim et al. 2008). Nevertheless, diagnosis of both primary and secondary hypogonadism primarily involves the investigation of clinical features such as delayed pubertal development, decreases in sexual desire or erectile function, fertility status, change in body hair distribution, gynecomastia, decreased musculature, small testicular volume and the exclusion of known testicular pathologies. Drug use and the occupation of the individual are also considered in the diagnosis. The measurement of serum T therefore is predominanty utilised in the clinic to support the overall diagnosis of hypogonadism (Handelsman et al. 2004).

Following taking of the patient history, blood samples are collected on two separate days in the morning and serum samples are measured for serum LH, FSH and T. While the diagnoses of individuals with congenital syndromes involve largely a clinical axamination, in males over 40 presenting with age-related androgen deficiency, the diagnosis of hypogonadism is not as well defined and very controversial. In such cases, the Endocine Society of Australia guidelines state that primary hypogonadism is diagnosed if the individual has clinical symptoms of androgen deficiency and low (below 8nmol/L) serum T and high LH levels (above 15 IU/L). Secondary hypogonadism is diagnosed if the individual has clinical symptoms of androgen deficiency and low (below 8nmol/L) total T and normal or low LH

levels, or between 8-15nmol/L total T with low LH levels (ESA 1999). However, similarly to the diagnosis of individuals with congenital disorders discussed above, these biochemical measures are utilised only to provide verification of a suspected diagnosis of hypogonadism based on clinical symptoms of androgen deficiency.

The predominant assays utilised for the measurement of serum T are a variety of automated platform immunoassays. Each assay has its own reported reference range, often established without clinical considerations and with variation as much as 350% between laboratories for the low and high values (Sikaris et al. 2005; Lazarou et al. 2006). The lack of standardisation of reference ranges leads to problematic inter-laboratory comparisons, and makes international guidelines for hypogonadism difficult to establish. Another disadvantage of automated immunoassays are their inability to accurately measure very low levels of androgens, such as those found in women and children (Moal et al. 2007), thereby generating a great deal of uncertainty about the androgenic status of these individuals and therefore the ability to diagnose androgenic disorders. Even though gas or liquid chromatography combined with mass spectroscopy displays the specificity of detection required and is advocated in these groups, there are no current methods that provide the sensitivity of detection required. Derivatives of this technique are currently being developed for more routine testing (Rauh et al. 2006; Kalhorn et al. 2007; Nishio et al. 2007). Given these current limitations, there is no current clinically acceptable routine method to measure serum T in individuals with low serum T such as in women, children and the male with frank hypogonadism (Wang et al. 2004).

Critically, there is also a lack of understanding of the physiological meaning of serum levels of T as they only weakly correlate with measures associated with androgen action measures such as grip strength, sexual function and erectile function (Rosen et al. 1997; Baumgartner et

al. 1999; Tsujimura et al. 2003; Seftel et al. 2004; Mohr et al. 2007; Schaap et al. 2007; Yassin et al. 2007).

There are some indications that serum T may not reflect the androgenic status within tissues. Medical castration of males for treatment of prostate cancer results in a decrease of 94% in serum T levels whilst prostate tissue levels only decrease by 75-80%, with the continued expression of androgen regulated genes in the prostate (Page et al. 2006; Mostaghel et al. 2007). Furthermore, testosterone replacement in individuals with adult onset hypogonadism increases serum levels of T but fails to increase intraprostatic androgens (Marks et al. 2006). Collectively, these results indicate that measurements of serum T levels do not reflect the physiological androgenic status within tissues.

In addition to the issues surrounding the physiological meaning of serum T measures, the symptoms of hypogonadism vary from individual to individual, the threshold at which males experience symptoms of androgen depletion differs between individuals, and many of the symptoms of androgen depletion reflect those of aging, making a diagnosis purely on the basis of symptoms of androgen depletion problematic (Kelleher et al. 2004; Kaufman et al. 2005). Furthermore, many different lifestyle or chronic conditions may impact on the level of serum T. For example, it has been demonstrated that serum T is lower in men that are obese (Allan et al. 2006) and in males that have 1 of a list of chronic conditions including cancer, coronary heart disease, hypertension, diabetes and ulcer (Gray et al. 1991). These issues highlight the need for a measure of androgen that better reflects the physiological actions of androgens in males.

1.3 Cellular androgen availability

To further complicate the above issues, once T enters the circulation, it binds to serum albumin or sex hormone binding globulin (SHBG) which may restrict the availability of T to target tissues. It has been postulated that steroid binding to albumin or SHBG provides a

buffer for T in the circulation so that cells do not become flooded with T during episodic secretion from the testis, that it allows circulation of larger amounts of hydrophobic T in circulation and also protects T from metabolism and degradation, particularly by the liver (Tait et al. 1991). However, because of this interaction with serum binding proteins, it is unclear how much of the total circulating levels of T may be able to enter cells and mediate biological effects. In other words, it is not known what proportion of the total T in serum is bioactive. The free hormone hypothesis involves the commonly-held assumption that the weakly bound albumin-T complex (53-55% of the total T in serum) is capable of dissociating and crossing the cell membrane to mediate cellular biological effects while in contrast, SHBG-bound T (43-45% of the total T in serum) is bound with such a high affinity it is unable to dissociate and pass the cell membrane and is therefore assumed to be unavailable to cells. The remaining 2% of testosterone is unbound and capable of crossing cell membranes by diffusion (Sodergard et al. 1982; Mendel 1992). Currently, in research studies, total T is measured by an automated platform immunoassay as described above while bioavailable T (BT) is either measured directly from the serum by precipitation (immunoassayed BT; iBT) or calculated as the non-SHBG bound portion of T (calculated BT; cBT)(Table 1.1)(O'Connor et al. 1973; Vermeulen et al. 1999). The proportion of T not bound by either serum binding protein (free T – FT) may be assayed by equilibrium dialysis or calculated based on the levels of total T, SHBG and albumin in serum and their binding coefficients (Vermeulen et al. 1999).

Evidence suggests that FT is most reliably calculated (cFT) in comparison to measurement by analog platform immunoassay (Table 1.1)(Vermeulen 2004). Various methods have been proposed to measure or calculate FT. Currently there is no universally agreed method for calculating the FT fraction. A recent report by Ho et al, 2006 reported that a commonly used cFT equation proposed by Vermeulen et al in 1999 may overestimate the FT fraction, while another empirical equation proposed by Ly et al in 2005 may underestimate the cFT fraction.

While the validity of utilising cFT measures to diagnose androgen insufficiency remains questionable, a recent research study of 2932 men aged between 70-89 years demonstrated that higher cFT is associated with better cognitive function in older men while higher total T is not (Yeap et al. 2008). Furthermore, the demonstrations that men with higher waist circumference or erectile function have been shown to have lower levels of cFT (Foresta et al. 2003; Derby et al. 2006) suggests that cFT may provide an independent measure of testosterone action in comparison to total T in the context of these large research studies.

Name	Abbreviation	Fraction	Measured by
Total T	total T	total	automated platform immunoassay
Bioavailable T	BT	non-SHBG bound	ammonium precipitation and automated platform immunoassay or calculated
Free T	FT	non protein bound	equilibrium dialysis or calculated
Bioactive T	BioT	???	bioassay

Table 1.1: Fractions of serum T and their methods of measurement

While it has not been conclusively demonstrated that any of the serum fractions of T are better than each other in describing the physiological status of an individual, it has been shown in one study that serum BT is more closely associated with muscle mass, strength and bone mineral density than total T (van den Beld et al. 2000). However, there is also evidence that the free hormone hypothesis may be an oversimplification of androgen bioavailability. SHBG-bound testosterone is capable of being actively transported into cells via a membrane bound low density lipoprotein receptor, megalin (gp330/LDLR2)(Hammes et al. 2005). This process involves the endocytosis of the SHBG-bound T, followed by the intracellular metabolism of SHBG and the release of T into the cell. In rat choriocarcinoma cells, this mechanism has been estimated to contribute more T to the cell than the remaining portions of serum T combined (Hammes et al. 2005).

Some T target tissues are capable of synthesizing their own T from adrenal precursors and different tissue types have different efficiencies with regard to the metabolism of T (Labrie et al. 1988). An example is the cell specific levels and activities of enzymes associated with the

metabolism of T such as the expression of 5α-reductase which reduces T to the more potent androgen DHT, will also influence the cellular actions of T. In addition to its role in the inactivation of serum T discussed in section 2.1.4, 5α-reductase may be able to increase the potency of T by conversion to DHT in tissues that require high levels of androgen action for differentiation functions. For example, males that posess an inactivating germline mutation in the gene for 5α-reductase type 2 have normal male internal genitalia at birth with male psychosexual behaviour and voice deepening at puberty but ambiguous or female exterior genitalia and undeveloped prostate glands at birth with female body hair growth at puberty. This indicates that while DHT is required for the development of the prostate gland and male body hair patterns at puberty and the development of the external genitalia, T itself is capable of initiating the development of the internal male genitalia, male psychosexual behaviour, muscle development, voice deepening and spermatogenesis (Randall 1994). Collectively, this data indicates that different tissues have differential requirements for the potency of androgen action and therefore differing requirements for the actions of T and DHT. The expression of 5α-reductase plays an essential role in governing tissue specific responses to T in target tissues and is also not considered by the free hormone hypothesis.

Further evidence against the assumptions of the free hormone hypothesis is that T binding to a putative membrane receptor results in the release of intracellular calcium leading to cell proliferation (Benten et al. 1999) and it has also been demonstrated that unbound SHBG binds to as yet unidentified cell surface receptors whereupon T binding results in the rapid induction of adenylyl cyclase (Hryb et al. 2002). These reports, combined with the findings that serum total T only weakly correlates with the levels of T within tissues (Jarow et al. 2005; Page et al. 2006; Mostaghel et al. 2007), suggest that current assumptions of hormone availability to cells, while useful in describing T binding dynamics within the circulation and at specific cell membranes, can not describe the actions of T within all target tissues and therefore the actions of T physiologically.

1.3.1 Serum androgen bioassays

In order to address this limitation, several serum androgen bioassays in mammalian cells have been developed and described in the literature (Raivio et al. 2001; Paris et al. 2002; Sonneveld et al. 2004; Chen et al. 2006; Roy et al. 2006). All of these bioassays involve utilising a luciferase-based transient or stable transactivation assay in order to quantify bioactive testosterone (BioT) in the serum. BioT is defined as the proportion of androgens in serum capable of binding to the AR and activating the expression of a quantifiable reporter protein (in all currently reported cases, luciferase)(Table 1.1)(Raivio et al. 2001). Despite being developed to measure bioactive androgens in serum, reports of current bioassays have not attempted to correlate their measure with physiological parameters associated with androgenic action as most currently developed bioassays are tested on very small sample sizes. In addition, while some of these bioassay measures show excellent correlations with serum total T (Raivio et al. 2001; Chen et al. 2006; Roy et al. 2006), these assays do not address the relationship of their assay measure to FT or BT. In summary, it is currently unknown what proportion of serum androgens these bioassays are measuring and if the resulting measure is more closely related to physiological parameters associated with androgenic action than the current routinely utilised measures of serum T.

1.4 Cellular androgen action

Once androgens enter the cell, genomic androgen action is mediated by the AR, which acts as a transcription factor to activate or repress transcription of androgen regulated genes in a cell and promoter-specific fashion. However, there is also good evidence that androgens are capable of mediating non genomic effects via signal transduction cascades within cells without the mediation of the AR. This non genomic steroid action involves the rapid induction of second messenger signal transduction cascades, and activation of protein kinase A (PKA) (Nazareth et al. 1996; Sadar 1999), protein kinase C (PKC) (Jasavala et al. 2006) and mitogen activated protein kinase (MAPK) (Gatson et al. 2006), as well as increases in intracellular calcium have been shown (Steinsapir et al. 1991; Lieberherr et al. 1994;

Gorczynska et al. 1995; Benten et al. 1999; Lyng et al. 2000; Sun et al. 2006). There are two classes of non-genomic androgen effects, those that are mediated by the AR, which can be blocked with androgen receptor antagonists such as bicalutamide (BIC) or hydroxyflutamide (OHF) and those non-genomic effects that can not be blocked with these agents and are therefore presumed to be independent of AR function. In both classes, inhibitors of transcription or translation fail to act directly on these pathways and these effects can be observed in the time frames of seconds or minutes rather than hours (Heinlein et al. 2002). Evidence indicates that overall androgen effects on a cell may be a balance between AR-genomic, AR-nongenomic and non-AR mediated signalling cascades (Gatson et al. 2006). This thesis focuses on the genomic effects of androgens as mediated by the AR.

1.4.1 The androgen receptor

The AR is a class III nuclear receptor, which comprises all of the steroid receptors including the estrogen receptors α and β (ERα and ERβ), progesterone receptor (PR), mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) as well as the orphan estrogen related receptors (ERR1 and ERR2) (Laudet 1997). All of these receptors diverged from an ancestral receptor which preferentially binds contemporary estrogens (Thornton et al. 2003). The AR signalling cascade involves association with chaperone proteins, ligand binding, nuclear translocation and DNA binding as well as association with coregulatory molecules resulting in subsequent regulation of the transcription of target genes.

1.4.2 Androgen receptor expression

The *AR* gene spans over 90kb at Xq11.2-12 and consists of 8 exons (Fig 1.3). The region -74 to +87 around the transcription start site of *AR* is necessary and sufficient to induce expression, and the gene utilises this promoter region for expression in all tissues (Tilley et al. 1990; Takane et al. 1996). However, there are two major transcription initiation sites within the promoter located 13 base pairs apart with each initiated by different regulatory sequences within the promoter (Faber et al. 1993). This *AR* promoter contains no TATA box for binding TATA binding protein (TBP) or CAAT sequence for Sp1 binding but does contain a GC-box

and a long homopurine stretch which form a Sp1 binding site (Fig 1.4)(Tilley et al. 1990; Faber et al. 1993; Takane et al. 1996; Chen et al. 1997). The region between -530 to -380 in the *AR* promoter is required for up regulation of AR mRNA by cAMP (Mizokami et al. 1994). In addition, the AR promoter contains three human T-cell factor 1/lymphoid enhancer factor (TCF/LEF) binding sites which, when stimulated by Wnt/β-catenin, confer up regulation of AR expression in prostate cells (Fig 1.3) (Yang et al. 2006). Paradoxically, the Wnt/β-catenin signalling pathway decreases AR protein levels via the up regulation of Mdm2, an E3 ligase involved in the ubiqutiniation and subsequent degradation of AR via the 26S proteasome (Yang et al. 2006). Autoregulation of the *AR* has also been demonstrated but the sequences for this self regulation do not lie within the *AR* promoter region. Four AR binding sites (ARBS) have been identified within *AR* exons that bind and up regulate AR mRNA levels in prostate and osteoblastic cell lines (Grad et al. 1999).

The AR protein contains 4 modular domains (Lubahn et al. 1988; Tilley et al. 1989) (Fig 1.3). The AR amino terminal domain (NTD) is the most divergent region of the receptor while the DNA binding domain (DBD) is highly conserved amongst the nuclear receptors and consists of two cysteine-rich zinc fingers that directly bind to specific DNA sequences in the genome (Thornton et al. 1998; Shaffer et al. 2004). A short non-conserved hinge region separates the DBD from the AR ligand binding domain (LBD) responsible for binding androgenic ligands. Each of these domains is discussed in more detail below.

1.4.3 Androgen receptor maturation

While direct evidence for the role of the chaperone system in AR signalling is sparse, inferences can be drawn from what has been discovered for the other nuclear receptors, and the general actions of the chaperone system within cells. During translation of a steroid receptor, it undergoes several cyclic periods of binding and release in an ATP-dependent manner to the constitutively expressed variant of heat shock protein 70 (Hsc70) and heat shock protein 40 (Hsp40/Ydj1), which induces local folding of the nascent protein

(Buchberger et al. 1996). After the binding of Hsc70 and Hsp40, Hsp interacting protein (Hip/p48) binds and stabilises the Hsc70/Hsp40/receptor complex. The subsequent binding of Hsp organizing protein (Hop/p60) allows the recruitment of a homodimer of Hsp90 to the complex, forming a bridge between the receptor and Hsp90 (Davies et al. 2005). The final mature complex is formed via the binding of p23 to Hsp90 and the dissociation of Hip, Hop and Hsc70 which allows for the recruitment of a tetratricopeptide repeat TPR-containing immunophilin to Hsp90, such as small glutamine-rich tetratricopeptide repeat containing protein α (SGTα) (Davies et al. 2005; Buchanan et al. 2007)(Fig 1.4). This complex holds the receptor in a relatively stable, active-ready state for ligand binding.

1.4.4 Androgen receptor ligand binding

The AR LBD consists of 9 α-helices, two 3_{10} helices and four short β-strands associated in two anti-parallel β-sheets arranged in an α-helical sandwich around the ligand binding pocket (Matias et al. 2000). Ligand binding to the LBD induces a conformational change in this domain which is similar for all of the nuclear receptors. The most carboxyl-terminal α-helix, helix 12, appears to alter in conformation with different agonists or antagonists, closing to form a lid on the ligand binding pocket with agonist binding, but is pushed away upon antagonist binding (Bohl et al. 2007). The closure of helix 12 on the ligand binding pocket by agonist binding leads to the formation of a hydrophobic cleft termed activation function 2 (AF2). Many coregulators have now been identified that interact with the AF2 surface of the nuclear receptors, including the AR. An important family of steroid receptor coactivator proteins that possess LxxLL motifs and interact with the AF2 surface is the p160 family of coregulators that includes the nuclear receptor coactivators NCOA1 (SRC-1), NCOA2 (GRIP1/TIF2) and NCOA3 (AIB1/RAC3). However, the utilization of this AF2 pocket by the AR is unique amongst the steroid receptors. For other steroid receptors, AF2 provides the majority of the capacity of the receptor to activate transcription by binding the LxxLL motif such as that found in the p160 coregulators with high affinity. In contrast, the AR AF2 surface preferentially binds bulky FxxLF moieties such as the ^{23}FQNLF^{27} peptide in the NTD due to

side chains that are unique to the AR (He et al. 2004; Estebanez-Perpina et al. 2005). Therefore, the AR AF2 is relatively weak in its potential to mediate gene expression in mammalian cells in comparison to the other steroid receptors (He et al. 2004). The AR LBD residues involved in the binding of these FxxLF peptides are conserved amongst species (Fig 1.5).

1.4.5 The AR amino-carboxyl (N/C) interaction

As described above, the ^{23}FQNLF^{27} peptide in the AR NTD interacts with the AF2 surface upon agonist binding, with this interaction termed the N/C interaction. Physiologically, the AR N/C interaction increases the stability of the agonist bound receptor (He et al. 2001), but is not required for the competency of AR to activate some exogenous reporter genes (He et al. 2002; Buchanan et al. 2004). Although it has been suggested that the N/C interaction may be required for transactivation activity of a chromatin integrated reporter gene, only one reporter has been investigated in such a context (Li et al. 2006). In addition, it has been demonstrated that the ^{432}WHTLF^{436} peptide plays a role in the N/C interaction as assessed by the mammalian 2-hybrid interaction assay. Deletion of this motif decreases the stability of the ligand bound complex but its specific role in the N/C interaction remains to be defined (He et al. 2000). The N/C interaction may play an important role in AR function as is evidenced in individuals with androgen insensitivity syndrome. Genetically male individuals with germ line missense mutations in the LBD (e.g. D695N, R774H and L907F) display phenotypic female characteristics despite having a receptor with normal or only slightly reduced ligand binding. These mutations abolish the N/C interaction perhaps leading to decreased AR transactivation activity, indicating that the N/C interaction may be vital for the function of the AR in males (Jaaskelainen et al. 2006).

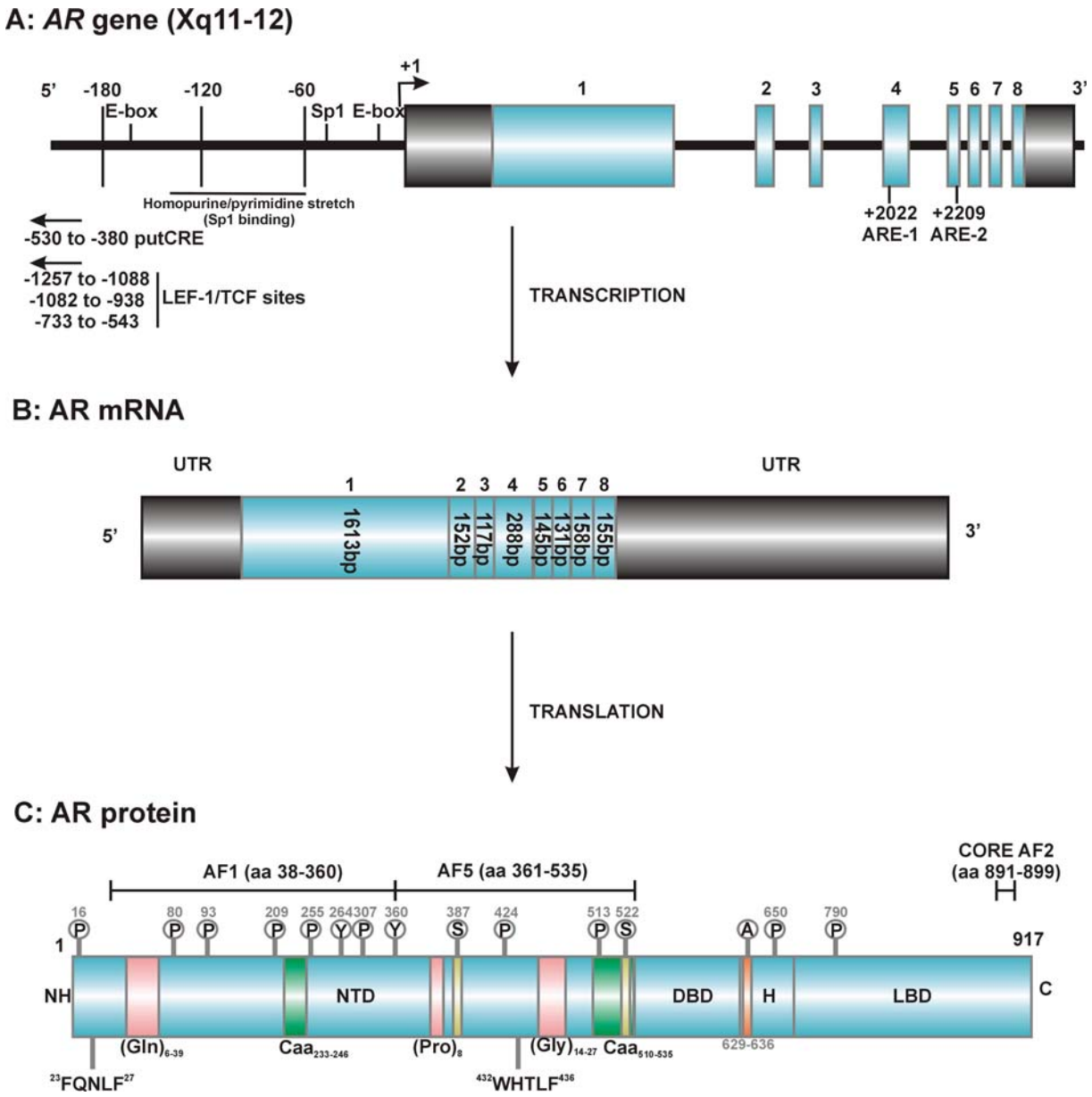


Figure 1.3 The *AR* gene, mRNA and protein structure. A: The *AR* gene consists of 8 exons, transcribed from a promoter containing no TATA or CAAT box. Instead, the promoter contains a long homopurine or pyrimidine stretch along with several Sp1 binding sites and two E boxes that have been implicated in binding an unknown protein containing helix-loop helix structures. In addition, there is a putative cAMP response element between -530 to -380 in the promoter (putCRE) as well as several βcatenin/Wnt binding motifs (LEF-1/TCF sites). Autoregulatory ARE sequences (ARE-1, ARE-2) within the coding sequence of the AR are within exons 4 and 5 and upregulate AR mRNA. **B:** The mature AR mRNA is 3569 bp long and contains a long 3' untranscribed region (UTR) and a short 5' UTR. **C:** The AR protein is 917 amino acids long and contains a long amino terminal domain (NTD), DNA binding domain (DBD), hinge region (H) and ligand binding domain (LBD). The AR LBD contains amino acids which form the activation function 2 (AF2) pocket upon ligand binding. The AR NTD contains two activation functions, 1 and 5 (AF1 and AF5). Caa: conserved amino acids in the AR NTD between species. $(Gln)_{6-39}$, polyglutmine repeat region; Pro_8 polyproline repeat region; $(Gly)_{14-27}$ polyglycine repeat region; A acetylation site; P serine phosphorylation site; Y tyrosine phosphorylation site, ^{23}FQNLF^{27} and ^{432}WHTLF^{436} motifs are shown in their respective locations.

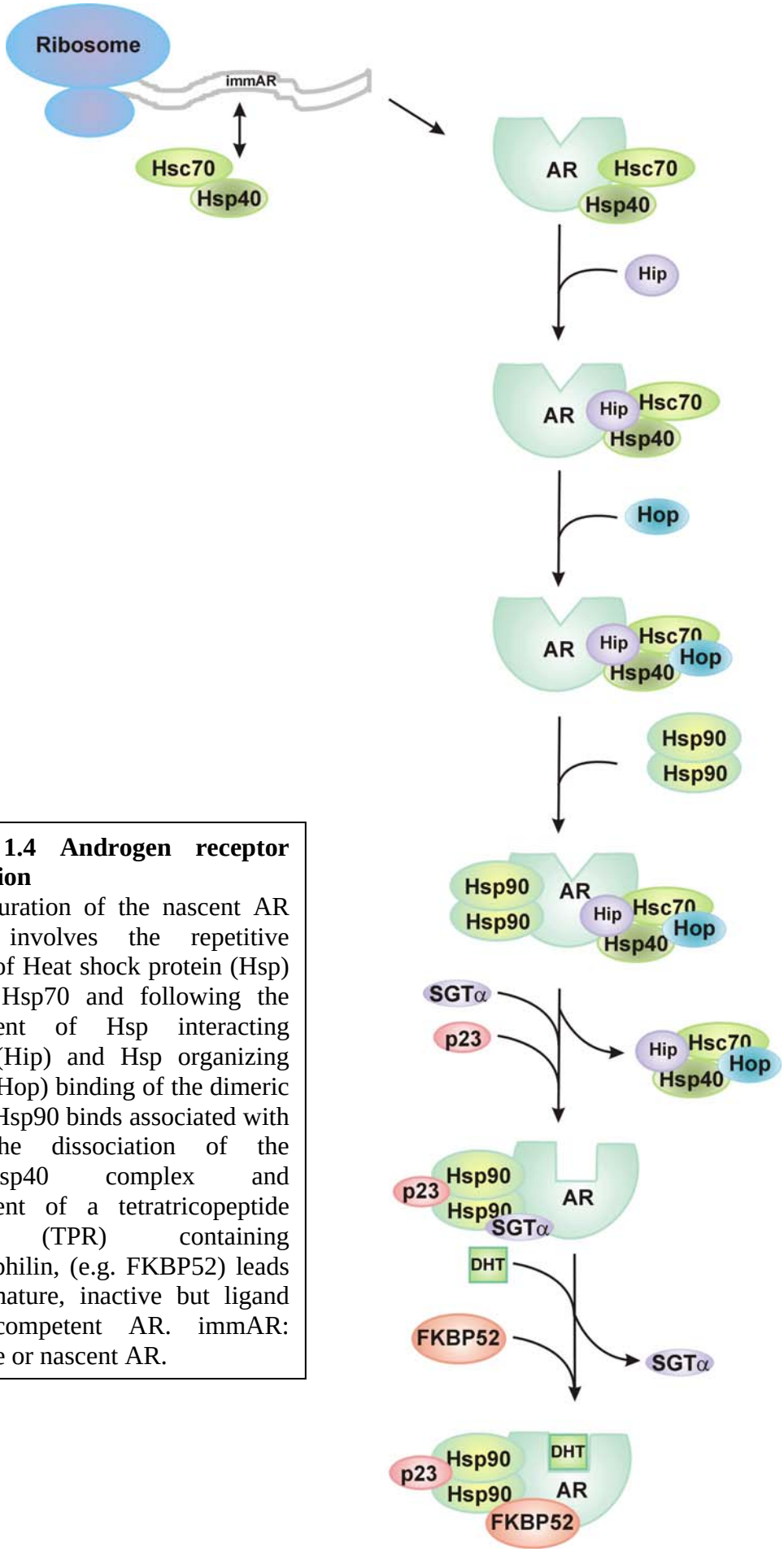


Figure 1.4 Androgen receptor maturation
The maturation of the nascent AR protein involves the repetitive binding of Heat shock protein (Hsp) 40 and Hsp70 and following the recruitment of Hsp interacting protein (Hip) and Hsp organizing protein (Hop) binding of the dimeric form of Hsp90 binds associated with p23. The dissociation of the Hsp70/Hsp40 complex and recruitment of a tetratricopeptide repeat (TPR) containing immunophilin, (e.g. FKBP52) leads to the mature, inactive but ligand binding-competent AR. immAR: immature or nascent AR.

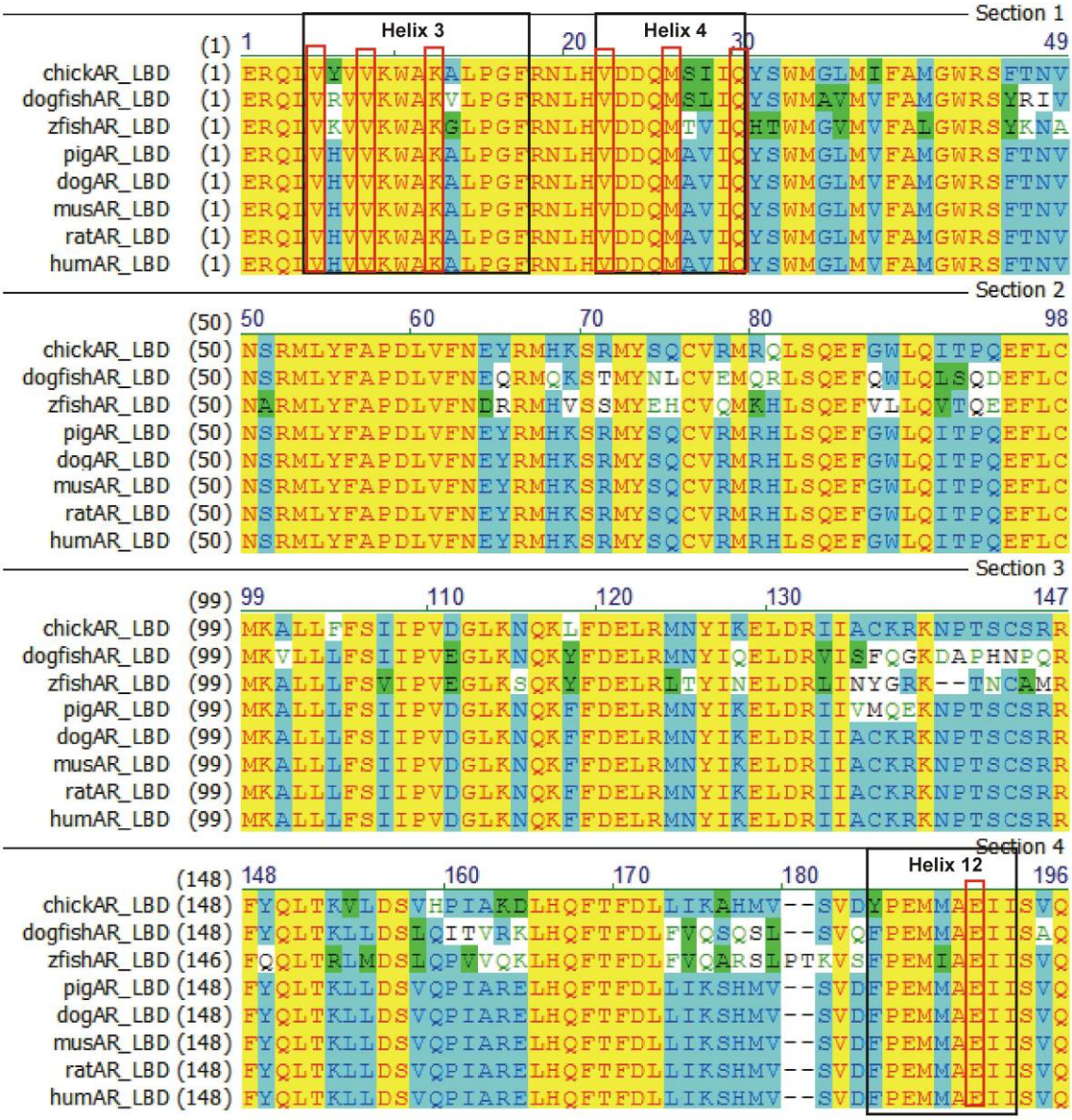


Fig 1.5: Conservation of activation function 2 amino acids in the AR ligand binding domain involved in LxxLL and FxxLF binding

While the AR LBD is well conserved amongst species, the absolute conservation of the residues involved in FxxLF and LxxLL binding according to crystallisation studies are boxed in red (helix 3 residues V713, V716 and K720; helix 5 resides V730, M734 and Q738 and helix 12 residue E895). Black boxes represent the portion of the helix involved in formation of the pocket. The reference sequence for each species was downloaded into the Vector NTi computer suite (Accession numbers: Tilley: human AH002607; rat: *Rattus norvegicus* NP_036634; mus: mouse *Mus musculus* NP_038504; dog: *Canis lupus familiaris* NP_001003053; pig: *Sus scrofa* accession number NP_999479; dogfish: *Squalus acanthias* AAP55843; chicken: *Gallus gallus* NP_001035179; zfish: zebrafish *Danio rerio* NP_001076592) and aligned utilising Align X, which operates on the ClustalW algorithm. Residues with a yellow background indicate complete identity between species, a green background indicates similar amino acids and blue indicates resides containing conservative amino acid differences between species. Weakly similar are shown as a green on white background while non similar amino acids between species are indicated with a black on white background.

Classically, the AR N/C interaction has been investigated utilizing partial AR proteins fused to yeast DNA binding and viral activation domains in an assay known as the mammalian 2-hybrid interaction assay. Due to the utilization of partial proteins in this assay, for many years there has been considerable confusion about the definition and therefore the role of the N/C interaction in AR signalling. For example, is the N/C interaction an interaction that occurs upon conformational change following ligand binding (intramolecular interaction) or is it an interaction that involves homodimerisation (intermolecular interaction) (Fig 1.6)?

Recent advances in protein-protein interaction imaging techniques in live cells with full length fluorescent protein tagged AR (fluorescent resonance energy transfer; FRET) have shown that the AR undergoes a rapid conformational change in response to agonist binding, bringing the NTD in close proximity with the AF2 surface (Schaufele et al. 2005; van Royen et al. 2007). This intramolecular N/C interaction was found to persist until the receptor bound to DNA. In fact, it was only observed while the AR molecule was mobile (van Royen et al. 2007), suggesting that the N/C interaction promotes stability and prevents undesirable interactions with coregulators in non-DNA bound ARs only (van Royen et al. 2007). This proposal is strengthened by the demonstration that interaction of the AR with two coregulators (RNF14/ARA54, NCOA4/ARA70) is increased for a receptor incapable of forming an N/C interaction, perhaps due to a vacant AF2 pocket to which LxxLL-containing coregulators can bind (Toumazou et al. 2007). It has also been reported utilizing the FRET method that an interaction could be measured in the nucleus between two AR molecules (intermolecular interaction) (Schaufele et al. 2005). However, the effect of AR amino- and carboxyl-terminal fluorescent tags on the ability of the AR to activate gene transcription has not been well documented, and questions remain as to the true nature of the N/C interaction in AR signalling.

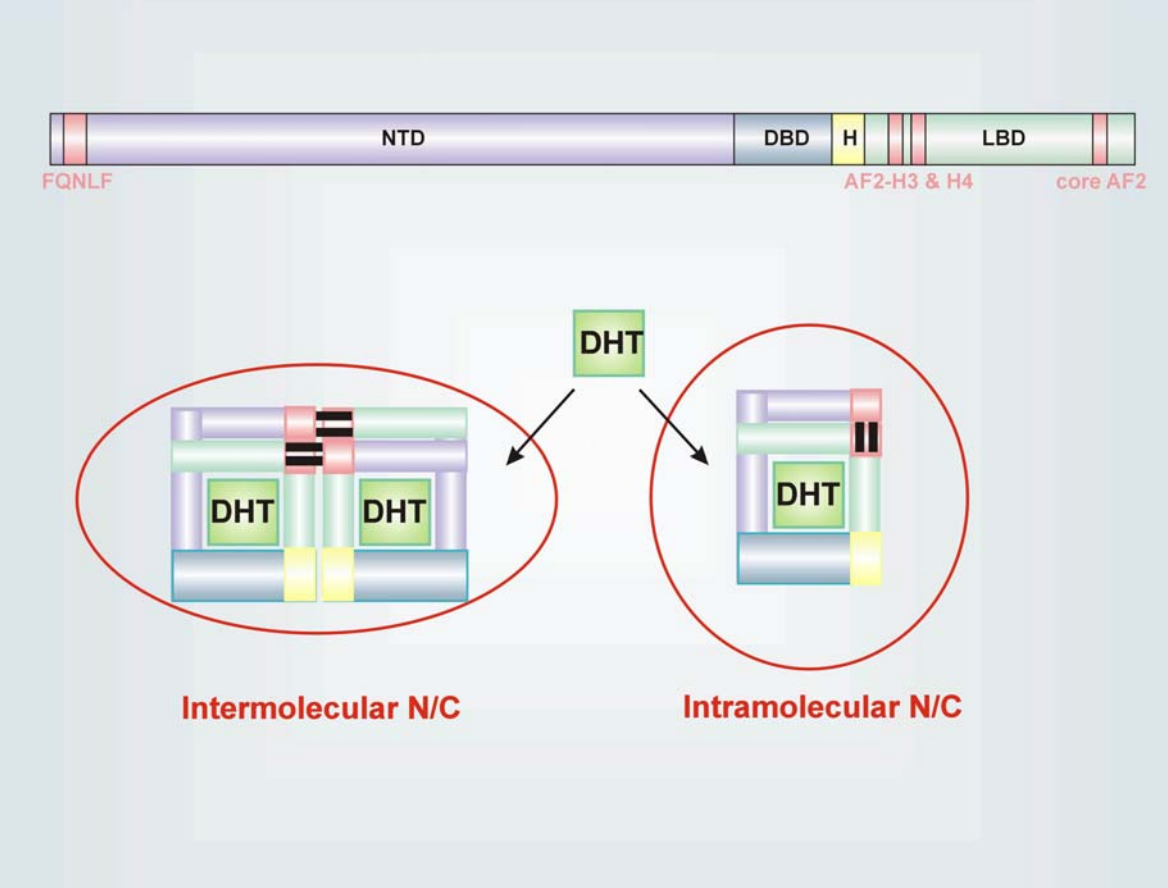


Figure 1.6: A model of two alternate forms of the AR N/C interaction. Ligand binding induces a rapid conformational change in AR structure, which has been proposed to be the N/C interaction (Intramolecular N/C). The N/C interaction has also been proposed to be a dimerisation event between two AR molecules (intermolecular N/C). AR is depicted at the top and domains within the AR are depicted via the indicated colours with FQNLF in the NTD and regions required for AF2 formation shown in pink. Double black lines indicate interaction between these domains of the AR. DHT: Dihydrotestosterone; AF2: Activation function 2; FQNLF: the ^{23}FQNLF^{27} moiety in the AR NTD, H3 helix 3 and H4 helix 4 of the ligand binding domain.

1.4.6 Nuclear-cytoplasmic shuttling

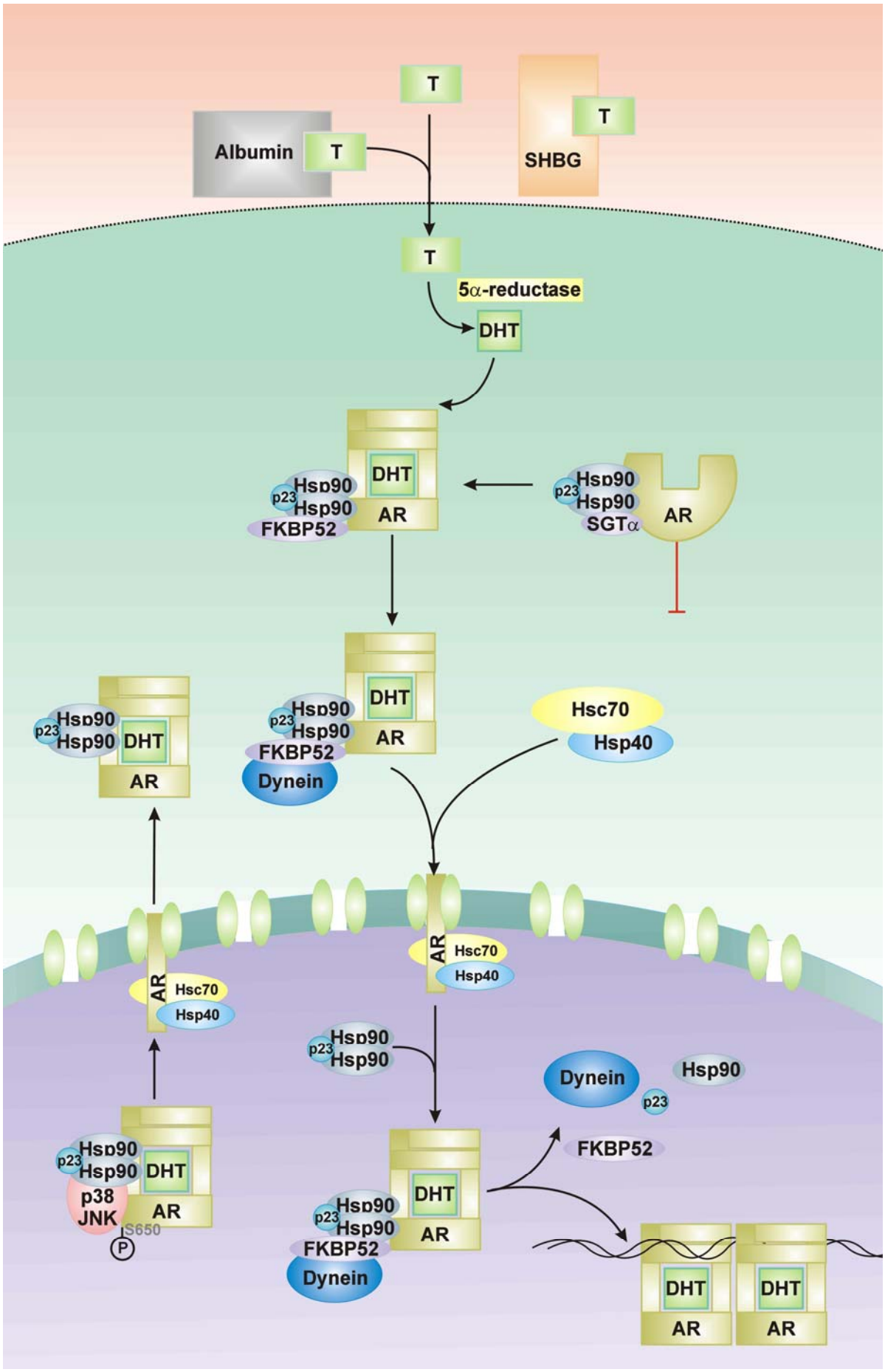
In order for the AR to exert its effect on the transcription of genes, the active receptor is required to translocate from the cytoplasm to the nucleus. The signal for nuclear localisation (NLS) in the hinge domain of the receptor contains a conserved sequence which when acetylated leads to receptor nuclear translocation (Haelens et al. 2007). While it was initially reported that hormone binding caused the dissociation of Hsp90 from the AR prior to nuclear translocation, it is now known that Hsp90 is required for translocation of the AR from the cytoplasm to the nucleus (Georget et al. 2002). Indeed, a general model for the steroid receptors has been proposed where the earliest event in translocation is not the complete dissociation of the multiprotein complex, but the exchange of a tetratricopeptide repeat-containing immunophilin (for example, FK506-binding protein 5 [FKBP51]) for any of the immunophilins FKBP52, cyclophilin 40 (Cyp40) or protein phosphatase 5 (PP5) on Hsp90, along with the recruitment of dynein, a transport protein linked to the cellular cytoskeleton (Fig 1.7) (Davies et al. 2002; Davies et al. 2005). This recruitment allows the retrograde transport of the receptor along the microtubules, with the specific immunophilin providing the guide as to the final destination of the complex within the nucleus (Owens-Grillo et al. 1996). In addition to the involvement of Hsp90 and immunophilins, Hsc70 along with Hsp40 is thought to provide the means for the receptor to cross the nuclear membrane via local unfolding and refolding (Caplan et al. 1991). Therefore, it is currently alleged that it is not until the receptor reaches the nucleus that it dissociates from the minimal complex (Davies et al. 2002)(Fig 1.7). While the exact mechanism of AR nucleo-cytoplasmic shuttling is still unclear, the relevance of this generalized mechanism to AR localization is supported by the discovery that small glutamine rich tetratricopeptide repeat-containing protein α (SGTα) specifically binds to the AR-chaperone complex and prevents nuclear localization until the receptor binds ligand and is then exchanged for the immunophilin FKBP52 (Fig 1.7)(Buchanan et al. 2007). Conversely, phosphorylation of AR at Ser 650 by p30 or JNK kinase induces nuclear export (Fig 1.7) (Gioeli et al. 2006).

1.4.7 Androgen receptor DNA binding and dimerisation

Binding of DNA by the AR is a homodimerisation event at specific sequences known as hormone response elements (HRE). There are two types of sites that the AR homodimer is capable of binding to, a more general type of HRE or consensus HRE and an androgen response element (ARE). The consensus HRE consists of a hexameric inverted repeat separated by a three nucleotide spacer to which the PR, GR and MR can also bind and has the general sequence 5'-AGAACAnnnTGTTCT-3'(n: any nucleotide). The AR is also uniquely capable of binding to direct repeats of the form 5'-AGAACAnnnAGAACA-3', by virtue of specific residues in the second cysteine-rich zinc finger of the DBD and a 12 amino acid carboxyl-terminal extension of the DBD (Haelens et al. 2003).

Structurally, the AR DBD is highly conserved in relation to the other nuclear receptors and consists of two cysteine rich zinc fingers, the first of which contains residues in the conserved P box responsible for the specificity of response element DNA binding and contacts the major groove of DNA (Shaffer et al. 2004). Amino acids in the D box of the second finger are responsible for the homodimerisation of the AR on response elements (Shaffer et al. 2004). In contrast to all of the steroid receptor DBD-DNA structures described, the AR homodimer binding to selective response elements does not reflect the same orientation of the DNA target; the AR homodimer bound to the direct repeat in the ARE displays instead a head-to-head arrangement which is similar to the configuration of GR bound to the inverted repeats in the consensus HRE. Amino acids Ser 580, Thr 585 and Ala 579 in the AR D box contribute to the ability of the AR to adopt this structure; mutation of the GR DBD from Gly to Ser or mutation of Ile to Thr in these positions results in GRs capable of binding to inverted and direct repeats (Shaffer et al. 2004). While this general model displays the mechanism of selective AR binding on HREs, the DNA sequences to which AR will bind vary greatly in the genome, as shown in several recent studies demonstrating a wide array of sequences and arrangements of potential AR binding sites (Bolton et al. 2007; Massie et al. 2007; Wang et al. 2007).

Figure 1.7 AR nucleocytoplasmic shuttling. The ligand bound AR is stabilised via the binding of a Hsp90 dimer along with p23 and one of the tertratricopeptide repeat (TPR) containing immunophilins FKBP52, Cyp40 or PP5; FKBP52 is shown here for clarity. The AR is also capable of binding small TPR containing protein α (SGTα), which inhibits non-hormone bound receptor translocation. The immunophilin provides the stimulus for the association of the receptor complex with dynein, which allows transport of the receptor complex along cytoskeleton microtubules. Hsc70 and Hsp40 are required for the transport of the receptor across the nuclear membrane, indicating that local folding and unfolding of the receptor complex is required for entry and exit into and out of the nucleus. p38 or JNK kinase activity phosphorylates S650 of the AR and leads to nuclear export of the receptor. FKBP52: FK506 binding protein, 52kDa; AR: androgen receptor; Hsp90: Heat shock protein 90; Hsc70: Constitutively expressed variant of Heat shock protein 70; Hsp40: Heat shock protein 40, Ydj1; SHBG: Sex hormone binding globulin; p50: Cdc37.


T
Albumin
T
SHBG
T
T
5α-reductase
DHT
DHT
Hsp90
p23
Hsp90
FKBP52
AR
Hsp90
p23
Hsp90
SGTα
AR
DHT
Hsp90
p23
Hsp90
AR
DHT
Hsp90
p23
Hsp90
FKBP52
AR
Dynein
Hsc70
Hsp40
AR
Hsc70
Hsp40
AR
Hsc70
Hsp40
Hsp90
p23
Hsp90
Dynein
Hsp90
p23
FKBP52
DHT
Hsp90
p23
Hsp90
p38
JNK
AR
S650
P
DHT
Hsp90
p23
Hsp90
FKBP52
Dynein
AR
DHT
AR
DHT
AR

1.4.8 Nuclear organization of the AR

The utilization of fluorescently tagged AR molecules has demonstrated that the AR, upon nuclear translocation, concentrates in discrete foci. Experiments have demonstrated that these spots are enriched for GRIP1 and the histone-acetyl transferase CREB binding protein (CBP) (Saitoh et al. 2002; Black et al. 2004). Thus these spots may be sites where AR undergoes association with coregulators. Additionally, it has been demonstrated that while agonist-bound AR is mobile within the nucleus, approximately 20% of the receptor pool is immobile for longer periods. In contrast, the agonist-bound AR DBD variant (A575D) unable to bind DNA and unliganded AR molecules are more freely mobile. Imaging of the whole nucleus also demonstrated that the nuclear distribution of this A575D variant was more homogenous than wildtype AR (wtAR), without the characteristic nuclear spots of wtAR (Farla et al. 2004). These results suggest that any immobile AR fraction in the nucleus may associate with coregulators whilst bound to DNA. The AR coactivators Creb-binding protein (CBP), NCOA2 and the transcription factor TFIIH show similar locations and kinetics as AR in the nucleus, further supporting this hypothesis that agonist bound AR nuclear speckles are sites of active transcription (Becker et al. 2002; Hoogstraten et al. 2002; Kang et al. 2002).

1.5 Androgen regulated genes

Despite being defined as a transcription factor, until the development of assays capable of high throughput measurement of AR binding (chromatin immunoprecipitation on microarray chip – ChIP on chip), there were only very few genes whose expression the AR had been shown to directly regulate. These included the human serine protease kallikreins *KLK2* and *KLK3* (*prostate specific antigen, PSA*) (Clements et al. 2001), *probasin* in the rat prostate (Rennie et al. 1993), mouse *sex limited protein* (Slp) (Adler et al. 1993; Verrijdt et al. 2002), mouse *C(3)* (Tan et al. 1992) and *TMPRSS2* (Tomlins et al. 2005). However, in several studies aimed at the identification of AR target genes, it has been revealed that AR binds directly to DNA surrounding the gene's open reading frame but regulates genes in a cell

specific manner, due to the function of complementary transcription factors which have been shown to be essential for the modulation of the AR target gene (Massie et al. 2007; Wang et al. 2007). Surprisingly, ARBS were found to reside up to 500kB from the start site of certain genes, and in one study, only 38% of genes had ARBS within 10kB of a gene start site (Wang et al. 2007). In another study in immortalised human prostate epithelial cells utilising ChIP on a chip containing promoter regions only (104kB of genomic sequence from the start site of 548 candidate hormone responsive genes) and microarray analysis of the same genes, 172 putative AR target genes were identified. Several reports have now identified that promoter-regulated AR target genes are involved in cellular proliferation, cell to cell communication, differentiation and development, metabolic activation, cell cycle, response to chemical stimulus and/or stress and cellular lipid metabolism including steroidogenesis (Nantermet et al. 2004; Bolton et al. 2007; Prescott et al. 2007).

1.5.1 AR-mediated transcriptional activation

The inability of transcription factors to mediate transcription independently of chromatin remodelling complexes indicates the importance of chromatin structure in the regulation of transcriptional activation. All of the evidence for transcriptional activation points to the dynamic assembly of multiunit protein complexes in a cellular, temporal and gene specific manner (McKenna et al. 1999; McKenna et al. 2002; Bebermeier et al. 2006). Indeed, it is the sequential order of assembly of unique multiunit complexes that is considered to be responsible for the subsequent action of the steroid receptor to regulate the expression of the target gene (Kang et al. 2002; Metivier et al. 2003; Kang et al. 2004). Transcription factors, such as AR, are believed to utilize different combinations of cofactors for regulation of gene expression depending on the cell type, promoter and DNA binding site.

1.5.1.1 AR coactivators

There are two general classes of coregulators involved in gene transcription. Class I consists of enzymes that covalently modify DNA or proteins within the transcriptional complex and

includes enzymes that modify histone tails via deacetylation or acetylation such as histone deacetylases (HDACs) and histone acetyltransferases (HATs), or enzymes that modify DNA via methylation or demethylation (e.g. HMT and LSD1). Also included in this class are protein phosphatases and kinases, ubiquitin ligases, SUMO ligases and poly(ADP)ribosylases. Class II contains enzymes that are capable of ATP-dependent DNA remodelling, such as the components of the SWI/SNF complex that mediate chromatin remodelling (McKenna et al. 1999).

The p160 family of transcriptional coactivators are important mediators of the transcriptional activation capacity of all of the nuclear receptors. Members of this evolutionarily conserved family of coactivators possess intrinsic HAT activity, but also bind directly to the nuclear receptor and provide an interaction interface with the CBP/p300 histone acetyl transferase complex or protein methyltransferases (e.g.CARM1) which then allows remodelling of the DNA to provide an active site of transcription (Edwards 2000). The primary site of interaction of these coactivators with steroid receptors is the agonist-induced AF2 pocket, via LxxLL motifs in the nuclear receptor interaction domain of the protein. However, interactions of the p160's via their glutamine-rich amino termini with the NTD of steroid receptors have also been described (Ma et al. 1999; Tetel et al. 1999; Benecke et al. 2000; Vottero et al. 2002).

1.5.2 AR-mediated transcriptional repression

While the kinetics and interactions of AR mediated transcriptional activation have generated much interest over the last decade, the role of AR-mediated repression of genes is less well documented. Originally isolated as retinoic acid receptor and thyroid receptor interacting proteins, the nuclear receptor corepressor (NCoR) and the related silencing mediator for retinoid and thyroid hormone receptors (SMRT) are now both known to play an intrinsic role in AR-mediated transcriptional repression. Both of these corepressors are recruited to endogenous androgen response elements in response to antagonist (OHF), agonist (DHT) or the synthetic androgen receptor agonist, R1881 (Hodgson et al. 2005; Yoon et al. 2006). Like

coactivators, these corepressors exist in large mutiprotein complexes that include histone deacetylase 3 (HDAC3) and a WD-40 repeat-containing protein transducin (β)-like 1 (TBL1), that play structural roles in the complex (Li et al. 2000). These corepressor complexes compete with coactivator complexes for binding to AR (Perissi et al. 2004; Jouravel et al. 2007). Binding of the corepressor complex requires the nicotinamide adenosine dinucleotide-dependent histone deacetylase sirtuin 1 (SIRT1) and results in a suppression of AR-mediated transcriptional activation (Yoon et al. 2006; Dai et al. 2007). Furthermore, binding of NCoR to AR results in a decrease in the AR N/C interaction which can be reversed by the over expression of the p160 coactivator NCOA1 (Wu et al. 2006). The p160 coactivators and these two corepressors may therefore play opposing roles with respect to AR function and thereby provide a further level of transcriptional control within cells. This thesis focuses on the role of AR in transcriptional activation.

1.6 The AR amino terminal domain (NTD)

The AR NTD is responsible for the majority of the transcriptional activity of the AR, and is the largest domain of the receptor forming over half the protein. The AR NTD is believed to compensate for the weaker activity of the LBD AF2 surface by providing the majority of the transcriptional activity of the AR. Despite this intrinsic role in AR function, not a lot is known about the specific actions of the NTD with respect to AR function, and has largely been attributed to the disordered and non conserved nature of this domain (Thornton et al. 1998). More recently, it has been shown that coregulator binding to the AR NTD induces the formation of secondary structure within this domain. This implies that this domain may serve as a flexible platform for coregulator interactions and that the conformation of specific regions of this domain may be more important that the sequence *per se* (McEwan 2004).

The AR NTD is a 555 amino acid domain that contains two functionally different activation functions (activation functions AF 1 and 5, Fig 1.3), each delineated indirectly via their role in AR transactivation activity as measured by transactivation assays. The AF1 domain between

amino acids 38-370 is required for AR transactivation activity (Chamberlain et al. 1996; Lavery et al. 2006). Activation function 5, which lies between amino acids 360-535 was originally defined as the region responsible for the transactivation activity of the constitutive AR variant truncated at amino acid 707 (Jenster et al. 1995; Chamberlain et al. 1996).

It has been proposed that the occupancy of the AF2 pocket in the agonist bound form of the receptor due to the AR N/C interaction has lead to the evolution of a large amino terminal domain which provides an alternative site of binding for coregulators (He et al. 2004). Indeed, the p160 family of coregulators has been demonstrated to also interact with the AF5 region of the AR (Ikonen et al. 1997; Moilanen et al. 1998; Bevan et al. 1999; Powell et al. 2004). While the naturally disordered nature of the AR NTD has precluded crystallization, there is good evidence that protein binding to the AR NTD following ligand binding induces conformational changes leading to a more ordered structure and the subsequent recruitment of other coregulators. Amino acids 142-485 (C-terminal portion of AF1 and the N-terminal portion of AF5) interact with transcription factor IIF (TFIIF), and binding of this factor induces an alpha-helical structure in the NTD well suited for interactions with the p160 family of coregulators (McEwan et al. 1997; Kumar et al. 2004). Interactions of AR with TFIIF are important for transcription complex assembly and subsequent promoter clearing (Choudhry et al. 2006). While AF1 and AF5 have been grossly defined on the basis of their ability to initiate transcription of exogenous reporter genes, the relative contribution of these domains to AR activity in response to androgens has not been thoroughly described. Nevertheless, in addition to their more general roles in AR transactivation assays, each of these domains contain a number of sequences and elements (in addition to the ^{23}FQNLF^{27} and the ^{432}WHTLF^{436} peptides discussed above) which are implicated in a wide variety of cellular processes (see below).

1.6.1 The polyglutamine repeat

Amino acids 58-77 of the AR encode a polymorphic trinucleotide CAG repeat (Fig 1.3) coding for glutamine which in healthy individuals contains between 6-39 repeats (Tilley et al. 1989; Giovannucci et al. 1997). The length of this repeat within the normal range has been inversely correlated to transactivation activity, and lower repeat number has been associated with prostate cancer risk, although this association has not been consistently demonstrated (Giovannucci et al. 1997; Buchanan et al. 2004). There is now a vast array of papers attempting to correlate many indices of androgen action with the polymorphic CAG repeat. For instance, there are reports of an inverse correlation of cognitive function with CAG repeat number (Yaffe et al. 2003), an inverse correlation of bone density with CAG length (positive with bone turnover) (Zitzmann et al. 2001) and a positive correlation between fat free mass and number of CAG repeats (Walsh et al. 2005).

1.6.2 The polyglycine repeat

The polyglycine repeat between amino acids 448-470 (Fig 1.3) of the AR AF5 region is polymorphic in the normal population with a range of 14 to 27 repeats being reported. The role of this repeat region in AR function is not well defined, but larger repeat number has been associated positively with AR transcriptional activity, particularly in the context of a mutation in the hinge domain of the receptor (Werner et al. 2006). It has been demonstrated that endometrial cancers with shorter repeat length tend to be lower grade (Rodriguez et al. 2006), and higher number of repeats may be associated with cryptorchidism and penile hypospadias in males (Radpour et al. 2007).

1.6.3 The polyproline repeat

The poly proline is a short stretch of 8 proline repeats in the AF5 region of the AR NTD (Fig 1.3). This region is required for interaction of AR with the Src homology 3 (SH3) domain of the non receptor tyrosine kinase, Src. Abrogation of this interaction results in inhibition of DNA synthesis independent of the ability of the AR to activate the transcription of genes in

breast and prostate cancer cells, indicating that this region is important in the function of AR as a non genomic intracellular signalling molecule (Migliaccio et al. 2007).

1.6.4 Conserved regions of the AR NTD

While the AR NTD is highly divergent, there are two regions of the AR NTD that are conserved amongst all known AR NTD sequences. The AR NTD signature sequence resides in the AF1 domain between amino acids 233-246 (Fig 1.3) and enforced expression in the mouse prostate of a mutation in this region (human E235G, mouse E231G) has been demonstrated to be oncogenic (Han et al. 2005). In addition the ubiquitin ligase C terminal of the Hsc70-interacting protein (CHIP) binds to this region of the AR and promotes the degradation of the receptor (He et al. 2004). Another region at the carboxyl-terminal end of the NTD conserved amongst species lies between amino acids 500-535 (Fig 1.3) and has been implicated in prostate cancer progression. Somatic mutations have been identified in this region in tumours from patients progressing following complete androgen ablation (Hyytinen et al. 2002). Interestingly, a peptide consisting of amino acids 477-558 of the AR has been shown to result in inhibition of DNA binding of the AR and impairment of transactivation activity, indicating that this region may be involved in the modulation of AR DNA binding (Liu et al. 2003). In addition, this region contains a putative MAPK phosphorylation site and sumoylation sites and is required for the optimal efficiency of a dominant negative AR (Butler et al. 2006).

1.7 Post translational modifications of the AR

In addition to the above motifs, it has been demonstrated that the AR undergoes various post-translational modifications. Sumoylation of the AR at amino acids 380-389 and 514-524 (Fig 1.3) by the E2 SUMO ubiquitin conjugating enzyme 9 (Ubc9) has been implicated in the co-operativity of AR transcriptional activation at sites containing more than one ARE and the association of the AR with the cytoplasmic signalling molecule Daxx (Callewaert et al. 2004;

Chang et al. 2007). This association negatively regulates AR transcriptional activity via inhibition of DNA binding capacity (Lin et al. 2004).

1.7.1 Phosphorylation

Although all of the hormone receptors can be phosphorylated the functional consequences and the kinases responsible for this phosphorylation are still largely unknown. Nevertheless, there is considerable evidence that phosphorylation can modulate receptor ligand sensitivity, cofactor interactions, degradation, nuclear translocation, and DNA binding (Lange 2004). Phosphorylation sites have been identified in the AR with 7 in the AR NTD and one each in the hinge and AR LBD (Fig 1.3) (Lin et al. 2001; Gioeli et al. 2002). Cyclin-dependent kinase 1 (cdk1) mediates phosphorylation at serine 82 resulting in increased AR protein levels and transactivation activity in prostate cancer cells with a large stoichiometric increase in response to agonist binding (Gioeli et al. 2002; Chen et al. 2006). Serine 650 phosphorylation is increased in response to the stress kinases, MAPK kinase 4 (MKK4), c-jun terminal kinase (JNK) or MKK6 (p38) and inhibits the expression of the AR regulated *kallikrein 3* (*KLK3/PSA*) gene via enhancement of nuclear export of the AR (Gioeli et al. 2006). Akt has been demonstrated to phosphorylate the AR at amino acids 210 and 790, and represses transcriptional activity by unknown mechanisms (Lin et al. 2001). The phosphorylation of amino acid 210 has been demonstrated to be confined to non-proliferating cells in vivo (Taneja et al. 2005). The heregulin-dependent HER2/HER3 receptor tyrosine signalling cascade mediates activation of the Cdc42-associated tyrosine kinase that phosphorylates the AR NTD on residues Y265 and Y361. These phosphorylations increase the maximal activity of AR using an exogenous reporter and mutation of these phosphorylation sites results in decreased prostate tumour growth in mice, suggesting an important role for this phosphorylation in AR function *in vivo* (Mahajan et al. 2007). Importantly, the AR has a preference for phosphorylation of S81, S256 and S308 in the nucleus, but S94 in the cytoplasm. This indicates that the kinases responsible for specific phosphorylation sites of the AR are cell site specific (Yang et al. 2007).

1.7.2 Acetylation

Acetylation of the AR by the histone acetyltransferases p300/cAMP-response element-binding protein at lysine residues in a conserved motif in the hinge domain 627 - RKLKKLGN- 636 leads to the enhancement of DHT-mediated AR activity. Substitution of lysine in this sequence leads to reduced p300 binding and enhances the binding of corepressors to the AR (Fu et al. 2003). Deletion of this motif results in enhanced receptor interaction with the p160 NCOA2 and N/C interaction (Haelens et al. 2007). The AR is deacetylated at this motif by an NAD-dependent histone deacetylase Sirtuin 4 (Fu et al. 2004). These results suggest that acetylation may influence specific protein interactions between AR and coregulators and that acetylation and deacetylation of the AR may be a method of fine-tuning AR signalling according to the cellular complement of coregulators. In addition, the processes of acetylation/deacetylation of the AR and phosphorylation may be linked, but the exact relationship between these two processes is yet to be elucidated.

1.7.3 Ubiquitinylation

The AR is also capable of being ubiquitinylated. This process entails the covalent attachment of a small, highly conserved 76 amino acid protein, ubiquitin, to specific regions within proteins which involves the actions of three enzymes in a sequential 3 step process. Mono- (the attachment of one ubiquitin molecule to the target protein) or bi-ubiquitinylation (two ubiquitin molecules) has been demonstrated to be involved in modulation of protein-protein interactions and/or subcellular localization while uniquitnylation of more than two ubiquitin molecules results in degradation of the protein target by the 26S proteosome, (Faus et al. 2006). Due to the transient nature of ubiquitinylation, sites of these modifications have been difficult to identify for the AR. The GR has one ubiquitinylation site bordering the NTD and DBD, and the MR has two ubiquitinylation sites, one in the NTD and one in the hinge region (Faus et al. 2006). There is now good evidence that the process of transcriptional activation and ubiquitinylation are linked, with ubiquitinylation allowing transient association of nuclear

receptors with DNA (Metivier et al. 2003) however this has not been fully investigated with respect to ubiquitinylation of AR on an endogenous AR regulated gene. Moreover, there is much conflicting data on the role of ubiquitinylation in AR signalling. For example, the E2 conjugating enzyme UbcH7 increases the transactivation activity of the AR, while the poly-ubiquitinylation of AR by the E3 ligase Mdm2 results in degradation of the receptor leading to decreased AR transactivation. Additionally, the action of tumour specific gene product 101 (TSG101) results in accumulation of mono-ubiquitinylated AR leading to enhanced stability and transactivation activity (Gaughan et al. 2005). Finally, AR ubiquitinylation can be reversed by the protease USP10, which increases AR transactivation activity (Faus et al. 2005).

1.8 The role of AR in prostate cancer

More recently, our enhanced understanding of AR signalling has been gained through the investigation of this receptor and naturally occurring mutations in prostate cancer. Prostate cancer is the most commonly diagnosed cancer and the second most common cause of cancer related death in Australian males, and comprised 23.4% of all new cancer cases diagnosed in 2001 (AIHW 2004). While the biggest risk factor for the development of prostate cancer is age, signalling via the AR is also required. A large majority of men who develop this cancer will be cured by surgery or radiation, however approximately 15-20% will progress with metastatic disease and require further treatment with systemic therapy (Scher et al. 2000; Soloway et al. 2005). Many of these treatments are aimed at reducing the level of androgens in serum, as functional androgen signalling via the AR is essential for the growth and maintenance of the prostate gland and also prostate cancer. In fact, the AR is expressed in nearly all prostate cancers (Bagatell et al. 1996; Culig et al. 2000; Buchanan et al. 2001). While androgen ablation initially leads to a reduction in disease burden and symptoms, the majority of patients eventually relapse on this treatment (Scher et al. 2004). An increase in AR expression and or stability, mutation of the AR, increase in the bioavailability of ligand,

alteration in the levels of AR coregulators and alterations in the post-translational state of the AR have all been implicated in continued AR signalling and prostate tumour progression.

1.8.1 Mutations in AR in advanced prostate cancer

Investigation of the sequence of AR in metastatic prostate tumours has revealed a high proportion of missense mutations of the *AR* gene. Furthermore, a large number of these mutations have been shown to result in a gain of function of the receptor, either with a higher affinity for androgens, promiscuous activation of the receptor by alternate ligands or higher transcriptional activity in response to androgens (Taplin et al. 1995; Buchanan et al. 2001; Thompson et al. 2003). These mutations have been shown to cluster in particular regions of the receptor, with AAT or CAB leading to the identification of mutations in the NTD of the AR while untreated cancers tend to have mutations in the LBD (Han et al. 2001). We have previously identified mutations in a prostate tumour cohort following CAB comprising chemical castration and administration of an AR antagonist, hydroxyflutamide (OHF). These mutations collocate to the most carboxyl-terminal end of the AF5 region, a conserved region of the AR NTD between amino acids 502-535 and to a region of the AR LBD between amino acids 749-769 (Buchanan 2002) (Table 3.1). While the functional consequences of many of the AR LBD somatic mutations have been determined or can be predicted based on the structure of the AR LBD, the functional consequences of mutations in the AR NTD have not been well described.

1.9 Summary

In summary, while androgens undoubtedly have effects independent of the actions of AR in cells, the requirement for the AR in the development of the male phenotype highlights its important role in cellular responses to androgens. Currently unknown is what portion of androgens in serum are measured by androgen bioassays and if this measure is more closely related to physiological parameters that have previously been associated with androgen action. Furthermore, the relative contribution of the domains responsible for overall AR

transcriptional activation and particularly the role of AR AF1 and AF5 in the N/C and p160 coregulator interactions are not well defined. Perhaps due to this limitation, the functional consequences of previously discovered somatic prostate cancer mutations that collocate to the AF5 region of the AR have not been well defined. Finally, the feasibility of utilising tagged AR's to investigate AR interactions in living cells in real time has not been comprehensively addressed.

1.10 Objectives of this thesis

The overall objective of this thesis was to determine the ability of an androgen bioassay to define the physiologically relevant fraction of androgens in serum and to characterise the structural requirements for the AR activation functions for sensitive AR signalling in androgen target tissues.

Therefore, the objectives of this thesis are to:

i) To characterise the effect of somatic prostate cancer mutations in the AF5 region of the AR NTD on the ability of the AR to activate genes

ii) To describe the relative roles of the activation functions AF1, AF2 and AF5 in AR transcriptional activation, interactions with p160 coregulators and the N/C interaction

iii) To assess the effect of tagging the AR with fluorescent proteins on the amino- or carboxyl terminus

iv) To develop an assay for serum T that utilises the intracellular effector of androgenic action, the AR

v) To assess if the measure from the assay developed above relates to any fraction of serum T or physiological parameter associated with androgen status in males

Chapter 2: General Materials and Methods

2.1 Materials

Reagent	***Reagent Supplier***
5α-dihydrotestosterone (DHT)	*Sigma (St Louis, MO, USA)*
17β-Estradiol (E2)	*Sigma (St Louis, MO, USA)*
Acrylamide/Bis solution	*BioRad (Hercules, CA, USA)*
Actin (I-19) Goat Polyclonal Antibody	*Santa Cruz Biotechnology (Santa Cruz, CA, USA)*
Agarose, analytical grade	*Promega Corporation (Madison, WI, USA)*
Agarose gel running apparatus	*Bio-Rad (Herculaes, CA, USA)*
Alexa 488-conjugated donkey anti sheep secondary antibody	*Molecular Probes (Eugene, OR, USA)*
Ammonium Persulfate	*Sigma (St Louis, MO, USA)*
Ampicillin	*Sigma (St Louis, MO, USA)*
Androstenediol (ASD)	*Steraloids (Newport, RI, USA)*
Anti-Calnexin Mouse Polyclonal Antibody	*Affinity Bioreagent (Golden, CO, USA)*
Anti-megalin (C-19) goat polyclonal antibody	*Santa Cruz Biotechnology (Santa Cruz, CA, USA)*
Anti-megalin (C-19) goat polyclonal antibody blocking peptide	*Santa Cruz Biotechnology (Santa Cruz, CA, USA)*
Anti-mouse IgG/HRP-conjugated	*DakoCytomation (Glostrup, Denmark)*
Anti-rabbit IgG/HRP-conjugated	*DakoCytomation (Glostrup, Denmark)*
AR N-20 Rabbit Polyclonal Antibody	*Santa Cruz Biotechnology (Santa Cruz, CA, USA)*
AR-U407 Rabbit Polyclonal Antibody	*Chiron (Clayton, VIC, Australia)*
BigDye™ Terminator	*Applied Biosystems (Boston, MA, USA)*
Bovine Serum Albumin (BSA)	*Sigma (St Louis, MO, USA)*
Bradford Protein Dye Reagent	*BioRad (Hercules, CA, USA)*
Bromophenol Blue	*Sigma (St Louis, MO, USA)*
Calcium Chloride	*Ajax Finechem (Seven Hills, NSW, Australia)*
Cells (COS-1, CHO-K1, PC-3)	*American Type Culture Collection (ATCC; Rockville MD, USA)*
Charcoal	*Asia Pacific Specialty Chemicals (Seven Hills, NSW, Australia)*
Complete Protease Inhibitor Cocktail Tablets	*Roche Applied Science (Penzberg, Germany)*
dATP, dCTP, dGTP, dTTP set of	*Promega Corporation (Madison, WI, USA)*

Dehydroepiandrosterone sodium sulphate (DHEA(S))	*Steraloids (Newport, RI, USA)*
Deoxynucleotide Triphosphates (dNTPs) (10 mM) each	*Promega Corporation (Madison, WI, USA)*
Developer and Rapid Fixer	*AGFA (Mortsel, Belgium)*
Dextran	*Amersham Biosciences (Buckinghamshire, England)*
DL-Dithiothreitol (DTT)	*Sigma (St Louis, MO, USA)*
DMSO	*BDH Laboratory Supplies (Kilsyth, Vic, Australia)*
DNA engine tetrad thermal cycler	*BioRad Laboratories (Hercules, CA, USA)*
ECL™ Hyperfilm™	*Promega Corporation (Madison, WI, USA)*
ECL™ Western Blotting Detection Reagents	*Amersham Biosciences (Buckinghamshire, England)*
EDTA (Ethylene-diamene tetra-acetic acid)	*Sigma (St Louis, MO, USA)*
Ethanol (molecular biology grade)	*Ajax Finechem (Seven Hills, NSW, Australia)*
Ethidium Bromide	*Pharmacia Biotech (Perkin-Elmer, Boston, MA, USA)*
Fluorescent Mounting Medium	*DAKO (Carpinteria, CA, USA)*
Foetal Calf Serum (FCS)	*Sigma (St Louis, MO, USA)*
Glycerol	*Sigma (St Louis, MO, USA)*
Glycine	*Sigma (St Louis, MO, USA)*
Glycogen	*Roche Applied Science (Penzberg, Germany)*
Hybond™-C Extra Nitrocellulose Transfer Membrane	*Amersham Biosciences (Buckinghamshire, England)*
Hydrochloric acid	*Ajax Finechem (Seven Hills, NSW, Australia)*
2 X iQ SYBR Green Supermix	*Bio-Rad Laboratories (Hercules, CA, USA)*
iQ real time PCR cycler	*Bio-Rad Laboratories (Hercules, CA, USA)*
Isopropanol (molecular biology grade)	*Ajax Finechem (Seven Hills, NSW, Australia)*
Kanamycin	*Sigma (St Louis, MO, USA)*
L-Glutamine (100x)	*Sigma (St Louis, MO, USA)*
Luria Broth components	*Becton Dickinson (Sparks, MD, USA)*
Lipofectamine™ 2000 Transfection Reagent	*Invitrogen (San Diego, CA, USA)*
Luciferase Assay System	*Promega Corporation (Madison, WI, USA)*
Magnesium Chloride	*Ajax Chemicals (Auburn, NSW, Australia)*
Methanol	*Ajax Finechem (Seven Hills, NSW, Australia)*
Microscope, inverted	*Olympus America Inc (Center Valley, Pennsylvania)*
Microscope, inverted fluorescent	*Olympus America Inc (Center Valley, Pennsylvania)*

Mineral Oil	*Sigma (St Louis, MO, USA)*
50mM $MgSO_4$ solution for PCR	*Invitrogen (San Diego, CA, USA)*
My iQ software	*Bio-Rad Laboratories (Hercules, CA, USA)*
Nanodrop spectrophotometer	*Nanodrop technologies (Wilmington, DE, USA)*
Nuclon Lab-Tek II Chamber Slide	*Lab-Tek (Naperville, IL, USA)*
Paraformaldehyde	*Ajax Chemicals (Auburn, NSW, Australia)*
Passive Lysis 5x buffer	*Promega Corporation (Madison, WI, USA)*
PfU DNA Polymerase, including 10x buffer	*Promega Corporation (Madison, WI, USA)*
Phenol Red Free RPMI 1640 Liquid Medium (1x solution)	*Sigma (St Louis, MO, USA)*
Phosphate Buffered Saline (PBS)	*Sigma (St Louis, MO, USA)*
Progesterone	*Sigma (St Louis, MO, USA)*
QIAquick Gel Extraction Kit	*QIAGEN (Hilden, Germany)*
QIAquick PCR Purification Kit	*QIAGEN (Hilden, Germany)*
QIAGEN Plasmid Maxi Kit	*QIAGEN (Hilden, Germany)*
QIAprep Spin Miniprep Kit	*QIAGEN (Hilden, Germany)*
Restriction Digest Enzymes, including 10x Buffer and 100x BSA	*New England Biolabs (Beverly, MA, USA)*
RPMI 1640 Liquid Medium (1x solution containing 2.05nM L-Glutamine)	*Sigma (St Louis, MO, USA)*
SeeBlue Plus2 Pre-stained Protein Standard	*Invitrogen (San Diego, CA, USA)*
Shrimp Alkaline Phosphatase	*Roche Applied Science (Penzberg, Germany)*
Skim milk powder	*Diploma (Melbourne, VIC, Australia)*
Sodium chloride	*Ajax Chemicals (Auburn, NSW, Australia)*
Sodium dodecyl sulphate	*Sigma (St Louis, MO, USA)*
Sodium hydroxide	*Ajax Chemicals (Auburn, NSW, Australia)*
Sodium molybdate	*Sigma (St Louis, MO, USA)*
Statistical package for the social sciences (SPSS)	*SPSS (Chicago, IL, USA)*
Superscript II^{TM} kit	*Invitrogen (San Diego, CA, USA)*
Syringe filters, 0.2 µm	*Nalgene (Rochester, NY, USA)*
T4 DNA Ligase including 10x Buffer	*Roche Applied Science (Penzberg, Germany)*
Taq DNA Polymerase	*New England Biolabs (Beverly, MA, USA)*

TEMED	*Sigma (St Louis, MO, USA)*
Testosterone (T)	*Sigma (St Louis, MO, USA)*
Tissue Culture Filter Units – 500 mL capacity	*Nalgene (Rochester, NY, USA)*
Top Count™ plate reading luminometer	*Packard (Mount Waverley, ACT, Australia)*
Tris	*Sigma (St Louis, MO, USA)*
Triton-X 100	*Sigma (St Louis, MO, USA)*
Trypsin (10x Solution)	*JRH Biosciences (Lenexa, KS, USA)*
Trypan blue	*Sigma (St Louis, MO, USA)*
TURBO DNA-*free*™	*Applied Biosystems (Foster City, CA, USA)*
Tween®20	*Sigma (St Louis, MO, USA)*
Vacuette serum collection tubes	*GreinerBio-One GmbH (Kremsmuenster, Austria)*
Vector NTi computer suite	*Invitrogen (San Diego, CA, USA)*
Whatman Filter	*Whatman International Ltd (Maidstone, England)*
Water	1) Sterilised reverse osmosis (RO) water 2) Baxter water *(Baxter Healthcare, NSW, Australia)* 3) Nuclease free water *(Applied Biosystems, CA, USA)*
Xylene Cyanol	*Sigma (St Louis, MO, USA)*

2.2 Buffers and solutions

All solutions were stored at room temperature and made utilising RO water unless otherwise stated

Dextran coated charcoal (DCC) in Tris/EDTA buffer

0.5 % Charcoal	5g
55 mM Dextran	0.5g
20 % Glycerol	100 mL

Made up to 1L in Tris/EDTA buffer and stirred overnight at 4^{o}C

4% Paraformaldehyde

Paraformaldehyde	2 g
Hot PBS	40 mL

pH to 7.5
stored at 4^{o}C for up to two weeks

6 x protein loading dye

4 x Tris-Cl/SDS	7 mL
Glycerol	3 mL
SDS	1 g
DTT	0.93 g
Bromophenol Blue	1.2 mg

RIPA lysis buffer

10 mM Tris (pH 7.4)
150 mM NaCl
1 mM EDTA
1% Triton X-100
Made up to 100 mL with water
2 x Protease and phosphatase inhibitor tablets added, allowed to dissolve and 1mL aliquots stored at -20^{o}C until use

10 x SDS-PAGE Glycine running buffer

Tris base	75.75 g
Glycine	360 g
SDS	25 g
H_2O	to 2.5 L

7.5 % SDS-PAGE Separating gel

1 M Tris pH 8.8	3.75 mL
Acrylamide/Bis	1.9 mL
20% SDS	50 µL
10% APS	50 µL
Temed	10 µL
H_2O	4.3 mL

SDS-PAGE Stacking gel

1 M Tris pH 6.8	625µL
Acrylamide/Bis	500 µL
20% SDS	25 µL
10% APS	25 µL
Temed	5 µL
H_2O	3.7 mL

10 X SDS-PAGE transfer buffer

25 mM Tris base	75.75 g
Glycine	360 g
H_2O	to 2.5 L

1 x SDS-PAGE transfer buffer

Methanol	800 mL
10 x transfer buffer	400 mL
H_2O	to 2.8 L

Stored at 4^{o}C

50 x Tris-acetate buffer (TAE)

Tris base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

To 1 L with water

10 x Tris buffered saline (TBS)

0.5 M Tris	75.75 g
1.5 M NaCl	109.5 g
H_2O	to 2.5 L

pH to 7.4

TBS + Tween 20 (TBST)

Tween 20	5 mL
TBS	to 2.5 L

4 x Tris-Cl/SDS

Tris-Cl	3.025 g
SDS	0.2 g

Made up Tris-Cl and pH to 6.8 prior to addition of SDS followed by the addition of H_2O to a final volume of 50mL

Tris/EDTA buffer

0.01 M Tris base	1.211 g
1.5 mM EDTA	0.588 g
0.01 Na Molybdate	2.42 g
10% Glycerol	100 mL
H_2O	to 1 L

pH to 7.4

Trypan blue

0.01% trypan blue in sterile PBS

6 x Type III load dye (Maniatis)

0.25%	bromophenol blue
0.25%	xylene cyanol FF
30%	glycerol in water

2.3 General Methods

Experimental procedures included in two or more chapters are included in this section while more specific experimental detail is provided within each chapter.

2.3.1 Cloning procedures

2.3.1.1 Creation of chemically competent cells

To create chemically competent bacterial cells for plasmid propagation, a glycerol stock of DH5α was streaked onto an antibiotic free LB-agar plate under aseptic conditions, and incubated overnight at 37^{o}C. One colony from the streak plate was selected and placed into 5 mL antibiotic free LB and shook at 225 rpm on a platform shaker overnight at 37^{o}C. The next morning, 200 mL of LB was inoculated with 1:500 of starter culture, and the culture was incubated for 3 h with shaking at 200 rpm at 37^{o}C. Thereafter, the OD_{600} of the cells was measured every 15 min by spectrophotometry until it reached 0.35 when the cells were placed into cold 50 mL centrifuge tubes and chilled for 15 min on ice with mixing by inversion every 5 min. Tubes were centrifuged at 2700 g for 10 min at 4^{o}C followed by decanting of the supernatant and inversion of the tubes on paper towel for 1 min. Cell pellets were gently resuspended by swirling and gentle pipetting in 30 mL sterile ice cold 80 mM $MgCl_2$, 20 mM $CaCl_2$ solution. This solution was centrifuged at 2700 g for 10 min at 4^{o}C and the solution drained from the pellet as described above. Pellets were then gently resuspended in 1 mL per pellet of sterile ice cold 30% glycerol in 0.1 M $CaCl_2$ solution. The cells were incubated on ice for 4 h and 100 µL aliquots were pipetted into microcentrifuge tubes using sterile, wide bore pipette tips and snap frozen in liquid nitrogen. Cells were stored at -80^{o}C until needed.

2.3.1.2 Transformation of chemically competent cells

Cells were thawed on ice for 10 min and transferred to ice cold 15 mL centrifuge tubes. Approximately 10 ng of plasmid DNA was added to the cells, incubated on ice for 10 to 30 min, heat shocked at exactly 42^{o}C for 45-50 sec and immediately incubated on ice for 2 min.

Thereafter 900 µL of LB was added to the cells and cells were incubated at 37°C for 1 h with shaking at 225 rpm. 300 µL of culture was spread onto LB-agar plates containing either 100 µg/mL ampicillin or 50 µg/mL kanamycin and incubated overnight at 37°C.

2.3.1.3 Isolation of plasmid DNA from bacterial cultures for sequencing

Liquid bacterial cultures were prepared by inoculation of 5 mL LB containing 50 µg/mL ampicillin or 50 µg/mL kanamycin with a single colony streaked from a glycerol stock or transformed according to the method described in section 2.3.1.2 and grown overnight at 37°C with shaking at 225 rpm. Plasmid DNA was prepared utilising the QIAprep spin Miniprep kit according to manufacturer's specifications. DNA was eluted in 40 µL TE pH 8.0, and quantified by UV spectrophotometry with the Nanodrop spectrophotometer.

2.3.1.4 Isolation of plasmid DNA from bacterial cultures for transfection

Depending on quantity of DNA required for transfection, small 10 mL LB cultures or starter 5 mL LB cultures containing 50 µg/mL of the required selection antibiotic were prepared from a single colony on an LB-agar selection plate and shook at 225 rpm at 37°C overnight or for 8 h, respectively. Plasmid DNA was isolated from the small culture using the QIAGEN plasmid mini kit according to manufacturer's instructions and DNA was dissolved in 40 µL TE pH 8.0. The 5 mL starter culture was utilised to inoculate 100 mL LB with required selection antibiotic at 1:1000. This culture was grown overnight at 37°C with shaking at 225 rpm. The QIAGEN plasmid maxi kit was utilised to isolate plasmid DNA from the culture according to manufacturer's instructions and plasmid DNA was dissolved in 200 µL TE pH 8.0. The plasmid DNA from both methods was quantified by UV spectrophotometry and diluted to the appropriate concentration for transfection into mammalian cells. Concentration of DNA was additionally verified by agarose gel electrophoresis.

2.3.1.5 Agarose gel electrophoresis

To make TAE-agarose gels, 100 mL TAE was added to the appropriate amount of analytical grade agarose (0.8 g for 0.8% gels and 1.5 g for 1.5 % gels) and microwaved on high for

approximately 1 min until all particles were dissolved. The TAE-agarose was allowed to cool to 60^{o}C, 0.5 µg/mL of ethidium bromide was added and the gel was pored into a gel casting apparatus. Gels were allowed to cool for at least 30 min whereupon they were placed into a gel running apparatus and submerged in TAE. DNA was loaded into the wells with 1 x load dye. The appropriate size DNA ladder (1 kB or 100 bp) was run in each gel. Typical running parameters were 100 volts for 30 min followed by visualisation under UV light.

2.3.1.6 PCR product purification

Products resulting from PCR were diagnosed by agarose gel electrophoresis, described in section 2.3.1.5. If the resulting product consisted of a single product without smearing, products were cleaned of PCR regents and primers utilising the QIAquick PCR purification kit, according to the manufacturer's instructions. Products were eluted from the column with 30 µL TE, pH 8.0 or water, depending upon the downstream application.

2.3.1.7 Gel purification of PCR products and restriction fragments

Gel purification of PCR products was necessary for PCR products with smearing or with multiple products, and for the purification of all restriction fragments. The digested or PCR product was run on an agarose gel according to section 2.3.1.5. The required fragments or products were excised from the gel under long wave UV light, with time of exposure kept to a minimum. Bands in the gel were purified utilising the QIAquick gel extraction kit according to manufacturer's instructions and eluted in 30 µL TE pH 8.0 or water depending on downstream application.

2.3.1.8 Phenol Chloroform extraction of genomic DNA from mammalian cells

To isolate genomic DNA from mammalian cells, an equal volume of isoamyl saturated phenol chloroform was added to 100 µL mammalian cells in PBS followed by vortexing for 15 sec and incubation at room temperature for 10 min. The solution was spun in a microcentrifuge at maximum speed for 10 min and the aqueous layer removed to a new tube. An equal volume of chloroform was added to the aqueous layer, vortexed for 15 sec and incubated at room

temperature for 10 min followed by centrifugation for a further 10 min at maximum speed in a microcentrifuge. The aqueous layer removed to a new tube and DNA was ethanol precipitated according to the method outlined in section 2.3.1.9.

2.3.1.9 Ethanol precipitation of DNA from aqueous samples

To precipitate DNA from aqueous samples, 1/10 of the volume of 3M sodium acetate pH 5.2 was added to the samples, followed by 2.5 times the volume of 100% EtOH. The sample was incubated at -20^{o}C for at least 2 hours then pelleted by centrifugation at maximum speed for 30 min and washed once in 500 µL 70% EtOH. The DNA pellet was air dried and resuspended in sterile 40 µL TE pH 8.0.

2.3.1.10 Sequencing of plasmid DNA

Plasmids were sequenced utilising AmpliTaq^{TM} DNA polymerase FS dye terminator cycle sequencing chemistry with the ABI PRISM^{TM} BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit. Each sequencing reaction contained 100 ng plasmid DNA and 5 pM primer along with the required kit components. Reaction parameters were 96^{o}C for 3 min, followed by the addition of the BigDye enzyme mix, then 25 cycles of 95^{o}C for 10 sec, 55^{o}C for 15 sec and 60^{o}C for 4 min. Sequencing products were cleaned and precipitated by isopropanol precipitation (Section 2.3.1.11).

2.3.1.11 Isopropanol precipitation of sequencing reactions

Sequencing reactions were precipitated and cleaned with the addition of 80 µL 100% isopropanol to the 20 µL sequencing reaction containing 1 µL glycogen as a DNA carrier. Reactions were incubated for 20 min at room temperature and then centrifuged at maximum speed in a microcentrifuge for 20 min. The supernatant was aspirated and the DNA was washed with 250 µL 100% isopropanol. The reactions were centrifuged for a further 5 min at maximum speed in a microcentrifuge, the supernatant was aspirated and pellets air-dried for 10 mins. The IMVS molecular pathology service dissolved and ran the reactions on an ABI

377 automated sequencer, an in-house service offered to IMVS and University of Adelaide research students and staff.

2.3.1.12 Creation of point mutations and deletions in expression vectors

Point mutations or deletions in the AR cDNA within the expression vector pCMV were created utilising the two sided splicing by overlap extension PCR method (Horton et al., 1989). Overlapping sense and antisense primers each containing the mutation or deletion were designed over the region to be mutagenised. These primers were used separately in individual PCR reactions utilising a proofreading polymerase, *Pfu* DNA polymerase, with wildtype AR downstream or upstream antisense and sense primers, respectively. PCR reactions of 50 µL contained 1.5 mM $MgCl_2$, 10 mM each dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton® X-100, 0.1 mg/mL BSA, 100 ng each primer and 20 ng of pCMV-AR as template. These reactions were all denatured for 5 min at 96^oC before the addition of 1 unit of *Pfu* DNA polymerase. Reactions were then cycled 40 times at with the conditions listed in appendix A1 for each specific primer pair. Both PCR products were gel purified (section 2.3.1.7) and utilised as the template in a third PCR reaction containing the above reagents but using only the wildtype sense and antisense primers with the cycling conditions as outlined in Appendix A1. This PCR reaction was either PCR or gel purified (sections 2.3.1.6 and 2.3.1.7) and digested overnight with the appropriate restriction enzymes, along with the AR expression plasmid whose terminal phosphate groups were cleaved with shrimp alkaline phosphatase treatment for 1 hour to prevent the plasmid re-annealing. The required length products were purified via gel purification and the fragment was ligated into the expression vector overnight at 16^oC utilising T4 DNA polymerase. The resulting plasmid mixture was transformed into DH5α and plasmid DNA was prepared utilising the procedures outlined in section 2.3.1.2 and 2.3.1.3. All plasmids were restriction verified followed by sequence verification on both strands over the entire length of the PCR product and insertion sites. Sequence verified plasmids were then maxi prepped or miniprepped for transfection according to the method outlined in section 2.3.1.4. Point

mutations made in expression vectors are notated with the wildtype amino acid, amino acid number and the variant amino acid, for example, E895Q – glutamic acid, amino acid number 895 mutated to glutamine.

2.3.1.13 Cloning of cDNA into expression vectors utilising adapter primer PCR

Fusion proteins were created utilising the adapter primer method. Primers were designed with the required restriction endonucelase recognition site at the 5' end of the primer, along with a 3 base pair nonsense overhang at the very 5' end of the primer. Different restriction enzyme sites were placed on the sense and antisense primers to aid insertion in the correct orientation. PCR was performed utilising a proofreading DNA polymerase, *Pfu* DNA polymerase, with the reaction components as outlined in section 2.3.1.12. All reactions were initially incubated for 5 min at 96^{o}C before the addition of 1 unit of *Pfu* DNA polymerase. PCR conditions were the same as listed in section 2.3.1.12, with the specific PCR cycling parameters outlined in Appendix A1. Products were purified utilising PCR purification, outlined in section 2.3.1.6. The PCR product and the host plasmid was digested with the appropriate restriction enzymes and the appropriate sized products were gel purified. The resulting plasmid and insert were ligated overnight at 16^{o}C in the presence of T4 DNA ligase, according to the supplier's protocol. The ligated plasmids were transformed into bacteria utilising the method outlined in section 2.3.1.2 and plasmid DNA for sequence verification was prepared according to the method outlined in section 2.3.1.3. Linearised plasmids were run on a 0.8% TAE agarose gel to determine insertion of the PCR product. DNA was sequence verified in both directions according to the method outlined in section 2.3.1.10 and sequence verified plasmids were then maxi prepped or miniprepped for transfection according to the method outlined in section 2.3.1.4.

2.3.2 Mammalian cell culture

2.3.2.1 Cell culture maintenance

Cell lines were maintained in growth medium which contained RPMI 1640 medium supplemented with 5% foetal calf serum (FCS) at 37^{o}C in 5% CO_2. All procedures were performed utilising aseptic conditions in a laminar flow cabinet. The PC3^{AR+} subline has been previously described and contains AR mRNA but non-functional AR protein (Buchanan et al. 2004).

2.3.2.2 Cell passaging

When cells reached 95% confluency, medium was aspirated from tissue culture flasks, cells were washed with 5 mL room temperature sterile PBS, replaced with 5 mL 1 x trypsin and incubated at 37^{o}C for 5 min. Once cells were detached from the flask, 20 mL growth medium was added and the cell suspension was centrifuged at 450 g for 5 min. The supernatant was aspirated and cells were resuspended in 5 mL growth medium. An aliquot of cells was taken for cell counting utilising a haemocytometer with Trypan blue exclusion, and 1mL of cell suspension was placed into a new flask with 20 mL or 35 mL of medium for a T-75 or a T-150 flask respectively.

2.3.2.3 Freezing of cell lines

Cells were trypsinised as described above and resuspended in 4 mL medium containing 45% FCS, 45% growth medium with 10% DMSO. Cell suspension was frozen in a Nalgene freezing apparatus at -1^{o}C per minute down to -80^{o}C. For long term storage, cells were placed in liquid nitrogen.

2.3.2.4 Thawing of cell lines

Stored frozen vials of cells were quickly thawed in a waterbath at 37^{o}C and placed in 10mL growth medium containing double the concentration of FCS (10%). The cell suspension was

centrifuged for 5 min at 450 g, supernatant was aspirated and cells were resuspended in growth medium containing 10% FCS before being placed into a T-25 tissue culture flask.

2.3.2.5 Charcoal stripping of foetal calf serum (DCC FCS) or human serum (DCC HS)

Dextran coated charcoal was centrifuged at 3200g for 30 min at 4^{o}C. Supernatant was discarded and 50mL foetal calf serum of human serum was added to the charcoal pellets and rotated for 2 hours at room temperature. The serum-charcoal was centrifuged at 3200g for 30 min and serum transferred to new tubes containing fresh dextran-coated charcoal pellets, followed by 2 hours rotation at room temperature. The serum-charcoal was centrifuged at 3200g for 30 mins at 4^{o}C and FCS was filter sterilised and stored in 25mL aliquots at -20^{o}C.

2.3.3 General transfection methods

2.3.3.1 General Transfection protocol and analysis of luciferase activity

AR negative human prostate cancer cells, PC3, transformed monkey kidney cells COS-1, T47D breast cancer cell line or chinese hamster ovary cells (CHO-K1) were seeded in 96-well plates in RPMI1640 medium containing 5% foetal calf serum (FCS) at a density of 10,000 cells/well and incubated for 24 hours. Immediately prior to transfection, cells were washed with serum free, phenol red free RPMI 1640 media. Transfection was performed for four hours in the same medium with the appropriate vectors (see sections below) mixed with LipofectAMINE™ (0.4μL for each 96 well) according to the manufacturer's protocol. Four hours after transfection, the reaction mix was carefully removed, and cells overlaid with stripped growth medium (phenol red free RPMI 1640 medium supplemented with 5% dextran coated charcoal stripped foetal bovine serum [DCC-FCS]) and 0.01-100nM of the appropriate steroid (stock solution was dissolved in ethanol and added to the medium at no greater than 1 μl/mL) or ethanol carrier alone. Cells were incubated for 24 hours and then harvested directly from plates by adding 30μL 1 x Passive Lysis Buffer (PLB) per well and freezing overnight at -80^{o}C. To assay for luciferase activity, plates were thawed on ice and 10μL from each well

was transferred to an optical plate and assayed immediately for reporter activity with the Luciferase™ Assay System using a plate reading luminometer. The remaining sample for each experimental variable was pooled and cleared via centrifugation for 10 min at 10,000 g at 4°C. Lysate was transferred to fresh tubes and stored at -80°C until immunoblotting.

2.3.3.2 AR transactivation assays

For assays of AR transactivation, the plasmid mix included 0-20 ng of wtAR or AR variant expression vectors (pCMV-AR) and 100 ng of the appropriate androgen responsive reporter construct (ARR3-tk-luc, MMTV-luc, pGL3-5.8PSA, pGL3-PSA540-luc). For transfection of deletion variants, molar concentration of vector was kept constant via the addition of empty expression vector to account for equal numbers of promoters per well and total DNA was kept constant by the addition of the prokaryotic plasmid pBS(sk-) to the transfection mixes.

2.3.3.3 In vivo luciferase assay

For the in vivo luciferase assay, stable cell lines C1 and C2 were plated at a density of 10,000 cells/well in 96 well plates and allowed to adhere for 48 h. Cells were transfected with 100 ng of pCMV-AR, AR variant or an equivalent molar amount of the AR deletion expression plasmids while controlling for the total DNA transfected with the prokaryotic plasmid pBS(sk-). Cells were treated with 0.01-100nM DHT or vehicle (EtOH) for 24 h and assayed for luciferase activity as described in section 2.3.3.1.

2.3.3.4 Coactivator experiments

The effect of cofactors on the activity of wildtype AR and AR variants was examined by transfection with a plasmid mix containing 0.5 ng of wildtype AR or AR variant expression vectors (pCMV-AR), 100 ng of ARR3-tk-luc, and 25 ng of the appropriate coactivator expression vector (pGS5:GRIP1, pSG5:AIB1 or pSG5:SRC-1). As above, transfections were carefully balanced with respect to the molar ratio of expression vectors (using the appropriate empty vector) and total plasmid [using the prokaryotic vector, pBS(sk-)] to control for any

spurious activity due to squelching of basal transcription factors or secondary co-regulatory proteins.

2.3.3.5 Mammalian 2-hybrid interaction assays

2.3.3.5.1 N/C interaction assay

N/C interaction assays were performed in cultured COS-1 cells or $PC3^{AR+}$ cells plated at a density of 10,000 cells/well in 96 well plates and allowed to adhere for 24 h. Cells were transfected with 5 ng each of pVP16-AR(1-538) and pM-ARLBD or equivalent molar amounts of the nominated AR variants and 25 ng of pGK-1 utilising 0.4 µL per well of Lipofectamine 2000 reagent. Each of the AR expression vectors were co-transfected with expression vectors containing a protein not known to interact with AR (e.g. pVP16-AR(1-538) was cotransfected into cells with pM-p53) as a control for intrinsic activity of each of the vectors containing the AR activation domain, or inappropriate interaction due to over expression. DNA and molar concentration of expression vector was kept constant throughout all of the mammalian 2-hybrid interaction assays. Cells were transfected for 4 h, after which the transfection medium was replaced with treatment medium containing the nominated concentration of DHT or the equivalent vehicle control (EtOH). Cells were left for 24 hours to allow luciferase expression and lysed in 30 µL passive lysis buffer per well and immediately placed at -80^{o}C overnight. Plates were thawed on ice and 10 µL per well was measured for luciferase activity according to the method outlined in section 2.3.3.1

2.3.3.5.2 AR-coactivator interaction assay

Coactivator interaction assays were performed in COS-1 cells utilising the expression of the p160 coactivator protein NCOA2 (TIF2/ GRIP1). Cells were plated at a density of 10,000 cells per well in 96 well plates and allowed to adhere for 24 h. Thereafter, cells were transfected with the 5ng each of the expression plasmid pVP16-AR(1-538) or AR variant and pM-GRIP1 and 25 ng of the luciferase reporter plasmid pGK-1 utilising 0.4 µL Lipofectamine 2000^{TM} per well. Vectors were also co-transfected with vectors expressing non-interacting

proteins to control for intrinsic activity of each of the vectors or opportunistic interactions due to over expression of the proteins. DNA concentration and molar concentration of expression plasmid was kept constant for each well of the assay. Cells were transfected for 4 h, after which the transfection medium was replaced with growth medium and cells were left for 24 hours to allow luciferase expression. Cells were lysed and assayed as described above in section 2.3.3.5.1.

2.3.3.5.3 Measurement of the intrinsic activity of the AR NTD variants

For analysis of the intrinsic activity of each of the AR fragments expressed in the mammalian 2-hybrid assays, they were created fused to the GAL4-DNA binding domain in the mammalian 2-hybrid expression plasmid pM. 5 ng of each of these expression plasmids were transfected into COS-1 cells as described above, along with the pGK-1 reporter plasmid containing 5 adjacent Gal4 response elements. Cells were transfected, lysed and assayed as described above in section 2.3.3.5.1.

2.3.4 Stable cell line creation

Two separate stable cell lines were created in this thesis. One involved the integration of an androgen responsive reporter gene (luciferase) and the other involved the integration of a constitutively expressed AR cDNA into the chromatin of the $PC3^{AR+}$ subline. The general method for the isolation of stable transformants is outlined below.

2.3.4.1 Cloning of an androgen responsive reporter vector for stable integration

The ARR3 response element cassette consisting of three copies of the minimal probasin promoter was cloned into a vector with a coding sequence which confers resistance to hygromycin via the adapter primer method. The primers PB3LUCS and PB3LUCAS and the cycling parameters are supplied in appendix A1. The amplified ARR3 reporter region was restricted utilising *NheI* and *EcoRV* along with the reporter vector pGL4.14 (plasmid map in Appendix B2). The amplified region was sequence verified in both directions along the entire length and over the ligation sites of the PCR product.

2.3.4.2 Cloning of the AR cDNA into a stable integration mammalian expression vector

The commercially available plasmid pIRESpuro3 was selected for the creation of a constitutively expressing AR cDNA vector. This vector contains an IRES sequence separating the gene conferring antibiotic resistance from the multiple cloning site. The AR cDNA was amplified from pCMV-AR3.1 utilising the adapter primer PCR method outlined in section 2.3.1.11. The strategy involved the amplification of each domain separately with adapter sites and then ligation of each domain into the required vector.The primers ARNTDS1, ARNTDAS1, ARDBDS1 ARDBDAS1, ARLBDS1 and ARLBDAS1; cycle parameters and restriction endonucleases utilised are provided in appendix A1. Once the insertion sites were verified, a portion of the AR was restriction digested and replaced with the corresponding fragment from pCMV-AR3.1 utilising the restriction enzymes *AflII* and *BstBI*. The resulting clones were sequence verified twice over all PCR and insertion sites.

2.3.4.3 Electroporation of PC3^{AR+} cells with stable transfection vectors

PC3^{AR+} cells were harvested at log phase by trypsinisation, resuspended in growth medium and pelleted by centrifugation at 2000 g for 5 minutes. Cells were washed once with double the volume of room temperature PBS and pelleted as above. Viable cells were counted by haemocytometer with trypan blue dye exclusion and resuspended at approximately 1 x 10^7 cells/ml in PBS. An aliquot of 0.5 mL of cells was mixed with 20ug of the pGL4.14-ARR3LUC plasmid, empty pGL4.14 or pCMV-AREGFP and 400 µL of the samples transferred to a 2 mm gap cuvette (BTX International, Massachusetts, USA) and electroporated on a square wave electroporator ECM 630 (BTX International) at 280 V for 2 msec. The cell mixtures were immediately resuspended in 15 mL of charcoal stripped growth medium and plated on a 10 cm tissue culture plates. After 48 h, AREGFP plates were visualised to assess electroporation efficiency.

2.3.4.4 Selection of stable transformants

Forty eight hours after transfection, growth medium containing 120 µg/mL hygromycin B in charcoal stripped growth medium was placed on each of the plates. Medium was changed every 48 h and plates were left until all cells on the pCMV-AREGFP transformed plate were dead, and visible colonies had formed on the pGL4.14 and pGL4.14-ARR3LUC plates, approximately 4 weeks. Single colonies were transferred to each well on 24 well plates utilising sterile cloning cylinders, vacuum grease and gentle trypsinisation. Colonies were subsequently propagated into T25 then T75 flasks and cell lines were frozen stripped growth medium utilising the method outlined in section 2.3.2.3. Empty vector clones were diagnosed via PCR amplification of genomic DNA. Genomic DNA was collected utilising phenol chloroform extraction (sections 2.3.1.8 and 2.3.1.9) and then amplified utilising the primers LUCS and LUCAS for the pGL4.14 clones and pCMVS1 and IRESp3AS for the pIRESpuro3 clones. Cycling parameters and primer sequences are supplied in appendix A1. Reporter or AR overexpressing clones were verified by transient transfection of AR and luciferase assay followed by immunoblotting as outlined in section 2.3.3.1 or 2.3.5.1, respectively.

2.3.5 Western blotting procedures

Except were explicitly stated, all western blots presented in this thesis were performed directly on the luciferase assay lysates, which provides a transfection control for these experiments.

2.3.5.1 Western blotting utilising luciferase assay lysates

Luciferase assay lysate was prepared according to the method outlined in 2.3.3.1 and an equal volume of this lysate (25 µL per well) was loaded onto 7.5 % SDS-PAGE gels. A protein standard was loaded into the left most lane on all western gels. These gels were run for 30 min at 15 mA, followed by approximately 1 h at 25 mA, until the dye front left the bottom of the gel. Gels were then transferred to nitrocellulose membrane for 90 min at 250 mA. Membranes

were then blocked for at least 2 h in 3% skim milk powder in TBST (Blotto). The primary antibody was diluted in 3% Blotto and placed on the membrane overnight at 4^{o}C. Three 10 min washes of the membrane in TBST were performed before horse radish peroxidase (HRP) – conjugated immunoglobulins diluted 1:2000 in 3% Blotto were placed on the membrane for 30 min at room temperature. The membrane was washed three times for 10 minutes each in TBST and bands were detected via ECL^{TM} western blotting detection reagents and exposure to film according to the manufacturer's recommendations.

2.3.5.2 Transfection of cells to over express protein or mRNA for western blotting or real time PCR

To prepare lysate of soluble proteins for western blot, or mRNA for real time PCR, cells were transfected in 6 well plates (500,000 cells/well) according to the general method outlined in section 2.3.3.1 but scaled up to involve the transfection of at least 33 ng DNA per well and Lipofectamine^{TM} 2000 at 10 µL per well. Cells for western blotting were treated with 10 nM DHT or the equivalent amount of vehicle (EtOH) and cells were left to over express the protein for at least 24 h. Cells for real time PCR were allowed to express the protein for 24 h and treated with 10 nM DHT or equivalent vehicle for 4 hours prior to lysis. Cells were lysed according to the methods outlined in section 2.3.5.3 or section 2.3.7.1., depending on downstream application.

2.3.5.3 Preparation of lysates in RIPA buffer

Cells for lysis were placed onto ice and growth medium was aspirated and replaced with 5 mL cold PBS to wash the cells. The wash was aspirated fully and replaced with 150 µL cold RIPA buffer with phosphatase and protease inhibitors added. Cells were scraped from the surface of the dish utilising a sterile cell scraper and collected utilising a 23 gauge needle and syringe. Lysate was syringed 8 to 10 times and placed into cold 1.5 mL microcentrifuge tubes. Lysates were cleared via centrifugation for 10 min at 10,000 g at 4^{o}C. The supernatant was

collected and placed into fresh cold sterile microcentrifuge tubes. Protein concentration was determined utilising the method of Bradford, outlined in section 2.3.5.4.

2.3.5.4 Bradford method to quantify protein concentration

Protein concentration in lysates was estimated according to the method of Bradford. This method involves the addition of a dye which binds primarily to basic and aromatic amino acid residues and upon binding of proteins causes a colour shift in the dye. Two hundred and fifty µL of Protein assay dye reagent concentrate was added to 750 µL RO water for the blank standard and also to duplicates of 749, 748, 746, 742, and 736 µL water for each of the standards to be created in duplicate. Standards were then created by the addition of 1, 2, 4, 8, and 16 µL of BSA at 0.1 mg/mL to each of these samples respectively. Lysates were prepared by the addition of 2 µL lysate to 250 µL protein assay dye concentrate with 748 µL RO water. Samples were vortexed and allowed to sit at room temperature for 5 minutes before reading in a spectrophotometer in polystyrene cuvettes at an absorbance of 595 nm. A standard curve was generated utilising the readings from the BSA standards and lysate protein concentration calculated from this curve. An equal concentration of protein was loaded onto 7.5% SDS-PAGE gels and immunoblotted according to the method outlined in section 2.3.5.1.

2.3.6 Fluorescent microscopy methods

This method was utilised on both transfected and non transfected COS-1 mammalian cells and $PC3^{AR+}$ prostate cancer cells to determine localisation of both over expressed protein and endogenous protein.

2.3.6.1 Plating of cells on chamber slides

COS-1 or $PC3^{AR+}$ cells were plated at a density of 54,000 cells/chamber in Lab-tek II 8 well chamber slides in growth medium and allowed to adhere for at least 48 hours before fixation (section 2.3.6.3) or transfection (section 2.3.6.2).

2.3.6.2 Transfection of cells on chamber slides

Cells on chamber slides were transfected with 800 ng of the indicated plasmid/well and 2.4 µL Lipofectamine™ 2000, according to the general method outlined in 2.3.1.1. After transfection, cells were treated with 10 nM DHT or the equivalent amount of vehicle (EtOH) and left for 4 h to allow localisation, after which the were fixed according to the methods outlined below.

2.3.6.3 Fixing of cells on chamber slides

Cells were fixed in ice cold 4.5% paraformaldehyde in PBS at pH 7.4 for 10 min at room temperature followed by two 5 min washes in PBS at room temperature. Cells were incubated for 3 min at -20°C in 100% methanol followed by 1 min incubation in 100% acetone at -20°C. Slides were washed twice in PBS and stained according to the methods outlined below.

2.3.6.4 Staining of cells on chamber slides

Primary antibody was diluted in PBS containing 5% serum of the animal in which the secondary immunoglobulin to be used was raised. Primary antibody and a no primary antibody control was placed onto the cells and incubated overnight at 4°C in a humid chamber. Following incubation, slides were washed twice for 5 min each in PBS and the indicated Alexa Fluor™ secondary antibody was placed onto the slides for 30 min in the dark at a concentration of 1:400 in PBS containing 5% blocking serum. Slides were washed twice for 5 min in PBS and DNA in nuclei was stained with bisbenzimide H 33258 (Hoechst) at a concentration of 200 ng/µL for 15 min in the dark. Cells were washed twice for 5 min in PBS, slides were rinsed in double distilled water and allowed to dry in the dark. When dry, coverslips were mounted onto the slides with DAKO fluorescent mounting medium and allowed to dry for a further 2 hours. Slides were then sealed with clear nail varnish and left overnight to dry.

2.3.6.5 Imaging of cells on chamber slides

All images presented in this thesis were taken at 60x magnification utilising an Olympus IX71 microscope with an Olympus DP70 digital camera. Bisbenzimide H 33258 (Hoeschst) stained nuclei were excited at a wavelength of 356 nm and visualised with a DAPI filter and proteins labelled with the Alexa Fluor 488 antibody excited with a wavelength of 495 nm and visualised with a FITC filter. For each field, both frames were taken and then overlayed.

2.3.7 Real time PCR of transfected cells

2.3.7.1 TRIZOL isolation of RNA from transfected cells

After transfection of cells according to the method outlined in 2.3.5.2, 1 mL of TRIZOL reagent was added to each well of a 6 well dish and passed several times through a filtered, sterile pipette tip to homogenise the solution. The sample was vortexed for 15 sec followed by the addition of 200 µL of chloroform. The samples were vigorously shaken by hand for 15 sec and left at room temperature for 2-3 min. Samples were spun for 15 min at 12,000 g at 4^{o}C. The top phase was taken from the mixture and placed into a fresh microcentrifuge tube, taking care not to disturb the interphase layer. RNA was precipitated with the addition of 500 µL room temperature 100% isopropanol and incubation at room temperature for 10 min. Samples were spun for 10 min at 12,000 g at 4^{o}C, and the supernatant removed carefully. The RNA pellet was washed once in 75% EtOH and the pellet air dried for 10 min. The RNA was redissolved in 20 µL RNAse free water, and 1 µL was utilised for spectrophotometry and run on an agarose gel to determine RNA integrity.

2.3.7.2 DNAse treatment from transfected cells

Upon RNA isolation, the RNA contained considerable plasmid contamination, as measured by direct PCR of the RNA sample, so all samples were subjected to a rigorous DNAse treatment utilising the TURBO DNA-*free*^{TM} kit and following manufacturer's guidelines. Two sequential DNAse treatments with double the amount of recommended DNase were

required to abolish any amplification of the gene-specific product on the RNA by PCR. This double DNAse treated RNA was then utilised for all subsequent steps.

2.3.7.4 First strand cDNA synthesis

Superscript II^{TM} was utilised for all cDNA synthesis reactions, and reactions were performed on 2 µg RNA according to manufacturer's instructions. All cDNA samples were quantified by spectrophotometry and diluted to 250 ng/µL. Control samples lacking RNA and one lacking reverse transcriptase were included in the synthesis reactions.

2.3.7.5 Real time PCR reactions

For real time PCR, one sample was chosen to create a standard curve which was run on each PCR plate. This sample was diluted serially, 1:5, 1:25, 1:125. 1:625 and 1:3125 in RNAse free water. 2 µL of each RNA sample (500 ng) was used per well on the 96 well reaction plate. RT-PCR was performed using the iQ SYBR green super mix and 10pmol of the primers: ARCS1 5' TGAAGCAGGGATGACTCTGGG 3' and X4AS 5' ATTGAGCCAGGTGTAGTGTGT 3' for AR specific amplification. The housekeeping gene *S27a* was used as a normalisation control and 5pmol of each primer (S27aF 5' CCAGGATAAGAAAGGAATTCCTCCTG 3' and S27aR 5' CCAGCACCACATTCATCAGAAGG 3') were used in separate standard reactions of the same cDNA samples. All reactions were performed in triplicate on the same plate with the following cycling parameters: 95^{o}C for 3min followed by 55 cycles of 95^{o}C for 15 sec, 55^{o}C for 15 sec and 72^{o}C for 30 sec, and a melt curve performed from 55^{o}C to 95^{o}C at 0.5^{o}C per 10 sec. Data were analysed using iQ5 software.

2.3.8 Statistical analysis

Statistical analysis was performed where indicated in the figure legends or specific methods section utilising the Statistics Package for the Social Sciences (SPSS) or Graphpad prism. More detailed statistical analysis as in Chapter 7 was performed with the assistance of the

Data Management and Analysis Centre at the University of Adelaide, School of Health Sciences.

Chapter 3: Characterisation of somatic mutations in the AR NTD in advanced prostate cancer

3.1 Introduction

In 2003, prostate cancer was the most commonly diagnosed cancer and the third leading cause of cancer related death in Australian males. The current treatment for prostate cancer that has not extended beyond the gland involves the surgical removal of the gland and/or radiotherapy. The latter treatment may also include adjuvant hormonal therapy. A large number of men are cured by these treatments, but 15-20% develop metastatic disease requiring subsequent therapy aimed at reducing or blocking AR signalling, typically involving medical castration via blockade of the HPG axis (androgen ablation therapy [AAT]). In some instances, AAT is combined with the administration of an AR specific antagonist to block the actions of the AR at a cellular level (combined androgen blockade [CAB]). Patients initially respond well to these hormonal treatments and exhibit a period of tumour regression but then progress biochemically (via measurement in the serum of an androgen regulated secreted protein, prostate specific albumin, PSA) followed closely by an increase in detectable metastatic disease.

Patient relapse whilst on AAT or CAB initially led to the assumption that the progression to advanced or metastatic prostate cancer was due to activation of AR-independent survival pathways. However, several lines of evidence point at continued AR signalling in these tumours despite AAT or CAB. Firstly, the promoter and enhancer of the *PSA* gene contain several AREs and expression of PSA is stimulated by AR binding to these sites (Luke et al. 1994; Schuur et al. 1996; Cleutjens et al. 1997). The levels of PSA in the blood increase in virtually all cases when resistance to CAB or AAT becomes evident (Loberg et al. 2003; Scholz et al. 2007). Secondly, a proportion of relapsed metastatic cancers (20-40%) have been demonstrated to respond biochemically to second line hormonal therapy, such as a different AR antagonist following failure of initial AAT or CAB (Kojima et al. 2004; Miyake et al. 2005; Nishimura et al. 2007). Thirdly, some patients exhibit biochemical regression upon the

termination of treatment with an AR antagonist, a syndrome called antiandrogen withdrawal syndrome (Kelly et al. 1993; Wirth et al. 1997). Finally, exogenous androgens result in an abrupt increase in the symptoms of metastatic prostate cancer, which cease or reduce when administration of exogenous T is discontinued (Fowler et al. 1981; Manni et al. 1988). Collectively, this demonstrates that AR signalling continues to be active despite comprehensive AAT or CAB therapies.

Many mechanisms that allow continued AR signalling despite AAT or CAB have been proposed. Whereas AAT significantly reduces circulating levels of androgens, two studies have now demonstrated measurable levels of androgen in the tumour, and shown that the transcription of androgen regulated genes persists despite AAT (Titus et al. 2005; Mostaghel et al. 2007). This could be due to intra-tumoral conversion of adrenal androgens or progresterone (Labrie et al. 1988) and/or altered expression of enzymes involved in androgen biosynthesis and/or catabolism (Rizner et al. 2003; Ji et al. 2007) (Figure 1.2). Additionally, prostate cells have been shown to be capable of synthesising SHBG, which acts to sequester testosterone in the tissue (Hryb et al. 2002). Secondly, AR protein levels are increased in a large number of prostate cancers (up to 93%) possibly due to amplification of the *AR* gene, increased activation of the *AR* gene promoter or increased AR protein stability (Tilley et al. 1995; Gregory et al. 2001; Lee et al. 2003; Chen et al. 2004). Thirdly, increased expression of a subset of AR coactivators or decreased expression of AR corepressors has been demonstrated in prostate tumours (reviewed in Chmelar et al., 2007). For example, the level of NCOA1 (SRC1) and NCOA2 (TIF2/GRIP1) which are members of the p160 family of coactivators is elevated in many prostate cancers that have relapsed following endocrine therapy compared to primary prostate cancer or benign prostatic hypoplasia. Over expression of p160 coactivators is sufficient to allow the activation of androgen responsive genes by AR in the presence of an adrenal androgenic precursor, DHEA (Gregory et al. 2001). Emerging evidence also suggests that the levels of cochaperones may be important in prostate cancer

progression as they have the potential to affect AR stability or the receptors ability to translocate from the cytoplasm to the nucleus (Buchanan et al. 2007; Nakauchi et al. 2007; Robzyk et al. 2007). Finally, the structure or function of the AR itself can be altered by spontaneous somatic mutations in prostate tumours and is discussed in detail below.

Whereas the number of somatic *AR* gene mutations identified to date in untreated localised prostate cancers is relatively small, those that result in a promiscuous, more sensitive or a super active receptor have been detected in up to 36% of tumours from patients with progressive disease following treatment with CAB. These mutations colocate to discrete regions of the NTD, hinge or LBD and the majority result in a gain of function of the receptor (Taplin et al. 1995; Tilley et al. 1996; Taplin et al. 1999; Buchanan et al. 2001; Hyytinen et al. 2002; Thompson et al. 2003; Chen et al. 2005). Conversely germ line mutations of the AR that confer loss of function and androgen insensitivity syndrome (AIS) which manifests as a female phenotype in a male genotype collocate to alternate discrete regions of the receptor (Buchanan et al. 2001).

There is emerging evidence that particular prostate cancer treatments result in the selection of somatic *AR* gene mutations that collocate in specific, functionally important regions of the receptor. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, mutations tend to collocate to the LBD in untreated prostate tumours but to the NTD in castrated mice (Han et al. 2001). A similar trend has been observed in humans (Haapala et al. 2001). The frequency of mutations in the NTD in prostate cancer however, is still controversial and has not been well defined, in a large part due to the omission of sequencing of this large domain in mutation studies. Nevertheless, four somatic *AR* gene mutations that colocate to a distinct and evolutionarily conserved region of the NTD between amino acids 514 to 533 were identified in five locally recurrent prostate cancers following CAB (orchiectomy plus the AR antagonist estramustine phosphate). Conversely, in that study, patients treated with AAT

alone had mutations that predominantly localized to the LBD (Hyytinen et al. 2002). Moreover, our laboratory has previously demonstrated collocation of somatic AR gene mutations in a prostate cancer cohort of men who relapsed following CAB (AAT and hydroxyflutamide (OHF)). These mutations collocate either to a discrete region of the LBD between amino acids 749-769 or to the most carboxyl terminus region of the NTD between amino acids 502-535 (Buchanan 2002) (Table 3.1). Amino acids 749-769 in the LBD have been demonstrated to be involved in ligand binding and the formation of the AF2 surface upon ligand binding and are important for stability of the receptor – ligand complex and the AR N/C interaction (Langley et al. 1998; Thompson et al. 2001; Jaaskelainen et al. 2006). Therefore, these mutations may be predicted to modulate ligand binding leading to a more stable receptor-ligand complex.

Tumour	Site of biopsy	Clinical state*	Duration of flutamide (months)	Survival (months)**	AR gene mutation
J2	node	non-castrate met	28	45	
J3	node	castrate met	8	127	W749R
J4	prostate	castrate met	n/a	n/a	
J5	bone	castrate met	24	85	
J6	retroperit LN	castrate met	14	23	M535V
J7	bone marrow	castrate met	1.5	9	M535R
					M521V
J8	liver	castrate met	7	18	L558P
J9	spine	castrate met	38	54	L760P
					N769S
J10	bone	naïve	22	113	
J11	node	naïve	11	111	
J12	prostate	castrate met	18	29	P502L
					S513G
					F762L

*Clinical state determined at time of biopsy

**After initiation of flutamide treatment (months)

Table 3.1: AR mutations detected by our laboratory in clinical tumour samples following treatment with combined androgen blockade (CAB) comprising surgical castration and treatment with flutamide.

The functional importance of *AR* gene mutations in prostate cancer, in particular those in the NTD, is most clearly illustrated by prostate specific over expression of an NTD variant (E231G, human E235G) in a transgenic murine model. This mutation results in an AR variant with increased basal activity and increased transactivation capacity in comparison to wildtype AR (wtAR) in the presence of the appropriate coactivator (e.g. NCOA4/ARA70). One hundred percent of mice overexpressing the E231G AR variant develop prostate cancer and lung metastases by 50 weeks of age. In comparison, mice over expressing either wildtype AR (wtAR) or the AR T875A LBD variant which exhibits broadened specificity for ligand dependent activation fail to develop cancer of the prostate (Han et al. 2005). Similarly, a mutation at the border of the NTD and DBD at amino acid 580 has been shown to increase the oncogenic potential of prostate cancer cells (Hsiao et al. 1999). Collectively, these findings indicate that perturbation of AR signalling via altered NTD structure and function can promote prostate tumourigenesis, and suggest that the NTD is an important regulator of AR function in prostate cancer cells.

Whereas the function of LBD mutations identified previously by our laboratory and others have been relatively well characterised, with most affecting ligand binding specificity and/or transactivation capaity, the role of mutations that collocate in the NTD has not been well described. In particular, the functional consequences of mutations in the conserved region of the AR NTD between amino acids 502-535 and the role of this highly conserved region of the receptor have not been well characterised. Therefore, the aims of this chapter were to i) investigate the functional consequences of somatic prostate cancer mutations identified between amino acids 502 and 535 of the AR and ii) define the role of this 35 amino acid conserved region in AR function.

3.2 Materials and Methods

3.2.1 Prostate tissues

Prostate cancer mutations collocating between amino acids 500-535 previously identified in our laboratory and by others (Buchanan 2002; Hyytinen et al. 2002) were analysed in this study. The mutations previously identified by our laboratory (Table 3.1) were derived from metastatic prostate tumour samples that were collected as part of the routine clinical management of patients treated with LHRH antagonist or diethylstilbestrol (DES) and oral flutamide (median 16 months, range 1-38 months). Sites of tissue acquisition were heterogeneous and included lymph nodes (n=4), bone (n=3) bone marrow (n=1), liver (n=1) and prostate (n=2). The median duration of tumour exposure to castrate levels of testosterone at the time of tissue acquisition was 13.9 months (range 0-55 months). Castration therapies included luteinising hormone releasing hormone (LHRH) agonist in 10 patients and diethylstilbesterol (DES) in one. The median duration of oral flutamide exposure for the 11 patients prior to the acquisition of the tissue was 16.2 months (range 1 –39 months) and the median survival was 38 months (range 14-132 months).

3.2.2.Sequencing of AR in prostate tumour samples

Genomic DNA was isolated from 25μm sections of paraffin embedded tumour tissue and single-stranded conformational polymorphism (SSCP) mutational analysis was performed for the entire coding sequence of the AR using 16 sets of overlapping primer pairs. DNA fragments with a reproducible mobility shift on SSCP analysis that was confirmed on an independent PCR product were cloned and sequenced utilising the sequencing primers X4AS and N111 (Appendix A2) as previously described (Tilley et al. 1996).

3.2.3 Plasmids

Plasmids for mammalian 2-hybrid and transactivation assays are listed in Appendix B3.1 and B3.2 respectively. Plasmids constructed for this thesis are described in detail below and listed in Appendix B3.3. All constructs were sequenced verified on both strands by automated sequencing as described in section 2.3.1.10 before being prepared according to section 2.3.1.4 for use in transfections. The AR expression vectors pCMV-AR, TAG:AR(1-538), TAG:AR(644-917) (where TAG indicates either the VP16 activation domains or the Gal4 DNA binding domain), pBS-SK-, the androgen responsive luciferase reporter ARR3-tk-Luc and the mammalian 2-hybrid reporter pGK-1 have been described previously (Tilley et al. 1989; Buchanan et al. 2004). The pM-GRIP1, pSG5-GRIP1, pSG5-SRC1a and pSG5-AIB1 coregulator plasmids were a gift from Professor R Evans. The previously published sequence of Tilley et al, 1989 was utilised for all construct numbering.

3.2.3.1 Creation of AR substitution and deletion variants

AR point mutation variants were created via PCR mutagenesis utilising the megaprimer PCR method (Sarkar et al. 1990) by Dr Nicole Moore. Briefly, these substitutions were introduced into an expression vector containing the complete human AR gene coding sequence under the control of the cytomegalovirus promoter (i.e. pCMV-AR3.1) (Tilley, 1989). Designated primers are listed in Appendix A1. For mutations in the AR NTD region, megaprimers were created by PCR with a common downstream primer 3' to the required mutation sites (N166) and a mutagenic primer incorporating the required missense substitution (P502L, S513G, D526G, M535A, M535V). 25µl PCR reactions were set up with 1ng pCMV-AR as template, 50µM of each of the deoxyribonucleotide triphosphates, 1.5µM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 8.3), 0.45% (v/v) Triton-X 100, 200µg/ml gelatin, 0.25µM of the 3' primer (C1), 0.25µM of the appropriate mutagenic oligonucleotide primer, and 0.2 units of a 1:16 mix of *Pfu* DNA polymerase and *Taq* DNA polymerase. Following an initial denaturation step of 3 min at 95^{o}C, 30 cycles of PCR was performed, for the most part, with the following

parameters: denaturation for 45 sec at 95^{o}C, annealing for 1.0 min at 55-65^{o}C (with the temperature optimised for the particular primer pair), and extension for 1.0 min at 72^{o}C. PCR products were run on 0.8% agarose 1xTAE gels and the correct megaprimer fragment excised and purified using gel extraction (Section 2.3.1.7). PCRs for full length fragments incorporating the required base substitution were performed using the same reaction mix as listed above, but using approximately 50-200ng of the megaprimer generated above and a upstream oligonucleotide primer within the AR coding sequence of pCMV-AR3.1. PCR was performed using a longer denaturation step (5 min) at 95^{o}C, followed by 20 cycles of PCR: denaturation for 1 min at 95^{o}C, annealing for 1.0 min at 50^{o}C, and extension for 1.0 min at 72^{o}C. PCR products of correct length were excised from 0.8% 1xTAE gels as above, digested with *HindIII* and cloned into pCMV-AR3.1 partially digested with the same enzyme. Correct orientation of the fragment was determined by restriction digest analysis, and the presence of the desired base change confirmed by automated DNA sequencing of both strands utilising primers N166 and X4AS according to the method in section 2.3.1.10 (sequences in appendix A2).

Deletion of the region containing amino acids 501-535 (pCMV-ARΔ35αα) by overlap method (Section 2.3.1.12) was performed by Mr Albert Choong, utilising primers d35aaS and d35aaAS and PCR conditions listed in appendix A1. This method involved the use of two overlapping deletion mutagenesis oligonucleotides containing AR coding sequence either side of the deletion (d35aaS and d35aaAS) and two oligonucleotides 5' to the deletion site (N111) and 3' to the deletion site (X4AS). PCR components are listed in section 2.3.1.12. The first PCR reactions consisted of amplification of pCMV-AR3.1 with N111-d35aaAS and d35aaS-X4AS. These products were gel purified (section 2.3.1.5) and utilised in a third reaction with the primer pair N111-X4AS. This third reaction was digested with *BstEII/TthIII1* and ligated into similarly digested pCMV-AR3.1. The deletion was sequence verified over PCR and

insertion sites utilising 120727s and X4AS and the plasmid preparation for transfection was performed as described in section 2.3.1.4.

For mammalian 2-hybrid N/C interaction assays and p160 interaction analysis, the NTD mutations and deletion of 502-535 were reconstructed as fusion proteins in pVP16-AR(1-538). The mutations M535A, M535V and D526G and the deletion of amino acids 500-538 were created by overlap extension, as described above and in section 2.3.1.12, with the primers, PCR cycling conditions and restriction endonucleases as listed in Appendix A1. The P502L and S513G mutations were created via adapter primer PCR (section 2.3.13) on pCMV-ARP502L and pCMV-ARS513G with a sense primer downstream of the mutation (N166) and an adapter PCR oligonucleotide containing amino acids 532-538, a stop codon and an *XbaI* site (ptmutAS). The PCR product was restriction digested with *Bsu36I* and *XbaI* and ligated into similarly digested pVP16-AR(1-538). Oligonucleotide sequences and PCR cycling conditions are listed in Appendix A1. All plasmids were sequenced on both strands over the PCR and insertion sites (section 2.3.1.10) utilising primers N166 and pVP16AS1 and prepared for transfection into mammalian cells according to the method outlined in section 2.3.1.4.

3.2.4 Stable cell line creation and in vivo luciferase assay

To assess chromatin integrated transcriptional activation of AR and the variants, $PC3^{AR+}$ cell lines stably integrated with ARR3-tk-Luc were created as outlined in section 2.3.4. *In vivo* luciferase assays were performed according to the method outlined in Chapter 2.3.3.3.

3.2.5 In vitro luciferase assays and immunoblot analysis

In vitro AR transactivation assays were performed in cultured $PC\text{-}3^{AR+}$ cells (10,000 cells/well in 96 well plates) transfected with 0.5ng of pCMV-AR or an equivalent molar amount of the AR variant expression vector and 100ng of ARR3-tk-Luc, according to the method outlined in 2.3.3.1. NCOA2 (GRIP1) interaction and N/C interaction assays were

performed with wtAR, each of the mutations and ARΔ35αα according to the method outlined in sections 2.3.3.5.1, 2.3.3.5.2 and 2.3.3.5.3. In order to control for transfection efficiency and to directly relate the transactivation assay results to AR steady state protein levels, the lysates from the six replicates of each sample from the transactivation or interaction assays were pooled and an equal volume of lysate for each sample was immunoblotted for AR utilising the method described in section 2.3.5.1.

3.2.6 Statistical analyses

All data was analysed to compare the variant AR to similarly treated wildtype AR utilising Mann Whitney U analysis of medians as normality of the data could not be assumed. Significant comparisons $p<0.05$ are indicated with an asterisk (*). Variants without a denotion were not significantly different utilising this statistical test.

3.3 Results

3.3.1 Mutations in the AR NTD following complete androgen ablation colocate to a small carboxyl-terminal region of the AF5 domain

Five of the point mutations identified in prostate tumours of men following CAB comprising treatment with a LHRH antagonist or DES and hydroxyflutamide collocated to a discrete region of AF5 in the NTD (amino acids 500-535) (Fig 3.1). This distribution is consistent with a previous report identifying a high frequency of *AR* gene mutations identified in the same region in patients treated with CAB (Hyytinen et al. 2002). Notably, all of the point mutations identified in this study were found in tumours isolated from patients that had relapsed whilst on CAB (Table 1.1). This region between amino acids 502-535 is one of the most conserved regions across species in the AR NTD (Fig 3.1)(Thornton et al. 1998), implying that this region is critical for AR function as previously suggested (Buchanan et al. 2001).

3.3.3 Mutations in the AF5 region of the NTD result in variable transactivation activity in different cell lines

AR variants containing AF5 mutations with the exception of M535A exhibited a modestly increased DHT-induced or vehicle treated transactivation activity in comparison to wtAR in transient luciferase reporter assays in prostate cancer cells (Fig 3.2A). In contrast, all of the same variants exhibited reduced transactivation activity in comparison to wtAR in transfected monkey kidney COS-1 cells (Fig 3.2B). A similar response to the AR antagonists bicalutamide (BIC) or hydroxyflutamide (OHF) was observed compared to wtAR in PC3 cells (Fig. 3.2C). Steady state levels of AR variant proteins by immunoblotting lysates directly from the transactivation assay demonstrates similar steady state levels in wtAR and AR variant transfected cells.

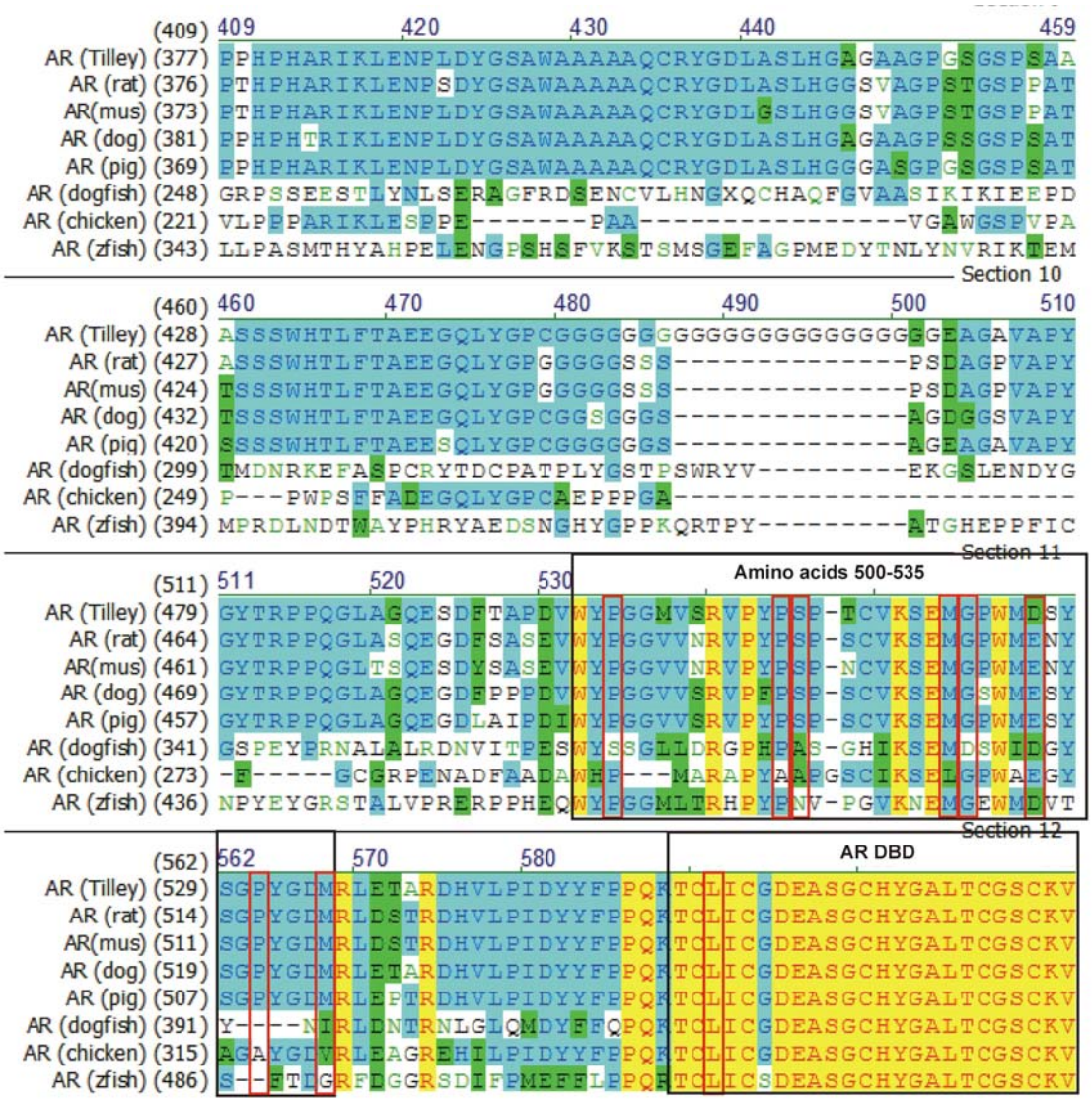
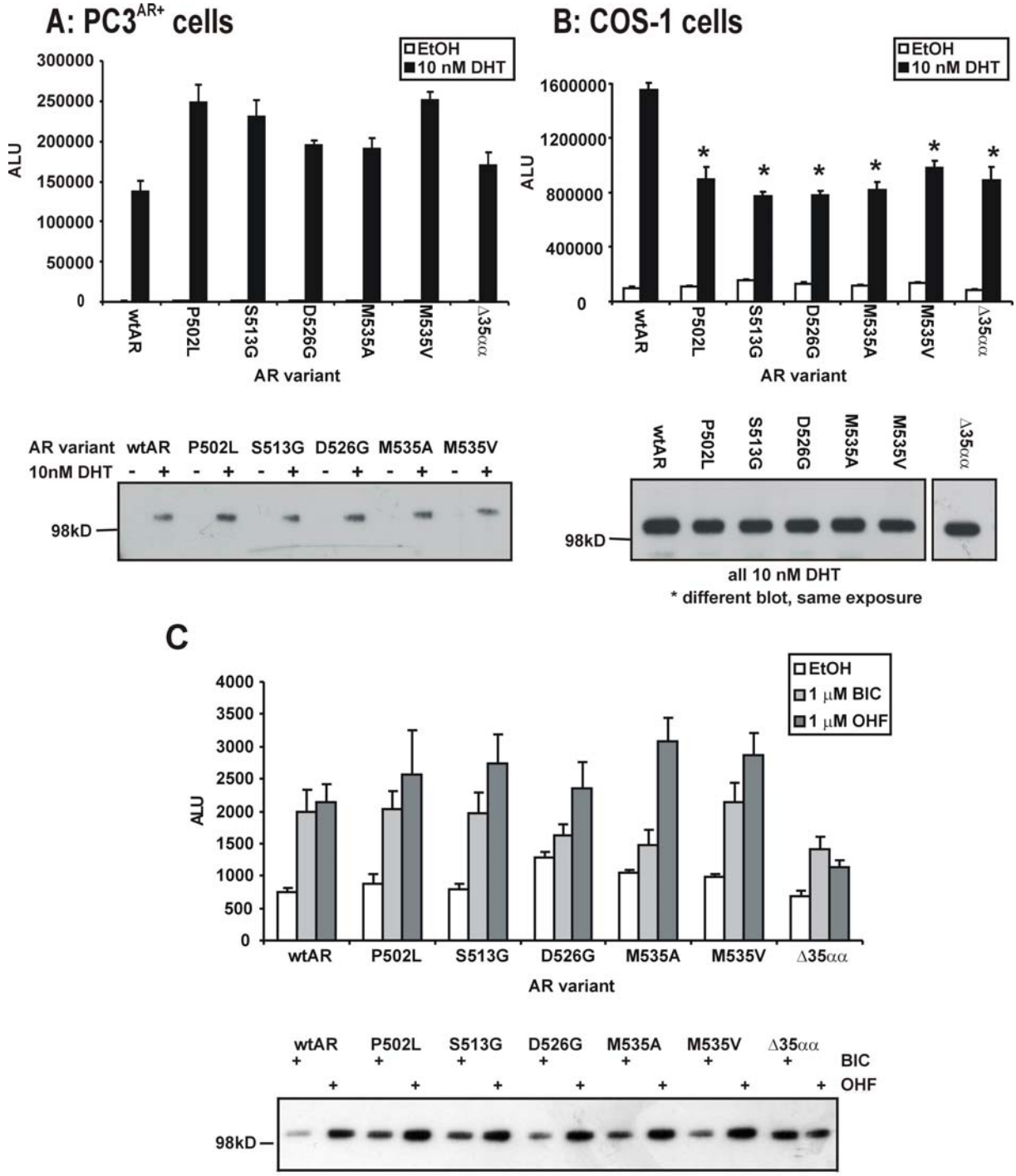


Figure 3.1: Somatic mutations identified in prostate tumours from men that relapsed following CAB collocate to a conserved region in the AR NTD AF5 region between amino acids 502-535. The reference sequence for each species was downloaded into the Vector NTi computer suite (Accession numbers: Tilley: human AH002607; rat: *Rattus norvegicus* NP_036634; mus: mouse *Mus musculus* NP_038504; dog: *Canis lupus familiaris* NP_001003053; pig: *Sus scrofa* accession number NP_999479; dogfish: *Squalus acanthias* AAP55843; chicken: *Gallus gallus* NP_001035179; zfish: zebrafish *Danio rerio* NP_001076592) and aligned utilising Align X, which operates on the ClustalW algorithm. Residues with a yellow background indicate complete identity between species, a green background indicates similar amino acids and blue indicates resides containing conservative amino acid differences between species. Weakly similar are shown as a green on white background while non similar amino acids between species are indicated with a black on white background. Red boxed resides are amino acids substitutions identified in prostate cancer by the Tilley and Kovisto laboratories (Buchanan 2002; Hyytinen et al. 2002).

3.3.4 Analysis of AR NTD mutations in a stably integrated reporter system demonstrates different phenotypes in comparison to the transient system.

As the region between amino acids 500-535 of the AR NTD has been implicated in DNA binding (Lin et al. 2004; Brodie et al. 2005), it was postulated that binding chromatin may be an important feature in the phenotype of NTD mutations. To address this possibility, a set of cell lines were created with a stably integrated androgen responsive probasin luciferase reporter. Two of these lines were then used to define the ability of the AR variants to activate this chromatin integrated reporter. In contrast to activation of the transiently transfected reporter, P502L and S513G AR variants had essentially similar capacity as wtAR to activate transactivation activity in both cell lines. In comparison, D526G and M535A consistently showed a decrease in DHT-induced transactivation activity concomitant with a small decrease in steady state AR levels (Fig 3.3A). A similar result was obtained on the second stable cell line (data not shown). The Δ35αα AR variant had lower steady state levels in comparison to wtAR on immunoblot analysis but had similar transactivation activity as wtAR (Fig 3.3B), with a similar result obtained on the second cell line examined (data not shown).

Figure 3.2: The AF5 mutations display increased transactivation activity in prostate cancer cells but decreased activity in non-prostate cancer cells. A: AR variants display increased transactivation activity in prostate cancer cells. Androgen receptor negative $PC3^{AR+}$ cells (10,000/well in 96 well plates) were transfected with 100ng ARR3-tk-LUC and 0.5 ng of pCMV-AR or molar equivalent of AR variant, with a total of 12 wells transfected per variant. DNA concentration per well was kept constant with cotransfection of the prokaryotic expression vector pBS(sk-). Six wells of each variant were treated with vehicle (EtOH) and 6 wells were treated with 10 nM DHT for 24 hours and lysed in 30 µL passive lysis buffer. Luciferase activity was evaluated on 10 µL lysate. Data is presented as mean + SEM luciferase activity from six independently transfected wells. The remaining lysate for each variant and treatment type was pooled, cleared by centrifugation and an equal volume of lysate (25 µL) resolved for AR by immunoblot analysis utilising AR N-20 antibody. **B:** AR variants display decreased transactivation activity in monkey kidney cells. Androgen receptor negative COS-1 cells were plated, transfected, treated, presented and immunoblotted as in A. **C:** AR variants display similar to wtAR activity in response to the AR antagonists bicalutamide (BIC) and hydroxyflutamide (OHF) in prostate cancer cells. Androgen receptor negative $PC3^{AR+}$ cells were plated and transfected as described in A, and were treated with vehicle (EtOH), 1 µM bicalutamide (BIC) or 1 µM hydroxyflutamide (OHF). Luciferase activity and immunoblot were performed as in A. All data is representative of at least 3 independently repeated experiments. All data in the figure was analysed by Mann Whitney U analysis of medians and * $p<0.05$ on when compared to similarly treated wtAR data for two independent repeats of the experiment.


A: PC3AR+ cells
EtOH
10 nM DHT
ALU
300000
250000
200000
150000
100000
50000
0
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
AR variant
B: COS-1 cells
EtOH
10 nM DHT
ALU
1600000
1200000
800000
400000
0
*
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
AR variant
AR variant wtAR P502L S513G D526G M535A M535V
10nM DHT - + - + - + - + - + - +
98kD
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
98kD
all 10 nM DHT
* different blot, same exposure
C
EtOH
1 μM BIC
1 μM OHF
ALU
4000
3500
3000
2500
2000
1500
1000
500
0
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
AR variant
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
BIC
OHF
98kD

3.3.5 The AR NTD variants display increased enhancement of transactivation activity in response to p160 coregulators in comparison to wtAR.

The cell specific phenotype of the AR variants and the ability of the AR AF5 region to interact with p160 coregulators led us to investigate of the ability of the p160 coactivators to enhance the transactivation activity of each of the AR variants utilising transient transactivation assays (Ma et al. 1999; Irvine et al. 2000). The over expression of NCOA2 and NCOA3 significantly increased DHT-induced transactivation activity of wtAR, and NCOA2 increased the basal activity of wtAR (Fig 3.4A). To measure the ability of each of the variants to be coactivated, results were expressed relative to the empty vector transfected controls for each variant. Each of the prostate cancer variants exhibited greater transactivation in response to NCOA1 compared to wtAR in the presence of DHT (Fig 3.4C). The more carboxyl-terminal substitutions (D526G, M535A and M535V) demonstrated increased enhancement than wtAR by NCOA1, NCOA2 and NCOA3 regardless of hormone treatment (Figs 3.4B and C respectively). In comparison, the Δ35αα variant demonstrated enhancement of transactivation similar to wtAR by all three p160 coactivators. Steady state levels were similar for all AR variants and transfections and did not relate to this increased enhancement of activity (Fig 3.4C).

3.3.6 The interaction of the AR NTD with the p160 coactivator NCOA2 is diminished by the deletion of amino acids 500-535

In order to investigate these results further, the ability of the AR variants to interact with NCOA2 was assessed by a mammalian 2-hybrid interaction assay. Strikingly the Δ35αα AR variant demonstrated a dramatically reduced interaction with NCOA2 in comparison to wtAR (Fig 3.5A). In comparison, all of the mutations demonstrated an interaction with NCOA2 similar to wtAR (Fig 3.5B). Immunoblotting of the assay lysates demonstrated similar steady state levels of AR protein for all of the variants.

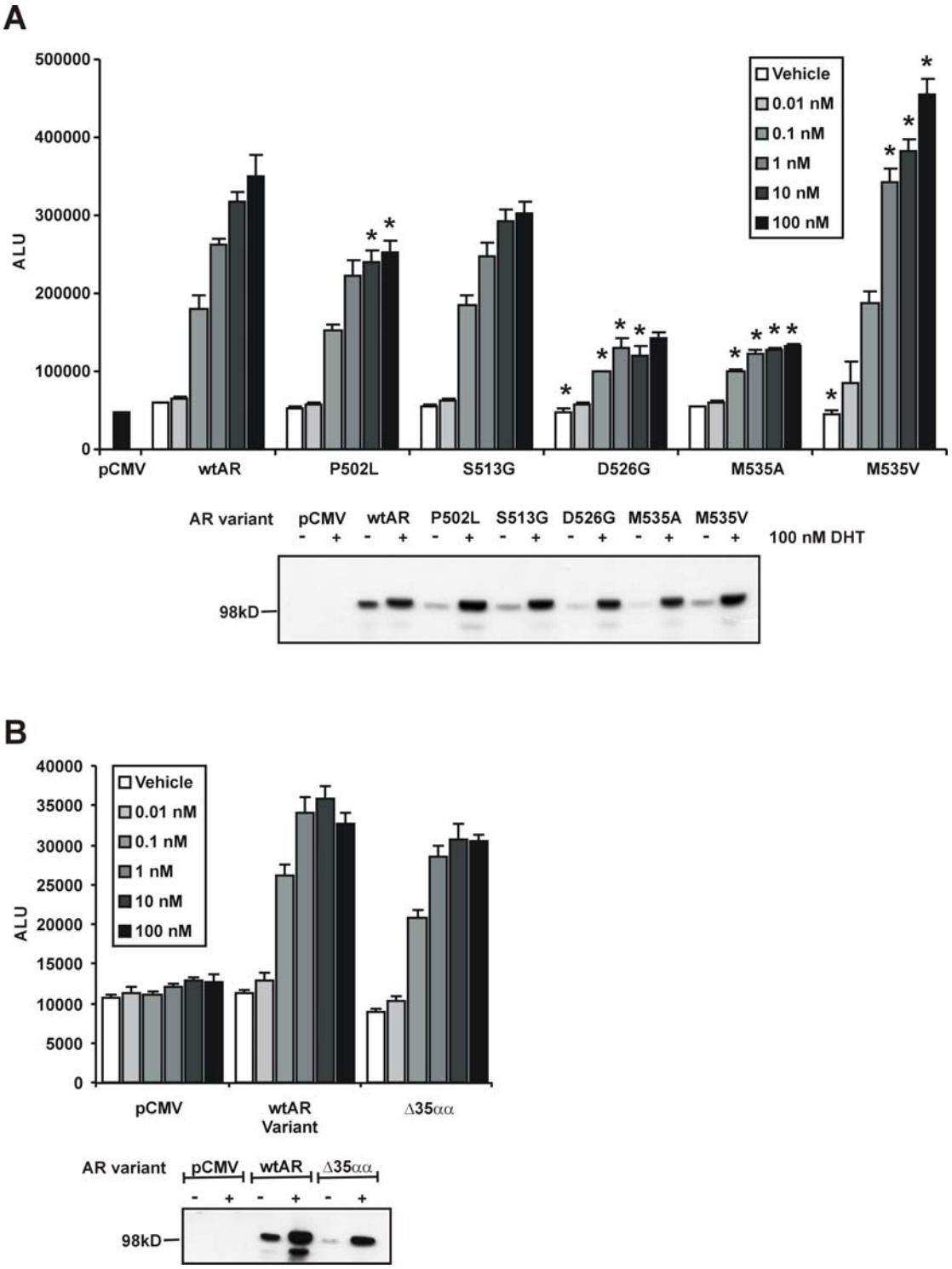
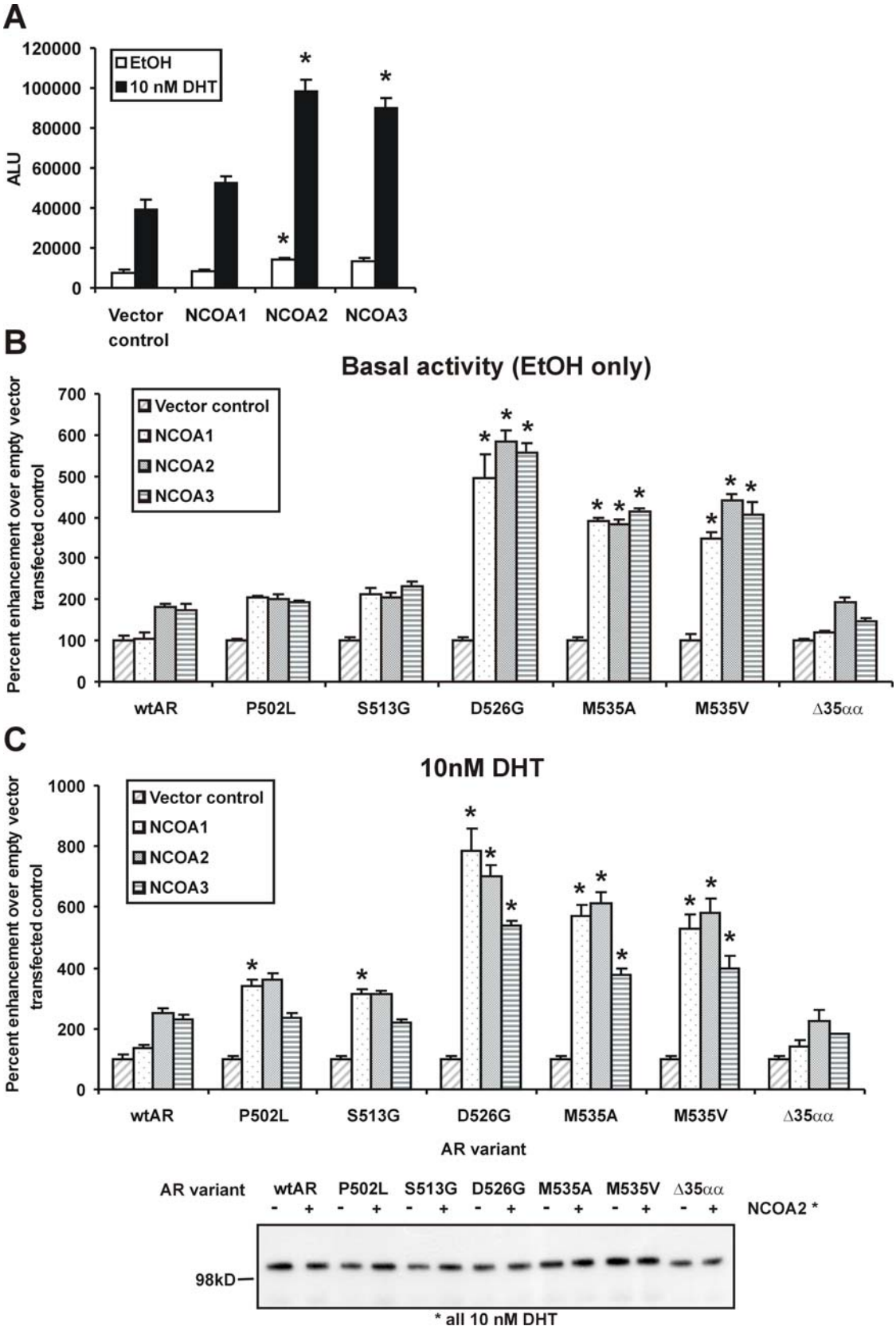


Figure 3.3: AR AF5 variants exhibit a different phenotype on a chromatin integrated reporter system compared with a transient assay. A, B: Stably transfected PC3^{AR+}-ARR3LUC clone 1 cells were plated (10,000 cells/well in 96 well plates) and transiently transfected with 100 ng AR per well, or molar equivalent of AR variant. Total DNA was controlled per well as described in Figure 3.2. Cells were treated, assayed and immunoblotted as described in figure 3.2. Data represent the mean + SEM of six independently transfected wells and is representative of at least 3 independent experiments for 2 different clonal cell lines. All data was analysed utilising Mann Whitney U analysis of medians and *$p<0.05$ compared to the similarly treated wtAR data. All data is representative of at least 3 independently repeated experiments.

Figure 3.4: The AF5 mutations demonstrate increased enhancement in comparison to wtAR in response to over expression of the p160 coactivators. A: AR DHT-induced transactivation activity is enhanced by the over expression of NCOA2 and NCOA3. $PC3^{AR+}$ cells (10,000 per well) were transfected with 100 ng ARR3-tk-LUC, 0.5 ng AR variant expression vector along with 25 ng of the indicated coregulator pSG5 mammalian expression plasmid or the equivalent molar concentration of the empty vector pSG5. Wells were treated, assayed and immunoblotted as described in Figure 3.2. Data represent the mean + SEM of six individually transfected wells and is representative of 2 independently repeated experiments. **B:** The carboxyl terminal AR AF5 mutations show increased basal enhancement compared to wtAR in response to the over expression of NCOA1, NCOA2 and NCOA3. Cells were transfected, treated with EtOH only and assayed as described in A. Data represent the percent enhancement of activity in the coregulator transfected wells compared to the respective empty vector control for each variant and is the mean ± SEM of four independently transfected wells. **C:** The most carboxyl terminal mutations demonstrate marked enhancement of activity when compared to wtAR when NCOA1, NCOA2 or NCOA3 are over expressed. Cells were transfected, treated with 10 nM DHT and assayed as described in A. Data presented as described in B. Immunoblot performed as described in Figure 3.2. Data is representative of 2 independently repeated experiments. All data was analysed by Mann Whitney U analysis of medians and *$p<0.05$ in comparison to wtAR transfected with the respective coactivator.


A
120000
100000
80000
60000
40000
20000
0
ALU
EtOH
10 nM DHT
Vector control
NCOA1
NCOA2
NCOA3
B
Basal activity (EtOH only)
Percent enhancement over empty vector transfected control
700
600
500
400
300
200
100
0
Vector control
NCOA1
NCOA2
NCOA3
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
C
10nM DHT
Percent enhancement over empty vector transfected control
1000
800
600
400
200
0
Vector control
NCOA1
NCOA2
NCOA3
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
AR variant
AR variant
wtAR
P502L
S513G
D526G
M535A
M535V
Δ35αα
- + - + - + - + - + - + - +
NCOA2 *
98kD
* all 10 nM DHT

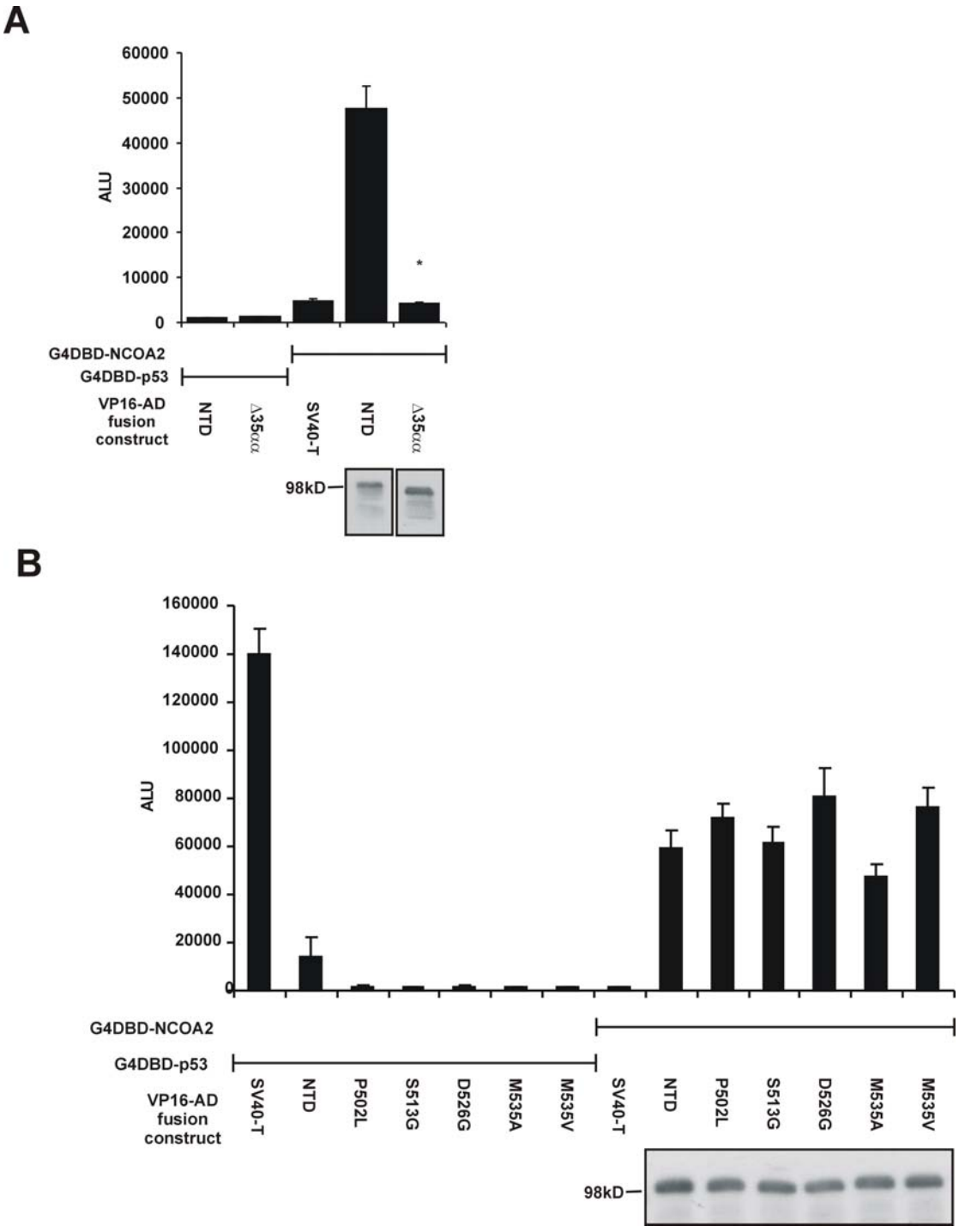
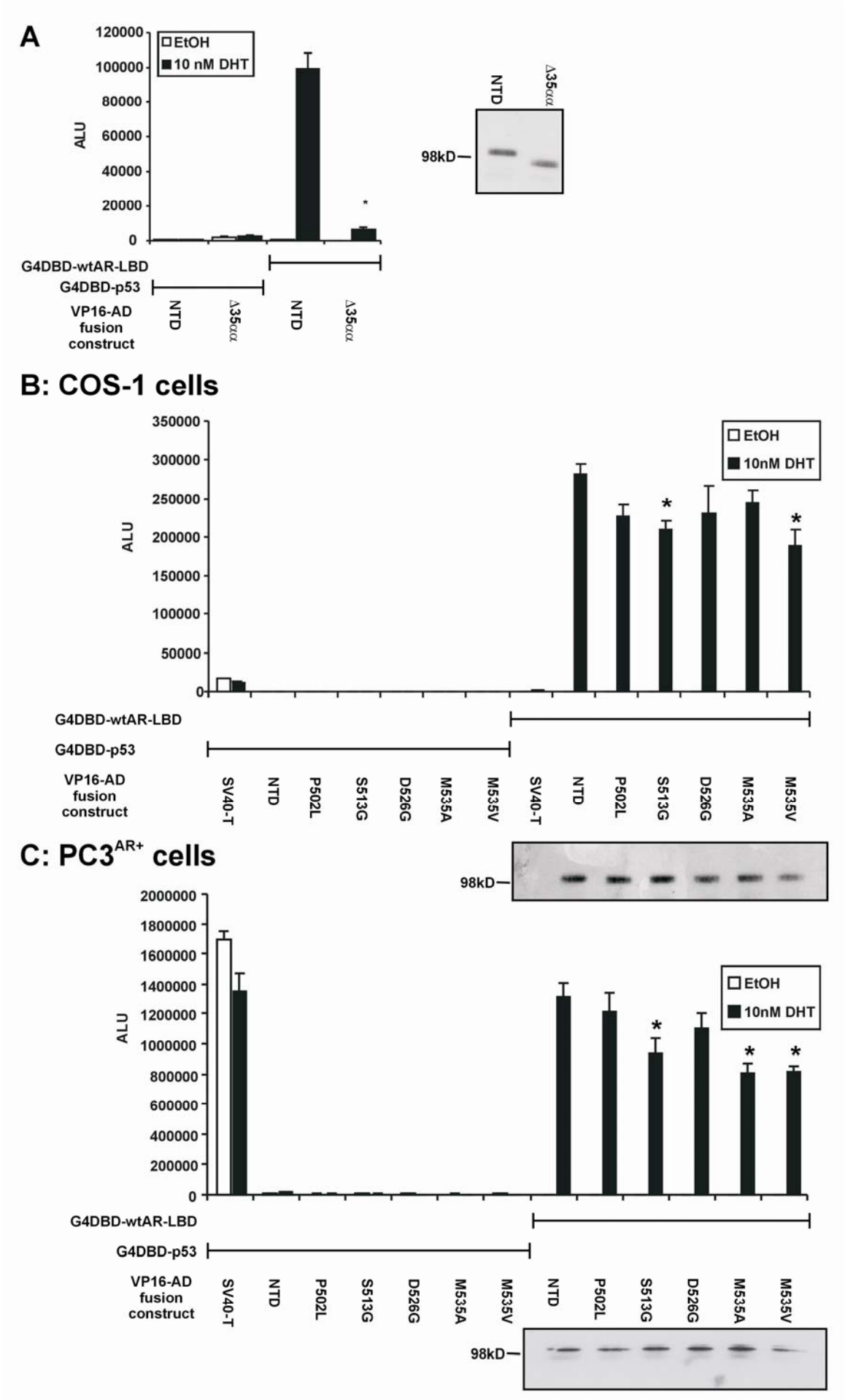


Figure 3.5 Deletion of amino acids 500-535 of the AR disrupts the interaction of the NTD with NCOA2 while mutations within this region do not effect the interaction. A: COS-1 cells (10,000) were transfected with 5 ng of VP16-tagged AR-NTD deletion variant (or molar equivalent) and 25ng of GAL4 DBD tagged NCOA2 with 100ng of the pGK-1 reporter plasmid. All wells were controlled for molar equivalent of expression plasmid and total DNA transfected. Data represent the mean + SEM of six individually transfected wells and is representative of at least 3 independently repeated experiments. Immunoblot performed as described in Figure 3.2. **B:** The AR point mutation variants display an interaction with NCOA2 similar to wtAR. COS-1 cells were plated, transfected and assayed as described in A. All data is representative of at least 3 independently repeated experiments. All data was analysed using Mann Whitney U analysis of medians *$p<0.05$ in comparison to wtAR data.

3.3.7 The N/C interaction is not disrupted for the AF5 AR variants in comparison to wtAR

As many reports have now demonstrated a link between the N/C interaction and coregulator interactions (section 1.4.5), the ability of each of the variants to modulate this interaction was assessed utilising the mammalian 2-hybrid assay. As observed for the interaction with NCOA2, the Δ35αα variant exhibited a large decrease in interaction with the LBD compared to the AR NTD (Fig 3.6A). In comparison, the N/C interaction was marginally decreased for all of the AR variants with the biggest decrease observed for M535V, which may be due to reduced steady state levels in both the COS-1 and $PC3^{AR+}$ cell lines (Fig 3.6B and C, respectively).

Figure 3.6: While the deletion of amino acids 500-535 ablates the AR N/C interaction, the AF5 mutations show an N/C interaction similar to wtAR. A: Deletion of amino acids 500-535 of the AR results in ablation of the N/C interaction. Androgen receptor negative COS-1 cells were plated (10,000 cells/well) in 96 well plates and 12 wells were transfected with 5ng each of the designated vectors, 5ng of GAL4-DBD tagged AR-LBD and 25ng of the pGK-1 reporter plasmid. After transfection, 6 wells were treated with vehicle (EtOH) and 6 wells were treated with 10 nM DHT. After 24 h, cells were lysed by the addition of 30 µL passive lysis buffer per well and assayed and immunoblotted as described in Figure 3.2. Data represent the mean + SEM of the 6 independently transfected wells and is representative of at least 3 independently repeated experiments. **B, C:** COS-1 (B) and $PC3^{AR+}$ (C) cells were transfected, treated, assayed and immunoblotted as in A. Data are presented as in A. All data is representative of at least 3 independently repeated experiments. All data was analysed with Mann Whitney U analysis of medians and *$p<0.05$ in comparison to similarly treated wtAR data.


A
120000
100000
80000
60000
40000
20000
0
ALU
EtOH
10 nM DHT
*
NTD
Δ35αα
98kD
G4DBD-wtAR-LBD
G4DBD-p53
VP16-AD fusion construct
NTD
Δ35αα
NTD
Δ35αα
B: COS-1 cells
350000
300000
250000
200000
150000
100000
50000
0
ALU
EtOH
10nM DHT
*
*
G4DBD-wtAR-LBD
G4DBD-p53
VP16-AD fusion construct
SV40-T
NTD
P502L
S513G
D526G
M535A
M535V
SV40-T
NTD
P502L
S513G
D526G
M535A
M535V
C: PC3AR+ cells
98kD
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
ALU
EtOH
10nM DHT
*
*
*
G4DBD-wtAR-LBD
G4DBD-p53
VP16-AD fusion construct
SV40-T
NTD
P502L
S513G
D526G
M535A
M535V
NTD
P502L
S513G
D526G
M535A
M535V
98kD

3.4 Discussion

Approximately 130 individual somatic missense mutations have been reported in the *AR* in prostate cancer (Gottlieb et al. 2004). Several regions of somatic *AR* mutation collocation have been defined in prostate cancer, and each of these defined regions has now been demonstrated to have an important functional role in AR signalling such as binding to coregulators, binding to ligand or binding to DNA (Newmark et al. 1992; Suzuki et al. 1993; Taplin et al. 1995; Tilley et al. 1996; Fenton et al. 1997; Taplin et al. 1999; Lim et al. 2000; Marcelli et al. 2000; Buchanan et al. 2001; Buchanan et al. 2001; Haapala et al. 2001; Han et al. 2001; Thin et al. 2003; Thompson et al. 2003; Duff et al. 2005; Steinkamp et al. 2007). These findings have lead to the theory of therapy mediated selection pressure, which holds that the particular therapy that an individual receives will provide a selection pressure for tumor cells with mutations that are permissive for continued survival of the prostate cancer cells while in the presence of that treatment (Scher et al. 2004).While these reports have identified several mutation hotspots in the LBD, hinge and DBD, no hotspots have previously been defined in the AR NTD. That another cohort treated specifically with CAB found somatic *AR* mutations that also collocate to a region between amino acids 514 to 533 provide further support to the suggestion that amino acids 500-535 play an important functional role in AR signalling (Hyytinen et al. 2002).

The conserved nature of the region between amino acids 500 to 535 indicates its importance in AR function. The broader AF5 region (amino acids 360-535) has been implicated in not only the modulation of DNA binding (Lin et al. 2004; Brodie et al. 2005) but also the interaction of AR with the p160 coregulators (Berrevoets et al. 1998; Alen et al. 1999; Bevan et al. 1999). The finding in this thesis that the amino acids between 500-535 are required for both the AR N/C interaction and p160 coactivator binding further defines a region important for inter- and intra-molecular communication in the AR, and is the first report of amino acids in the AF5 region being involved in the N/C interaction, which is essential for AR mediated

transcriptional activation of chromatin integrated targets (Li et al. 2006). The role of the N/C interaction and the effect of the AF5 region in this interaction and p160 coregulator interaction are explored further in chapter 4.

Somatic *AR* gene mutations have been consistently identified in prostate cancer specimens, especially in patients treated with metastatic disease following treatment with hormonal therapy and whereas the reported frequency of these AR gene mutations in prostate cancer cohorts is varied, this is likely due to cohorts with different clinical histories, sequencing only a portion of the receptor and methodological issues involved in mutation detection. In general, studies investigating mutation of the *AR* gene have focused on the LBD, and have consistently identified AR variants capable of being activated by alternative, typically non-androgenic ligands (Taplin et al. 1995; Chen et al. 2005; Sun et al. 2006; Bohl et al. 2007). The current findings are the second to show that prostate cancer mutations detected in patients following CAB collocate to a discrete region of the AR NTD between amino acids 502-535 (Hyytinen et al. 2002). This relationship suggests a biologically significant role for this region *in vivo*.

The mammalian 2-hybrid interaction assay is a convenient and efficient assay to quantitatively dissect protein interactions. However, a disadvantage of this approach is that interactions can be masked by any intrinsic activity of the protein fragments, particularly for transcription factors such as the AR. Therefore, it is a requirement of the particular assay employed in this thesis that partial AR fusion proteins be employed to circumvent this artefactual activity. The AR variants D526G, M535A and M535V demonstrated enhanced transactivation in the presence of increased NCOA2 without alteration in their interaction with NCOA2 in comparison to wtAR as assessed by the mammalian 2-hybrid interaction assay. NCOA2 is capable of binding to both the AF5 and AF2 regions of the receptor (Alen et al. 1999). While these findings do not initially support each other, the observation that the mammalian 2-hybrid interaction assay utilises partial proteins, thereby obscuring any

potential allosteric effects of p160 binding to the full length receptor provides an explanation for this apparent contradiction. The involvement of the AF2, AF5 and AF1 domains in p160 interactions are investigated further in chapter 4.

The findings here that mutations between amino acids 502-535 have an enhanced ability to be coactivated by all 3 p160 coactivators in contrast to wtAR indicates that this region is important in governing the overall response to these coactivators. In support of this hypothesis, we show that mutations collocating to this region had distinct phenotypes in prostate cancer PC-3 cells in comparison to monkey kidney COS-1 cells. Preliminary work in our laboratory indicates that PC-3 cells have higher levels of all 3 p160 coactivators in comparison to COS-1 cells, which may explain this difference between the cell lines. In addition to this finding, we show that the chromatin context of the promoter element may also lead to differential activity. Given that it is demonstrated here that amino acids 502-535 are required for the N/C interaction and that the N/C interaction has been shown to be required for the activity of the AR on a chromatin integrated response element (Li et al. 2006) it is possible that the decreased activity of the D526G and M535A variants observed on the chromatin integrated reporter may be a consequence of decreased N/C interaction in the context of a full length AR molecule. Collectively, these findings highlight the importance of chromatin and cellular context for AR signalling *in vivo*.

The phenotype of the more carboxyl AF5 mutations investigated in this study reflects the phenotype exhibited by the E235G substitution. This variant exhibits enhanced activity in *in-vitro* assays in comparison to wtAR in response to the AR coactivators NCOA4 (ARA70) and TATA element modulatory factor 1 (TMF1/ARA160). In addition, this increased cellular level of coactivator allows the mutant AR to have increased basal activity and be activated by estradiol (Han et al. 2001). The over expression of the E231G AR variant in the prostates of mice results in prostate cancer by 50 weeks of age, while the over expression of wtAR does

not (Han et al. 2005). Additionally, our laboratory has recently identified that the E231G AR variant induces a distinct gene expression profile both *in vivo* and *in vitro* in comparison to wtAR (unpublished results). Together, these results demonstrate the importance of this mutation and the cellular context in prostate tumourigenesis. It may be informative to investigate each of the AF5 mutations in a similar context.

While D526G, M535A and M535V confer enhanced transactivation in the presence of high levels of coactivators in comparison to wtAR, P502L and S513G showed similar to wtAR activity and enhancement. These two mutations in conjunction with the LBD mutation F762L were identified in the same prostate tumour sample (Table 3.1) but due to the technique utilised to identify the mutations, it is impossible to identify if they occurred in the same receptor or in different receptors within the sample. It remains possible that these somatic mutations occurred on the same receptor and that the creation of all three mutations in the same receptor is required in order to characterise their functional characteristics.

The level of the p160 coregulators NCOA1 and NCOA2 are up regulated in the majority of prostate tumours of individuals who have failed endocrine cancer therapy (Culig et al. 2000; Gregory et al. 2001; Fujimoto et al. 2007) and increased expression of these coactivators in prostate cancer cells results in a permissive environment for AR to be activated by the adrenal androgenic precursor DHEA (Culig et al. 1993; Culig et al. 2004). Similarly, steady state levels of NCOA4 (ARA70) are more frequently detected in prostate tumour specimens (91.74%) in comparison to benign tissues (64.64%) and NCOA4 has increased expression in higher grades of prostate cancers (Hu et al. 2004; Chmelar et al. 2007). Recently, it was also demonstrated NCOA1 can coactivate the amino terminus of the AR in response to the antiandrogen cyproterone acetate (Hodgson et al. 2007). Furthermore, reduction of the activity of an AR coregulator transforming growth factor β1-induced 1 (TGFB1I1/ARA55) utilising a dominant negative TGFB1I1 construct results in a loss of ability of 1μM

hydroxyflutamide to mediate AR transactivation activity (Rahman et al. 2003). Together, these results indicate that alternate ligands present in the tumor environment during hormone therapy can bind to the AR and, in the presence of increased levels of AR coactivators, lead to AR transactivation activity. The collocation of somatic mutations in prostate cancer following antiandrogen therapy in the region of the AR NTD required for interactions with the p160 coactivator NCOA2 in this study and the demonstration that NCOA1 can coactivate the NTD of the AR in the presence of CPA (Hodgson et al. 2007) suggests that the presence of increased levels of coactivators in castrate resistant prostate tumors may provide a permissive environment for AR transcriptional activation even in the presence of AR antagonists.

In conclusion, this chapter demonstrates that collocation of somatic prostate cancer mutations to amino acids to 500-535 define a region that is involved in the N/C interaction and in interactions of the NTD with the p160 coregulator GRIP1. These mutations result in an increased ability of the receptor to transactivate genes in the presence of high levels of p160 coactivators, which is found in the majority of tumours that have failed hormonal therapy. As prostate cancer is dependent on AR activity for growth, these mutations may therefore allow continued growth of prostate cancer cells harbouring these mutations in an environment deprived of androgens and with high circulating levels of AR antagonist such as that encountered during CAB.

Chapter 4: The AF5 domain in the AR NTD determines the transcriptional response of the receptor via modulation of p160 and N/C interactions.

4.1 Introduction

In the previous chapter it was demonstrated that a portion of the AR AF5 domain in the NTD is involved in interactions with AF2 and p160 coregulators. These results strongly implicate the NTD and, in particular AF5, as a key regulator of AR transcriptional activity.

The long, structurally flexible NTD interacts with the p160 coactivators and the general transcriptional machinery, thereby inducing conformational changes within this domain that provide a more closed, active conformation (McEwan 2004; McEwan et al. 2007). The transcription factor TFIIH has been shown to bind to the AF1 domain (amino acids 38-360) and the AF1 domain is required for the ability of the AR to transactivate genes (Jenster et al. 1995; Chamberlain et al. 1996; McEwan et al. 1997; Callewaert et al. 2006). The contribution of AF5 (amino acids 360-535) to AR transactivation was originally defined as essential for activity of a constitutive AR variant truncated in the carboxyl terminal portion of the LBD at amino acid 707. This finding suggested that AF5 was the predominant interaction surface for transcriptional activation in the absence of AF2 function (Jenster et al. 1995). Perhaps more importantly, the AR AF5 domain has been implicated in binding both p160 coregulators (Ikonen et al. 1997; Alen et al. 1999; Callewaert et al. 2006; Hodgson et al. 2007) and the corepressor SMRT (Dotzlaw et al. 2002; Dotzlaw et al. 2003) which play important roles in the regulation of AR transactivation activity. Interaction with one of the p160 coactivators is an initial step in the activation of the AR which is essential for transcription (Shang et al. 2002). Given the strong occupancy of AF2 by the ^{23}FQNLF^{27} peptide in the N/C interaction, AF5 may therefore a critical determinant of the AR transcriptional response.

There is evidence that p160 binding and N/C interaction of the AR are distinct processes required for AR transcriptional capacity. Firstly, both share the AF2 surface as a potential binding site (He et al. 2003). Secondly, interaction between the NTD and LBD of an N/C

deficient AR variant receptor (i.e. ^{23}FQNLF^{27} to ^{23}AQNAA^{27}) can be rescued by over expression of NCOA2 (Shen et al. 2005). Finally, it has been suggested that the N/C interaction may occur to prevent association of AR with LXXLL and FXXLF-containing coregulators prior to nuclear import (Schaufele et al. 2005; van Royen et al. 2007). In all three of these cases, AR N/C interaction and p160 binding can be seen to be mutually exclusive to each other. Overall, the involvement and relative contributions of AF5, AF2 and AF1 to p160 binding and the AR N/C interaction remain to be defined.

The role of the AR activation functions has not been studied comprehensively in a prostate cancer cell line on more than a single reporter. It has been demonstrated that the prostate cancer cell line PC3 has higher steady state levels of NCOA2 than the non prostate cancer cell line, COS-1 (Gregory et al. 2004). The findings of the previous chapter demonstrated that the cellular complement of coregulators plays an important role in AR transcriptional activity, particularly with respect to the function of amino acids within AF5. Therefore, the aims addressed in this chapter are i) to more comprehensively define the roles of AF1, AF5 and AF2 in AR transactivation activity on multiple reporters in both prostate cancer (PC3) cells and non prostate (COS-1) cell lines ii) to investigate the contribution of each of these domains to N/C and p160 interactions, and thereby their potential interplay in governing AR transactivation response.

4.2 Methods

4.2.1 Plasmids

Plasmids for transactivation assays and mammalian 2-hybrid are listed in Appendix A2.1.1 and A2.1.2 respectively. Plasmids constructed for this thesis are described in detail below and listed in Appendix A2.2. All constructs were sequenced verified on both strands by automated sequencing as described in section 2.3.1.10 before being prepared according to section 2.3.1.4 for use in transfections. AR expression vectors pCMV-AR, pCMVAR-Δ297-361, pCMV-

E895Q, pCMV-T707, TAG:AR(1-538), TAG:AR(644-917) (where TAG indicates either the VP16 activation domains or the Gal4 DNA binding domain), pBS-SK-, androgen responsive luciferase reporters ARR3-tk-Luc, PSA540, MMTV-Luc and mammalian 2-hybrid reporter pGK-1 have been described previously (Tilley et al. 1989; Buchanan et al. 2004; Butler et al. 2006). The 5.8PSA luciferase reporter plasmid was a gift from Dr J Clements. pM-GRIP1 (NCOA2), pSG5-GRIP1, pSG5-SRC1a (NCOA1), pSG5-AIB1 (NCOA3) and pCMX-hSMRT coregulator plasmids were a gift from Professor R Evans. Numbering of AR constructs is according to the published sequence of Tilley et al., 1989.

4.2.2 Creation of AR substitution and deletion variants

Deletion of regions containing amino acids 38-360 (ΔAF1), 38-100, 101-160, 161-220, 221-297, 360-535 (ΔAF5) and 360-494 (ΔcAF5) in pCMV-AR was performed by overlap extension (Section 2.3.1.12) utilising primers and PCR conditions listed in Appendix A1. Once the plasmids had been sequence verified, the 360-494 (ΔcAF5) and 360-535 (ΔAF5) deletions were subcloned into pCMV-ART707 and pCMV-ARE895Q with the restriction enzyme pair *BsmI* and *TthIII1*. The ΔAF5 or ΔcAF5 deletions were re-created in the TAG:AR(1-538) plasmids utilising the overlap method with the primers and conditions indicated in Appendix A1. The 38-360 (ΔAF1) deletion was subcloned into TAG:AR(1-538), pCMV-ART707 and pCMV-ARE895Q utilising the restriction endonuclease pair *EagI* and *Bsu36I.* Primer sequences, cycling conditions and restriction endonucleases utilised are supplied in appendix A1. PCR fragments were digested with *AflII* and *XbaI* and ligated into the expression vectors pVP16-AR(1-538) or pM-ARAF1 according to the method in section 2.3.1.12. All plasmids were sequenced (section 2.3.1.10) on both strands over the PCR and insertion sites and stored as glycerol stocks in 15% glycerol at -80^{o}C. Plasmids were prepared for transfection into mammalian cells according to the outlined method in section 2.3.1.4.

4.2.3 Cell Culture

COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD). The PC-3^{AR+} subline used in this study was derived from long term culture of PC3 cells as described in (Buchanan et al. 2004). Both cell lines were maintained in RPMI 1640 medium supplemented with 5% foetal bovine serum (FBS).

4.2.4 Chromatin integrated reporter luciferase assay

PC-3^{AR+} cells stably transfected with ARR3-tk-LUC (clones 1 and 2) were utilised to assess how AR and its variants altered in their capacity to activate a chromatin integrated reporter. The method for the creation of these stable cell lines is outlined in Chapter 2.3.4. *In vivo* luciferase assays were performed in these cells transfected with 100ng pCMV-AR or molar equivalent of AR variant according to the method outlined in Chapter 2.3.3.3.

4.2.5 Transient luciferase assay

AR transactivation assays were performed in cultured PC-3^{AR+} cells (10,000 cells/well in 96 well plates) transfected with 0.5ng of pCMV-AR or an equivalent molar amount of the AR variant expression vector and 100ng of an androgen responsive reporter according to the method outlined in 2.3.3.1. Two of the androgen responsive reporters were derived from the *KLK2* gene, one with the enhancer region from this gene placed immediately upstream from the proximal promoter element (PSA 540) and the other containing the full genomic enhancer/promoter region of 5.8 kb from this gene (5.8 PSA). In addition to these specific androgen response elements, the response of each of the variants was also tested on the more general hormone response element from the mammalian murine tumour virus (MMTV) which is also responsive to PR, GR and MR (Ham et al. 1988; Katso et al. 2005). NCOA2 interaction, N/C interaction and intrinsic activity assays were performed with each of the AR variants according to the methods outlined in sections 2.3.3.5.1, 2.3.3.5.2 and 2.3.3.5.3. In order to directly relate the transactivation assay results to AR steady state protein levels, the lysates from the six replicates of each sample from the transactivation or interactions assays

were pooled and an equal volume of lysate for each sample was immunoblotted for AR utilising the method described in section 2.3.5.1.

4.2.6 Statistical analysis

Where quantitative differences between wtAR and an AR variant are asserted, Mann Whitney U analyses were performed utilising or graphpad prism, as indicated in the figure legends. For figures containing mutipart panels, all of the panels were assessed and significant differences are denoted by an asterisk (*). $p < 0.05$ was set as the level of significance for all statistical tests.

4.3 Results

4.3.1. The AF1 region of the AR is required for transcriptional activity in vitro and on a chromatin integrated reporter

In agreement with previously published results, transfection of an AR variant deleted of AF1 (amino acids 39-360, Fig 4.1) was unable to activate transcription of an androgen responsive reporter in either $PC3^{AR+}$ or COS-1 cells, even at high concentrations of DHT (Chamberlain et al. 1996)(Fig 4.2A and B). The ΔAF1 was then tested on various AR-responsive reporter constructs. Interestingly, ΔAF1 exhibited a small, non significant increase in DHT-induced activity on the reporters derived from the PSA gene but little activity on the ARR3 or MMTV reporters (Fig 4.2C). Testing of the activity of ΔAF1 on the ARR3 chromatin integrated reporter in two separate cell lines demonstrated that AF1 is required for activity regardless of the reporter context (Fig 4.2D and data not shown). $PC3^{AR+}$ cells exhibit a small amount of non functional endogenous AR protein in the presence of DHT as previously reported (Buchanan et al. 2004). Steady state levels of the ΔAF1 variant were consistently decreased in comparison to wtAR (Fig4.2A, B and D). Increasing the amount of transfected expression plasmid resulted in a stepwise increase in the steady state levels of ΔAF1 but did not result in an increase in transactivation activity (Fig 4.3A). Quantititive RT-PCR suggests that the decreased steady state levels of the ΔAF1 variant protein is not due to decreased mRNA stability levels (Fig 4.3B). To test if a specific region of AF1 was responsible for the decreased steady state level and transactivation activity of ΔAF1, five deletion variants covering the complete area of the ΔAF1 deletion were created (Fig 4.1). None of these variants alone recapitulated the dramatic decrease in transcriptional activity or steady state levels of ΔAF1 (Fig 4.3C). These data suggest a requirement of an active AF1 surface rather than particular amino acids in these effects.

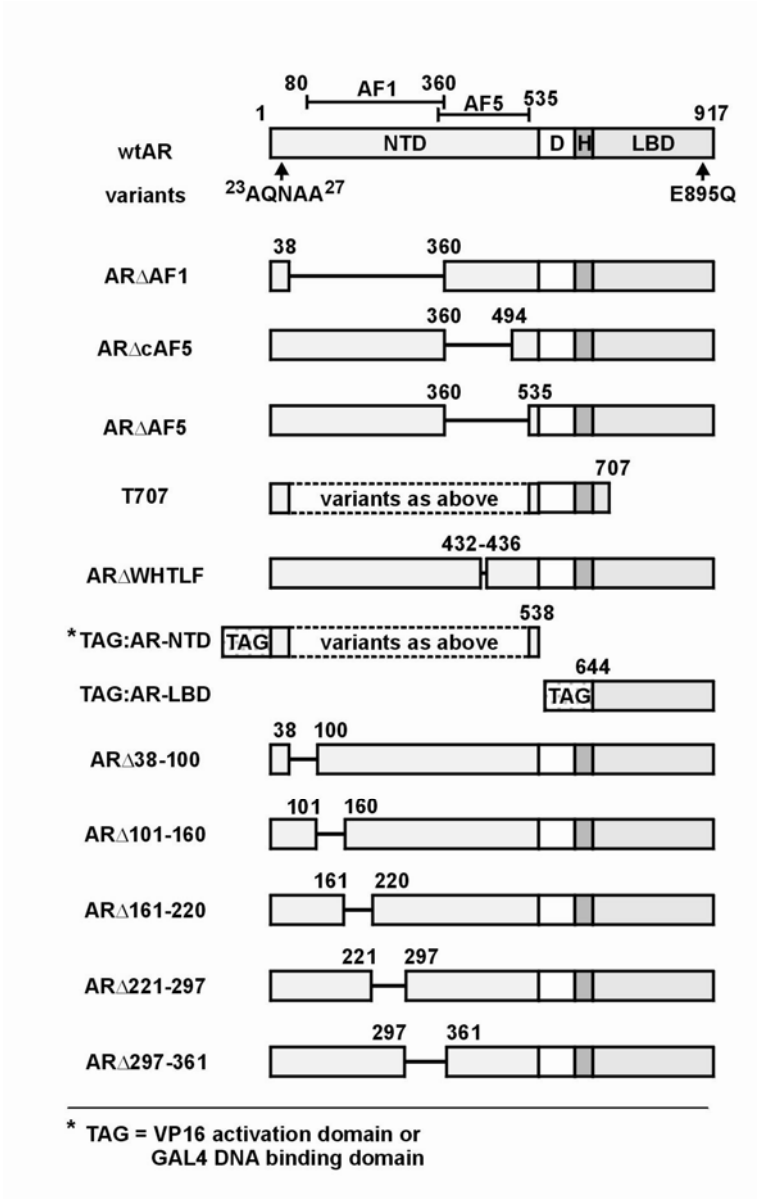


Figure 4.1: AR variants created and investigated in this study. Deletions of the AR coding sequence were created by two sided splicing with an overlap extension PCR method. AF1 = activation function 1, AF5 = activation function 5, T707 = constitutive AR variant truncated at amino acid 707, Δ = deletion.

Figure 4.2: Deletion of AF1 from the AR NTD disrupts DHT-induced transactivation activity in two cell lines, on four different reporters and on a chromatin integrated reporter. **A:** Androgen receptor negative $PC3^{AR+}$ cells **(A)** or monkey kidney COS-1 cells **(B)** (10,000/well in 96 well plates) were transfected with 100ng ARR3-tk-LUC reporter and 0.5 ng of pCMV-AR or molar equivalent of pCMV-ARΔAF1 with a total of 48 wells transfected per variant. DNA concentration per well was kept constant with cotransfection of the prokaryotic expression vector pBS(sk-). Six wells of each variant were treated with vehicle (EtOH), or increasing concentrations of DHT between 0.001 to 10 nM at 6 wells per concentration for 24 hours and lysed in 30 µL passive lysis buffer. Luciferase activity was evaluated on 10 µL lysate. Data represents the mean $\pm$ SEM luciferase activity in arbitrary light units (ALU) of 6 independently transfected wells, and is representative of at least 3 independently performed experiments. The remaining lysate for each treatment type was pooled, cleared by centrifugation and an equal volume of lysate (25 µL) resolved for AR by immunoblot analysis utilising AR N-20 antibody. **C:** PC3 cells (10,000 per well in 96 well plates) were transfected with 0.5 ng of pCMV-AR or molar equivalent of pCMV-ARΔAF1 and 100 ng of either ARR3-tk-Luc, PSA540-Luc, 5.8PSA-Luc or MMTV-Luc and treated for 24 hours either in the absence (vehicle) or presence (10 nM) of DHT. Cells were lysed and assayed as described in A. **D:** The cell line PC3-AR+PB3-LUC C13 (10,000 cells/well in 96 well plates) was transfected with 100 ng pCMV-AR or molar equivalent of pCMV-ARΔAF1. Cells were then treated, assayed and immunoblotted as described above. All data is representative of at least 3 independently performed experiments. n/a: lanes of AR variants not applicable to the displayed transactivation assay.

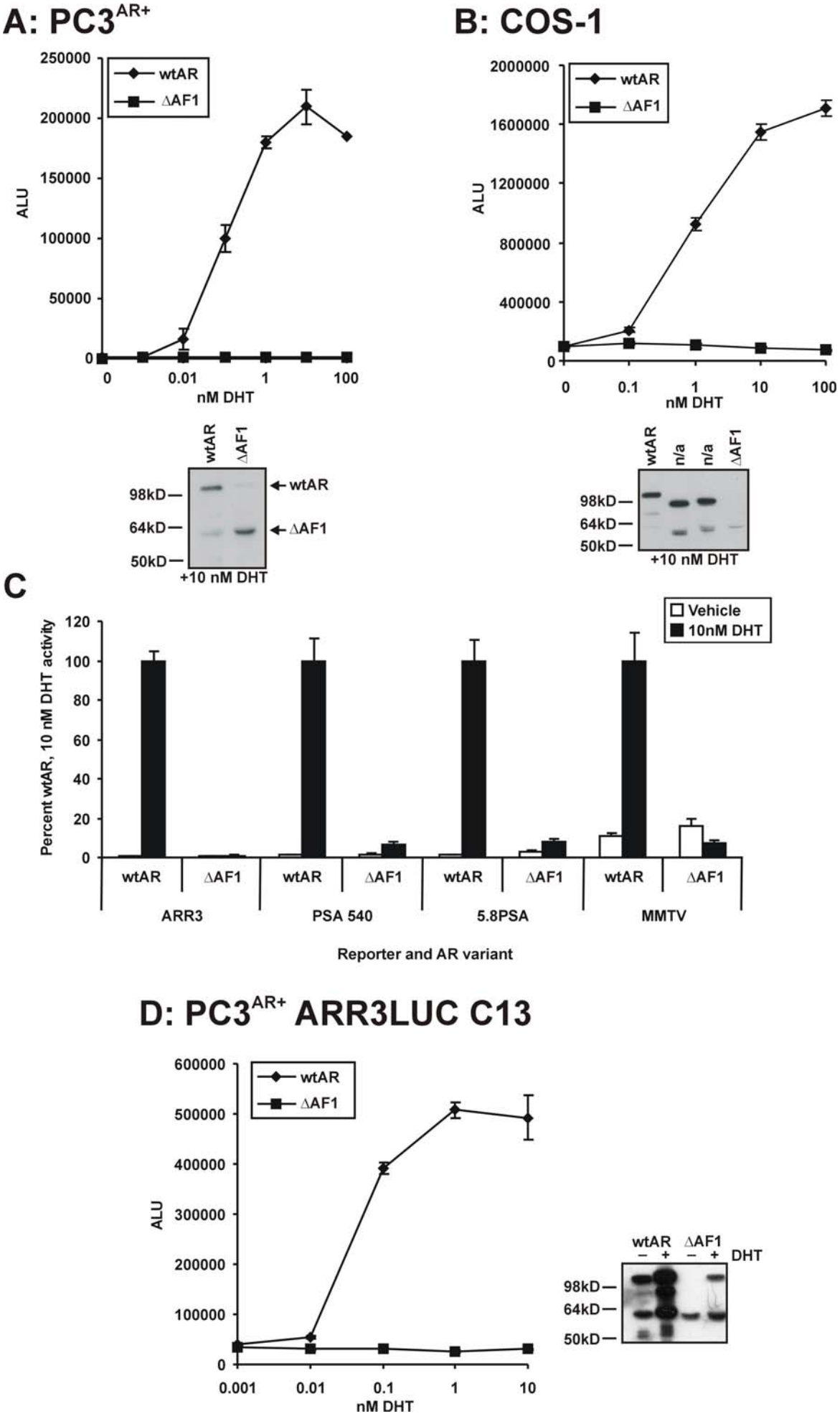
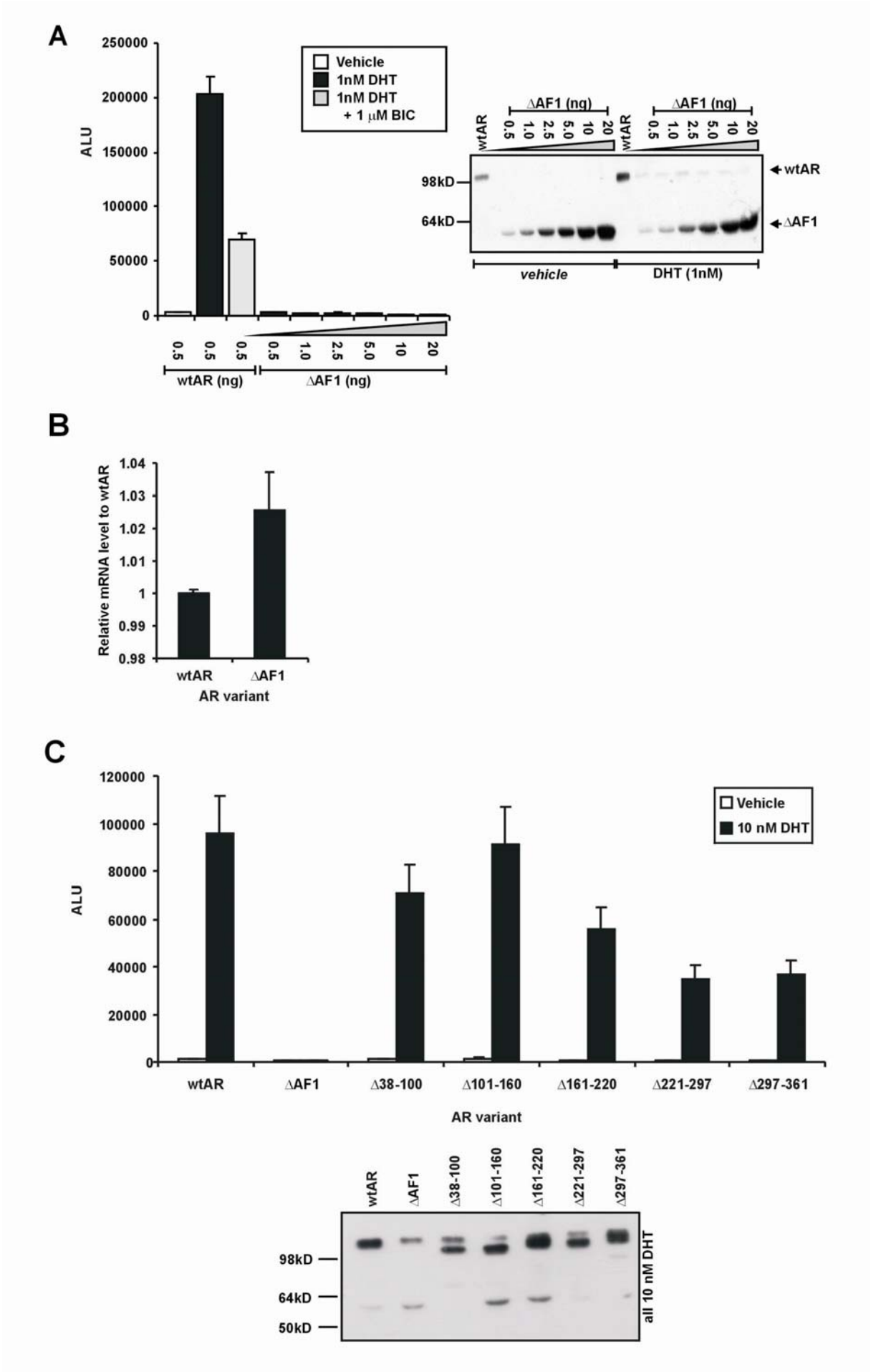

A: PC3AR+
B: COS-1
wtAR
ΔAF1
ALU
nM DHT
98kD
64kD
50kD
+10 nM DHT
n/a
C
Vehicle
10nM DHT
Percent wtAR, 10 nM DHT activity
ARR3
PSA 540
5.8PSA
MMTV
Reporter and AR variant
D: PC3AR+ ARR3LUC C13
DHT

Figure 4.3: AR ΔAF1 steady state levels are dependent on the amount of DNA transfected and are not affected by smaller deletions within this domain. A: $PC3^{AR+}$ cells were transfected with increasing amounts of pCMV-ARΔAF1 DNA while controlling for expression vector and DNA concentration per well with the vectors pCMV and pBS(sk-), along with 100 ng ARR3-tk-Luc per well. Cells were treated, lysed and assayed as described in Figure 4.2. **B:** $PC3^{AR+}$ cells (500,000/well in 6 well plates) were transfected with 33ng of pCMV-AR or molar equivalent of pCMV-ARΔAF1 per well. Wells were treated with 10 nM DHT and RNA isolated 24 hours later. RNA was extensively DNAse treated before cDNA synthesis. cDNA was amplified with AR-specific primers and normalised for expression with the housekeeping gene *S27a*. Data are expressed relative to pCMV-AR expression and represent the mean $\pm$ SD of three independently amplified wells of the same cDNA sample **C:** $PC3^{AR+}$ cells were transfected, treated and assayed with the ΔAF1 variants depicted as described in Figure 4.2A. All data is representative of at least 3 independently performed experiments.


A
250000
200000
150000
100000
50000
0
ALU
Vehicle
1nM DHT
1nM DHT
+ 1 μM BIC
0.5
0.5
0.5
0.5
1.0
2.5
5.0
10
20
wtAR (ng)
ΔAF1 (ng)
ΔAF1 (ng)
ΔAF1 (ng)
wtAR
wtAR
0.5
1.0
2.5
5.0
10
20
0.5
1.0
2.5
5.0
10
20
98kD
64kD
wtAR
ΔAF1
vehicle
DHT (1nM)
B
Relative mRNA level to wtAR
1.04
1.03
1.02
1.01
1
0.99
0.98
wtAR
ΔAF1
AR variant
C
120000
100000
80000
60000
40000
20000
0
ALU
Vehicle
10 nM DHT
wtAR
ΔAF1
Δ38-100
Δ101-160
Δ161-220
Δ221-297
Δ297-361
AR variant
wtAR
ΔAF1
Δ38-100
Δ101-160
Δ161-220
Δ221-297
Δ297-361
98kD
64kD
50kD
all 10 nM DHT

4.3.2. Activation of the constitutive AR variant T707 demonstrates redundancy in the usage of AF1 or AF5

In contrast to the wtAR receptor, it has traditionally been held that the activity of a constitutive AR is dependent on AF5 rather than AF1 (Jenster et al. 1995). In agreement with other studies, deletion of AF1 resulted in a reduction of activity of approximately 50% in comparison to T707. In contrast to these results however, the apparent loss of activity of T707 variants with a deletion of the core (T707ΔcAF5) or the entirety (T707ΔAF5) of AF5 can be rescued when the amount of transfected T707 expression plasmid is increased. Neither RNA or protein levels of these variants as assessed by quantitative RT-PCR or immunoblot, was responsible for these effects (Fig 4.4A and B). These data suggest a role for AF5 in the sensitivity of signalling of the constitutive AR variant rather than an absolute requirement for function.

4.3.3. Disruption of the AF2 surface does not lead to redundant AF domain usage in the AR NTD

To test whether the absence of an AF2 surface from T707 variants leads to redundancy in the requirement for transcriptional activation of the two activation surfaces in the AR NTD, NTD deletions were subcloned into full length AR harbouring the E895Q mutation, which abolishes the AR N/C interaction (Buchanan 2002). In contrast to the results with T707, E895QΔAF1 was unable to mediate transcription in $PC3^{AR+}$ cells whereas E895QΔAF5 and E895QΔcAF5 were functional albeit at much lower maximal activity in comparison to E895Q or wtAR (Fig 4.5A). Remarkably, when assessed in a different cell line, COS-1, E895Q, E895QΔAF5 and E895QΔcAF5 demonstrated identical sensitivity and maximal activation in response to DHT (Fig 4.5B). Consistent with published results demonstrating that N/C deficient AR variants have decreased stability (He et al. 2000), E895Q steady state protein

levels were lower than wtAR. The AR NTD deletion variants exhibited a similar effect on protein levels as observed for the full length or T707 AR variants (Fig 4.5A and B).

4.3.4. Deletion of AF5 results in a decrease in sensitivity of DHT-induced AR transcriptional activation

In full length AR with an intact AF2 surface, each of the deletion variants demonstrated reduced sensitivity of DHT-induced transcriptional activation in $PC3^{AR+}$ cells similar to that observed in the E895Q background (Fig 4.6A). While the requirement for ΔAF5 for maximal sensitivity of DHT-stimulated AR transactivation activity was consistent between $PC3^{AR+}$ and COS-1 cells as well as on a chromatin integrated ARR3 reporter, the relative effect of E895Q differed between the cell lineswhich may be due to different levels of coregulators and/or chromatin context in these cell lines (Fig 4.6A, B and C). RT-PCR indicated that these differences were not due to differences in mRNA levels (Fig 4.6D). These results indicate that the AF5 surface is required for maiximal sensitivity of DHT-stimulated AR activity regardless of cellular context. In comparison, the phenotype of the N/C disrupting E895Q mutation is cellular context dependent.

4.3.5. The AR deletion variants display similar responses on a variety of AR reporters

To assess if deletion variants exhibit response element specificity, activity was assessed on 2 *PSA* reporters and the MMTV promoter. Each of the deletion variants exhibited similar responses relative to wtAR on the PSA-based reporters (Fig 4.7). The response of the variants on the MMTV reporter was unique, with the ΔAF5 variant capable of the same level of transcriptional activation as the E895Q variant indicating that the MMTV promoter may have a stronger requirement for AF2 function than the other promoters. Relative steady state levels of the AR variants in comparison to wtAR remained consistent across the reporters (Fig 4.7).

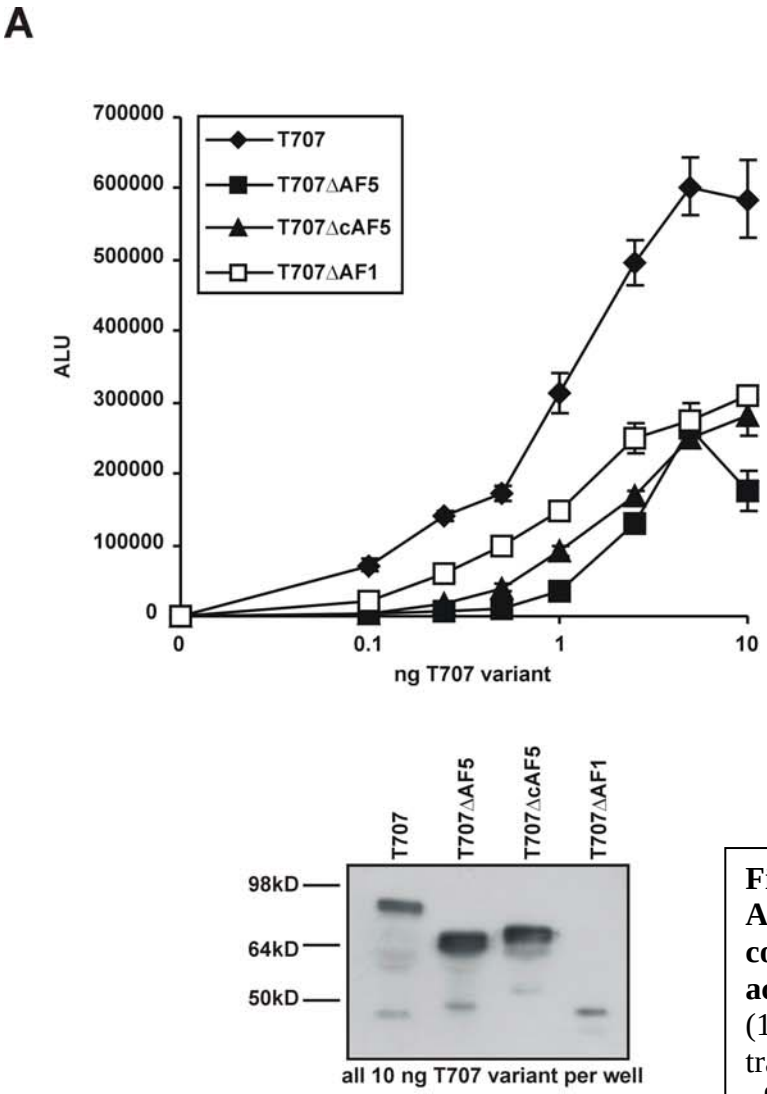


Figure 4.4: AR T707 utilises both AF1 and AF5 domains for constitutive transactivation activity. A: $PC3^{AR+}$ cells (10,000/well in 96 well plates) were transfected with increasing amounts of T707 or the molar equivalent of variants while controlling for expression vector and DNA concentration per well with pCMV and pBS(sk-), along with 100 ng ARR3-tk-Luc. Cells were left 24 hours before lysis, assay and immunoblotting as described in Figure 4.2. Data is representative of at least 3 independently performed experiments. **B:** RT-PCR, conducted for each of the variants was carried out as described in Figure 4.3B without hormone treatment. Data is representative of 2 independently performed experiments.

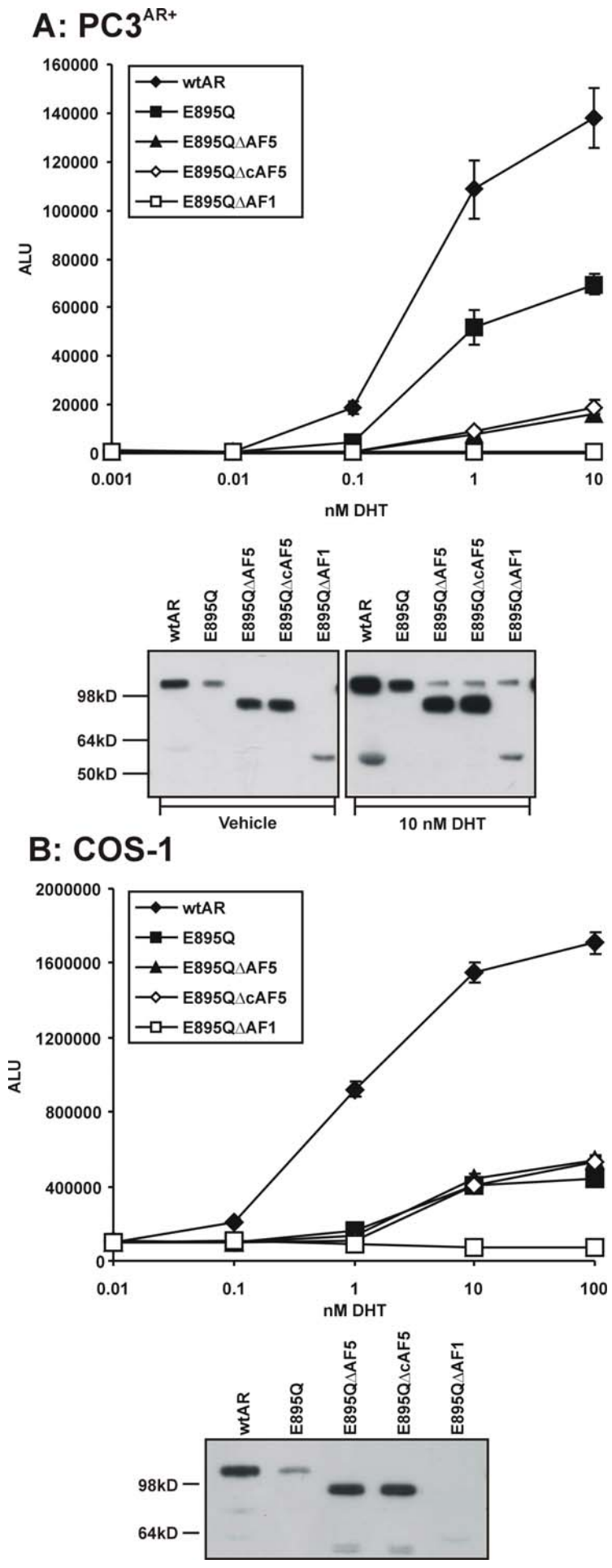
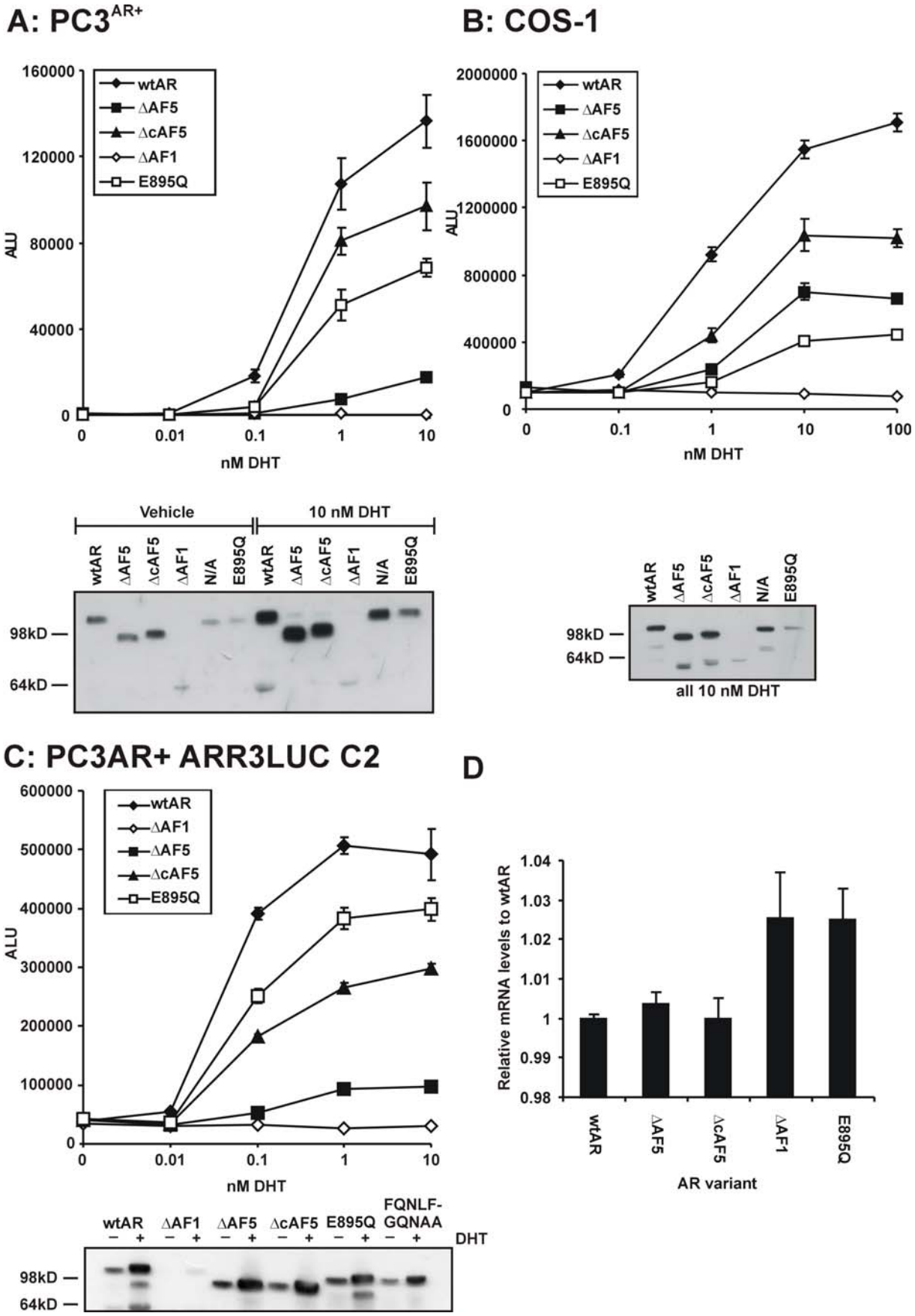


Figure 4.5: The disruption of the AF2 domain does not cause redundant AR NTD domain usage for transcriptional activation. A: PC-3^{AR+} cells (10,000/well in 96 well plates) were transfected, treated, assayed and immunoblotted utilising the AR variants depicted as described in Figure 4.2. **B:** COS-1 cells were transfected, treated, assayed and immunoblotted utilising the AR variants depicted as described in Figure 4.2. All data is representative of at least 3 independently performed experiments.

Figure 4.6: Deletion of AF5 results in a decrease in the sensitivity of AR transcriptional activation in response to DHT concomitant to that displayed for E895Q an N/C deficient AR variant. A: $PC3^{AR+}$ cells were transfected with the variants shown and treated, assayed and immunoblotted as described in Figure 4.2A **B:** Similar results were obtained when the assay was conducted in COS-1 cells **C:** Cell line $PC3^{AR+}$ ARR3 luciferase stable cell line C2 was transfected with the AR variants depicted as described in Figure 4.2C **D:** RT-PCR of the corresponding deletion variants was conducted as described in Figure 4.3B. All data is representative of at least 2 independently performed experiments.


A: PC3AR+
B: COS-1
wtAR
ΔAF5
ΔcAF5
ΔAF1
E895Q
ALU
nM DHT
Vehicle
10 nM DHT
98kD
64kD
all 10 nM DHT
C: PC3AR+ ARR3LUC C2
D
Relative mRNA levels to wtAR
AR variant
FQNLF-GQNAA
DHT

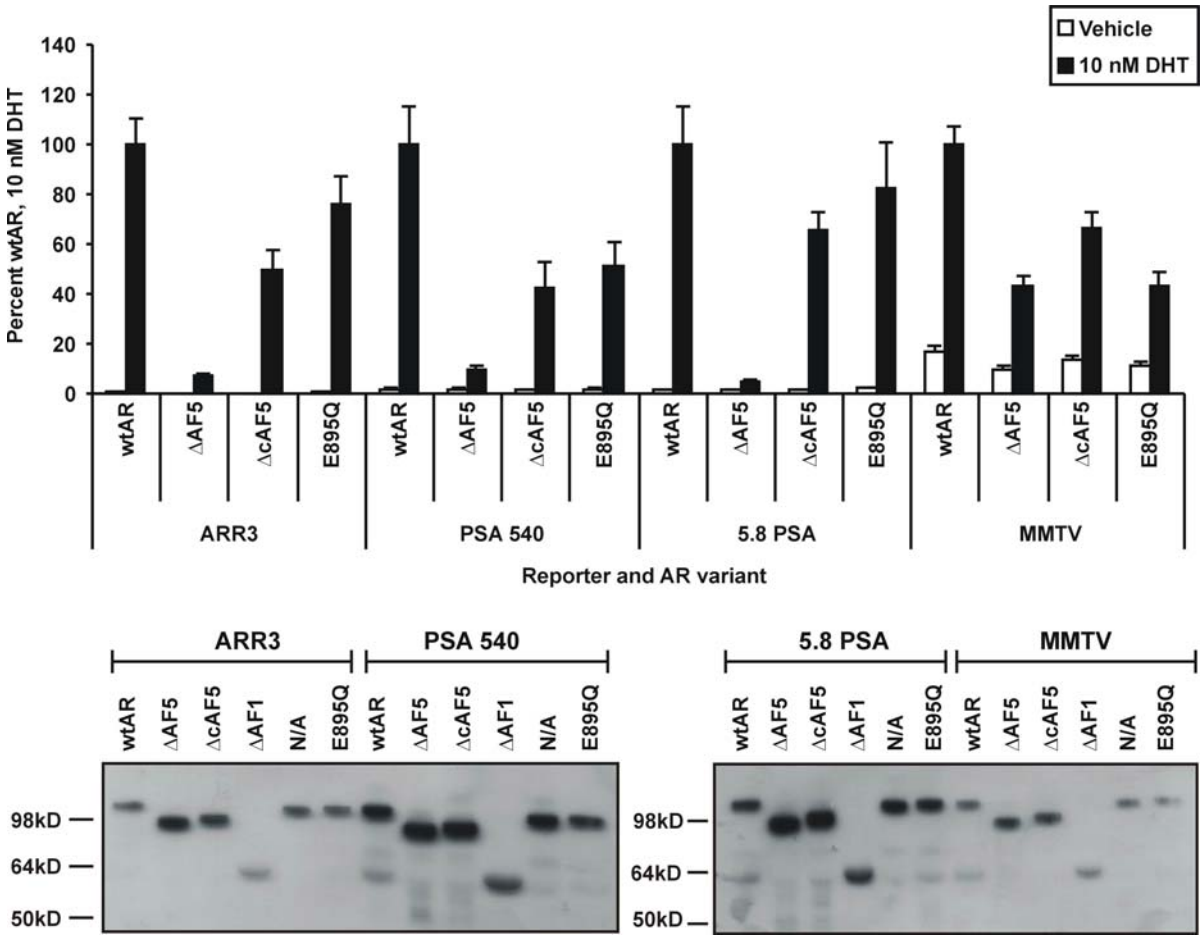
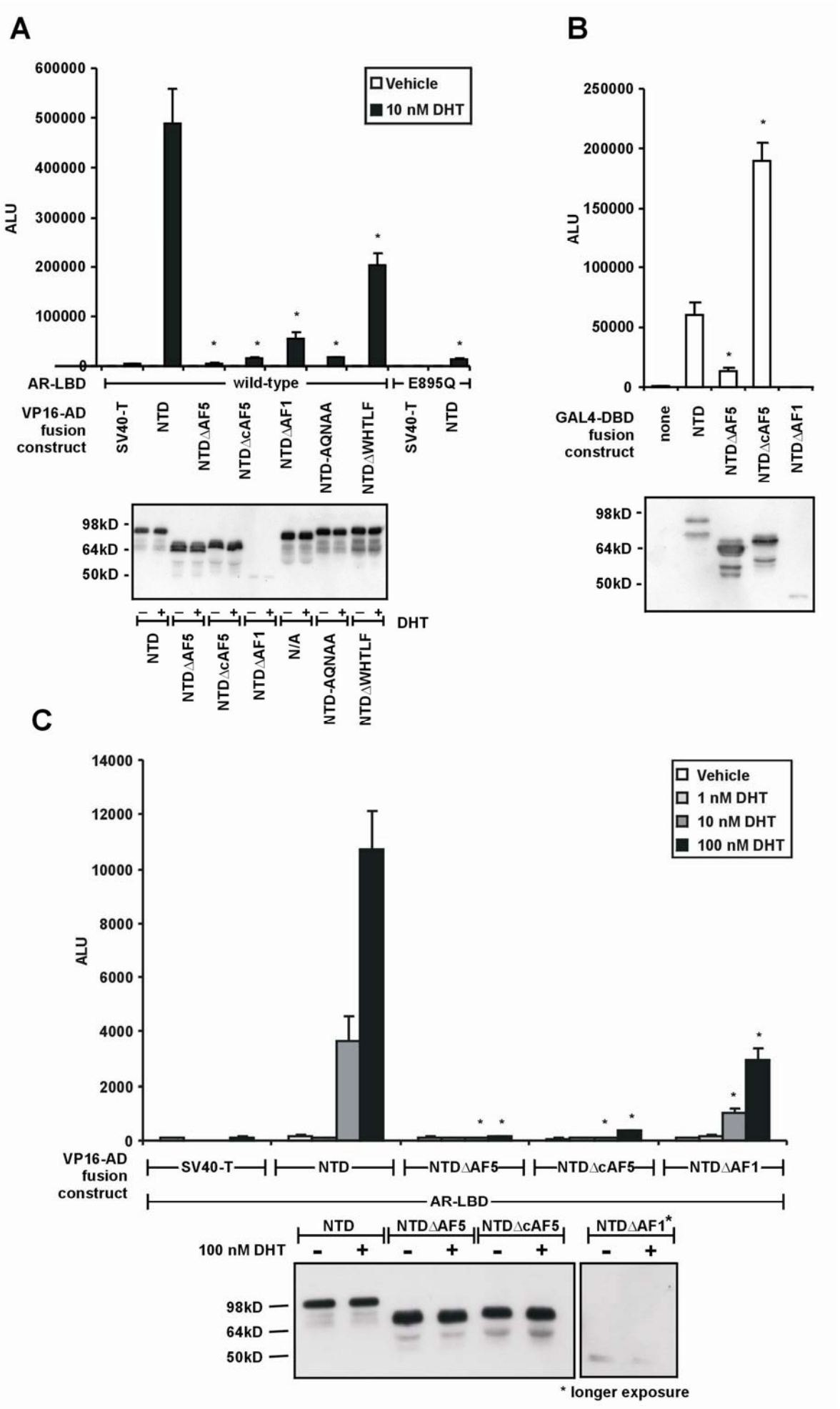


Figure 4.7: The AR deletion variants display similar responses between reporters. PC3^{AR+} cells (10,000 per well in 96 well plates) were transfected with 0.5 ng of pCMV-AR or molar equivalent of AR variant and 100 ng of either ARR3-tk-Luc, PSA540-Luc, 5.8PSA-LUC or MMTV-Luc and treated for 24 hours either in the absence (vehicle) or presence (10 nM) of DHT. Cells were lysed and assayed as described in Figure 4.2A. Immunobots show 10 nM DHT treated samples. Data is representative of 2 independently performed experiments.

4.3.6. Deletion of any of the activation functions in the AR NTD disrupts N/C interaction but can be rescued with increasing levels of NTDΔAF1

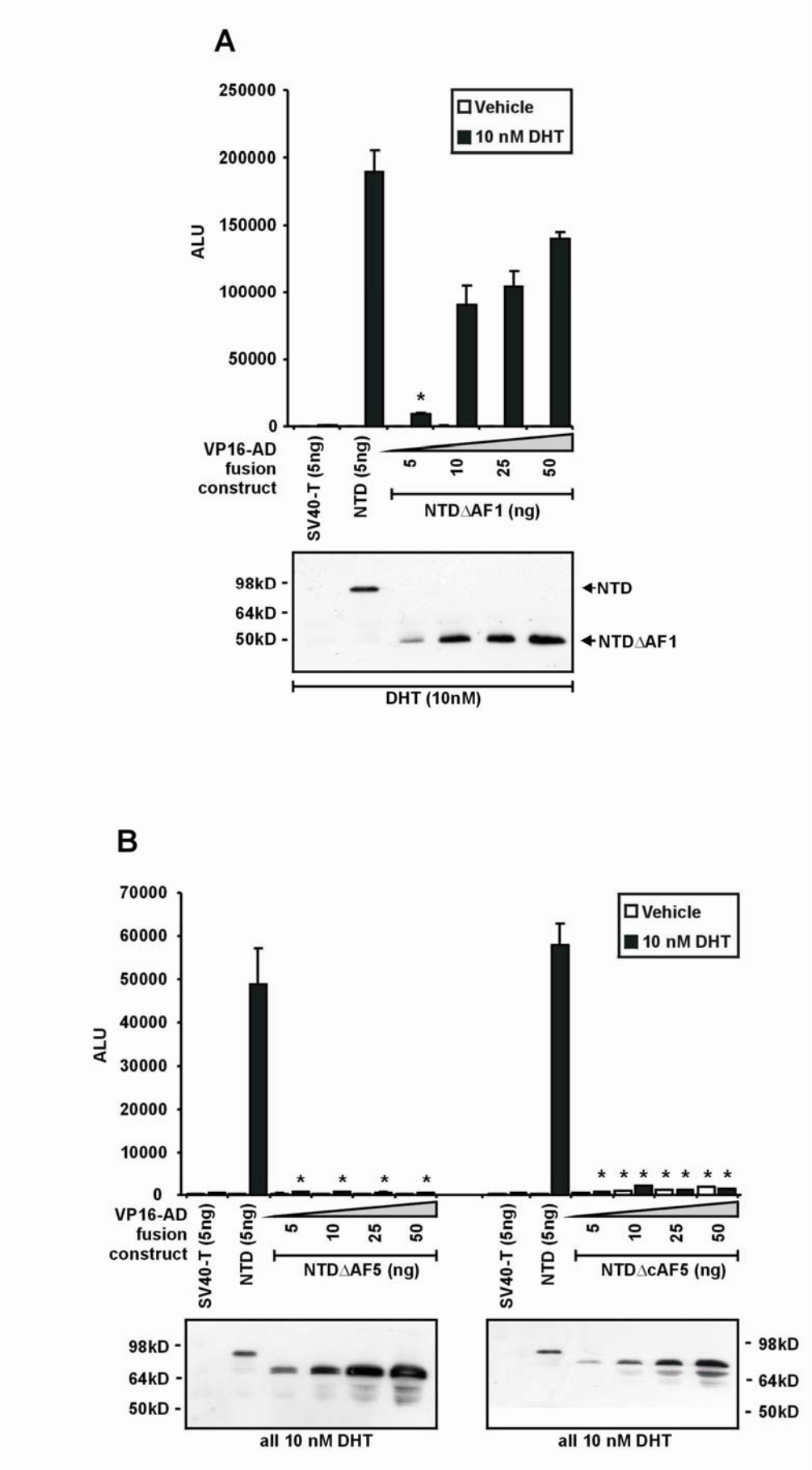
As ΔAF5 and ΔcAF5 exhibited decreased sensitivity to DHT similar to E895Q, it was postulated that deletion of AF5 may similarly interfere with the N/C interaction. To test this, these deletions were created in the AR NTD, fused to the VP16 activation domain and used in the classic AR N/C interaction assay (He et al. 2000; He et al. 2001; He et al. 2002). All NTD deletion variants exhibited a dramatic decrease in N/C interaction similar to that observed for the previously described N/C deficient ^{23}FQNLF^{27} to ^{23}AQNAA^{27} or E895Q mutations (Fig 4.8A). Deletion of the ^{432}WHTLF^{436} in AF5 decreased the N/C interaction activity, but not to the extent observed for the deletion of the complete AF5. These differences were not due to inherent differences in intrinsic transactivation activity of the NTD variants (Fig 4.8B), which exhibit activity independent of that observed in the N/C interaction assay, with NTDΔcAF5 having the greatest activity. Even in this context, NTDΔAF1 exhibited dramatically decreased steady state levels, whereas ΔAF5 and ΔcAF5 had increased protein in comparison to wtAR on immunoblot analysis (Fig 4.8B). While the N/C interaction exhibited by the ΔAF1 variant could be increased by increasing DHT concentration, this was not observed for the ΔAF5 and ΔcAF5 variants (Fig 4.8C). Furthermore, while the N/C interaction exhibited by the ΔAF1 variant could be rescued by increasing the amount of DNA transcted, the interaction exhibited by the ΔAF5 and ΔcAF5 variants could not be rescued in this manner (Fig 4.9A compared to 4.9B). Similar results were obtained in PC3^{AR+}cells (data not shown). Together, these results demonstrate a surprising and absolute requirement for AF5 for the N/C interaction of the AR, despite contemporary thought that only the ^{23}FQNLF^{27} and the AF2 surfaces are necessary.

Figure 4.8: Deletion of any portion of the NTD results in the disruption of the N/C interaction. A: The AR NTD deletions result in decreased N/C interaction in COS-1 cells. Androgen receptor negative COS-1 cells were plated (10,000 cells/well) in 96 well plates and 12 wells were transfected with 5ng each of the designated vectors or molar equivalent of variant, 5ng of GAL4-DBD tagged AR-LBD and 25ng of the pGK-1 reporter plasmid. After transfection, 6 wells were treated with vehicle (EtOH) and 6 wells were treated with 10 nM DHT. Cells were lysed, assayed and immunoblotted as described in Figure 4.2A. Data represent the mean $\pm$ SEM of the 6 independently transfected wells. **B:** N/C assay activity does not correlate to the intrinsic activity of the tagged receptor NTD's. AR NTD deletion variants were created fused to a Gal4 activation domain and 5ng of each (the molar equivalent of deletion variants) was co transfected into COS-1 cells (10,000/well in 96 well plates) with 25ng of the reporter pGK-1. Cells were lysed, assayed and immunoblotted 24 hours later as described in Figure 4.2A. . **C:** COS-1 cells were transfected with AR NTD or each of the deletion variants as described in Figure 4.8A. Six wells each were treated with increasing concentrations of DHT from 1-100 nM or with the equivalent amount of vehicle (EtOH). Wells were lysed, assayed and immunoblotted as described in Figure 4.8A. All data is representative of at least 3 independently performed experiments. *$p<0.05$ on Mann Whitney U comparison of medians compared to similarly treated non-variant AR NTD. Samples without an aterisk are not statistically significantly different from the respective similarly treated non-variant AR-NTD.



response of the receptor via modulation of p160 and N/C interactions.

Figure 4.9: The AF5 domain is required for the N/C interaction. A: COS-1 cells were transfected with AR NTD or increasing molar concentrations of NTDΔAF1 while controlling for molar concentration of expression plasmid and DNA concentration with pVP16 and pBS(sk-) respectively. Wells were treated with 10 nM DHT or the equivalent amount of vehicle (EtOH) for 24 hours before being assayed and immunoblotted as described in Figure 4.8A. **B:** Increasing steady state levels of delAF5 and delcAF5 utilising the method described in A fails to rescue the N/C interaction. All data is representative of at least 3 independently performed experiments.


A
250000
200000
150000
100000
50000
0
ALU
Vehicle
10 nM DHT
*
VP16-AD fusion construct
SV40-T (5ng)
NTD (5ng)
5
10
25
50
NTDΔAF1 (ng)
98kD
64kD
50kD
NTD
NTDΔAF1
DHT (10nM)
B
70000
60000
50000
40000
30000
20000
10000
0
ALU
Vehicle
10 nM DHT
VP16-AD fusion construct
SV40-T (5ng)
NTD (5ng)
NTDΔAF5 (ng)
NTDΔcAF5 (ng)
98kD
64kD
50kD
all 10 nM DHT
all 10 nM DHT

4.3.7 AF5 is required for interaction of the AR NTD with the p160 coactivator, NCOA2

While this chapter demonstrates that both the AF2 and AF5 surfaces are important for the N/C interaction, both of these surfaces have previously been implicated in the interaction with p160 coactivators (Irvine et al. 2000; He et al. 2003). Therefore, to assess if the region of the AR NTD required for N/C interaction is the same region required for p160 interaction, the ability of each of the deletion variants to interact with the p160 coregulator NCOA2 was assessed. The p160 coactivator NCOA2 was able to minimally enhance the control for the mammalian 2-hybrid assay, a vector overexpressing solely the VP16 activation domain (Figure 1A) while a stronger increase in luciferase activity was observed for the AR NTD (Figure 1A). In addition, each of these variants fused to the VP16 activation domain were also separately co-expressed at their highest levels with Gal4DBD fused to p53 to discount intrinsic activity of the AR NTD constructs themselves being a cause of the increased activity observed. Deletion of any portion of the NTD appeared to disrupt the interaction of the AR NTD with NCOA2, but similarly to the N/C interaction, could be rescued by increasing the amount of NTDΔAF1 but not NTDΔAF5 or NTDΔcAF5 (Fig 4.10A and B). The NTD-^{23}AQNAA^{27} variant, which is incapable of N/C interaction, exhibited similar interaction with NCOA2 as the wtAR NTD. Whereas the previous chapter demonstrated a requirement for amino acids 500-535 in the interaction of the AR NTD with NCOA2, these amino acids are not sufficient for interaction as demonstrated by the inability of NTDΔcAF5 to increase activity over baseline (Fig 4.10B). Therefore, amino acids 500 to 535 as well as amino acids within the core of AF5 are required for the formation of the p160 interaction surface in the AR NTD.

4.3.8 AF5 inhibits the ability of AF2 region to mediate AR activity via the p160 coregulators

The importance of utilising full length receptors to investigate p160 binding by the AR was demonstrated in the previous chapter. This is perhaps not surprising given that the p160 coregulators have been reported to interact with both AF2 and AF5 (Ma et al. 1999). Therefore, the ability of each of the p160 family members to enhance the transactivation activity of the full length AR deletion variants in comparison to wtAR was assessed. The basal activity of all of the variants in response to overexpression of the coregulators was similar to wtAR (Fig 4.11A). Similarly, the DHT-treated AR variants with an intact AF2 or AF5 domain did not exhibit enhancement above the level of wtAR by the overexpression of NCOA1, NCOA2 and NCOA3 except for the ΔAF5 variant which exhibited significantly increased enhancement compared to wtAR with the overexpression of NCOA2 (Fig 4.11B). In comparison, E895QΔAF5 and E895QΔcAF5 exhibited a significant enhancement of DHT-induced activity in comparison to wtAR for all three p160 coactivators (Fig 4.11B).

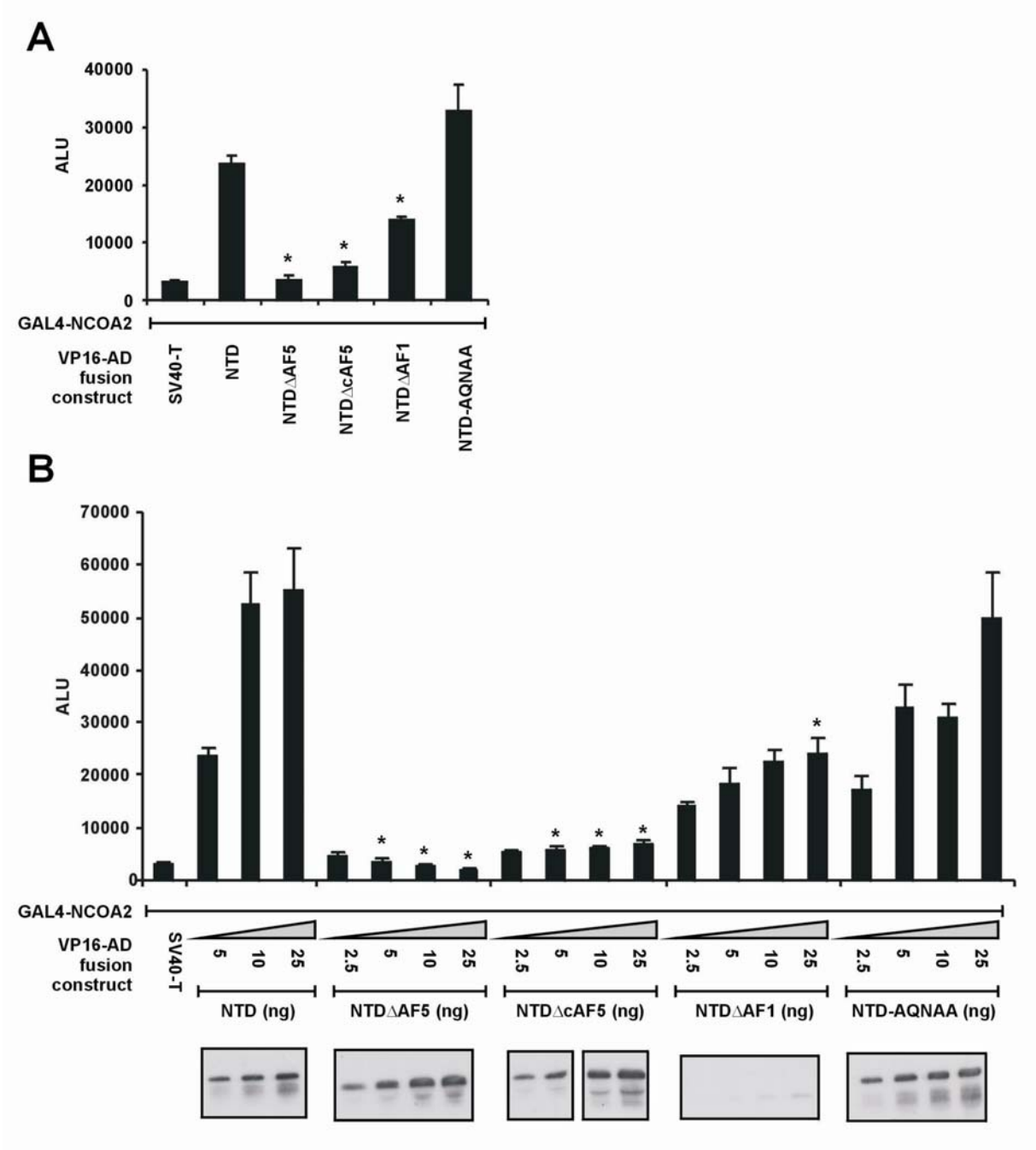


Figure 4.10: Deletion of AF5 results in disruption of the AR NTD interaction with the p160 coregulator NCOA2. A: COS-1 cells (10,000) were transfected with 5 ng of VP16-tagged AR-NTD deletion variant (or molar equivalent) and 25ng of GAL4 DBD tagged NCOA2 with 100 ng of the pGK-1 reporter plasmid. All wells were controlled for molar equivalent of expression plasmid and total DNA transfected. Data represent the mean ± SEM of six individually transfected wells. Immunoblot performed as described in Figure 4.2A. **B:** COS-1 cells were transfected with increasing concentrations of each of the deletion variants depicted as described in Figure 4.9C. All data is representative of at least 3 independently performed experiments. All variants were compared by Mann Whitney U comparison of medians to similar level of intact AR NTD in the NCOA2 interaction assay. * = $p<0.05$.

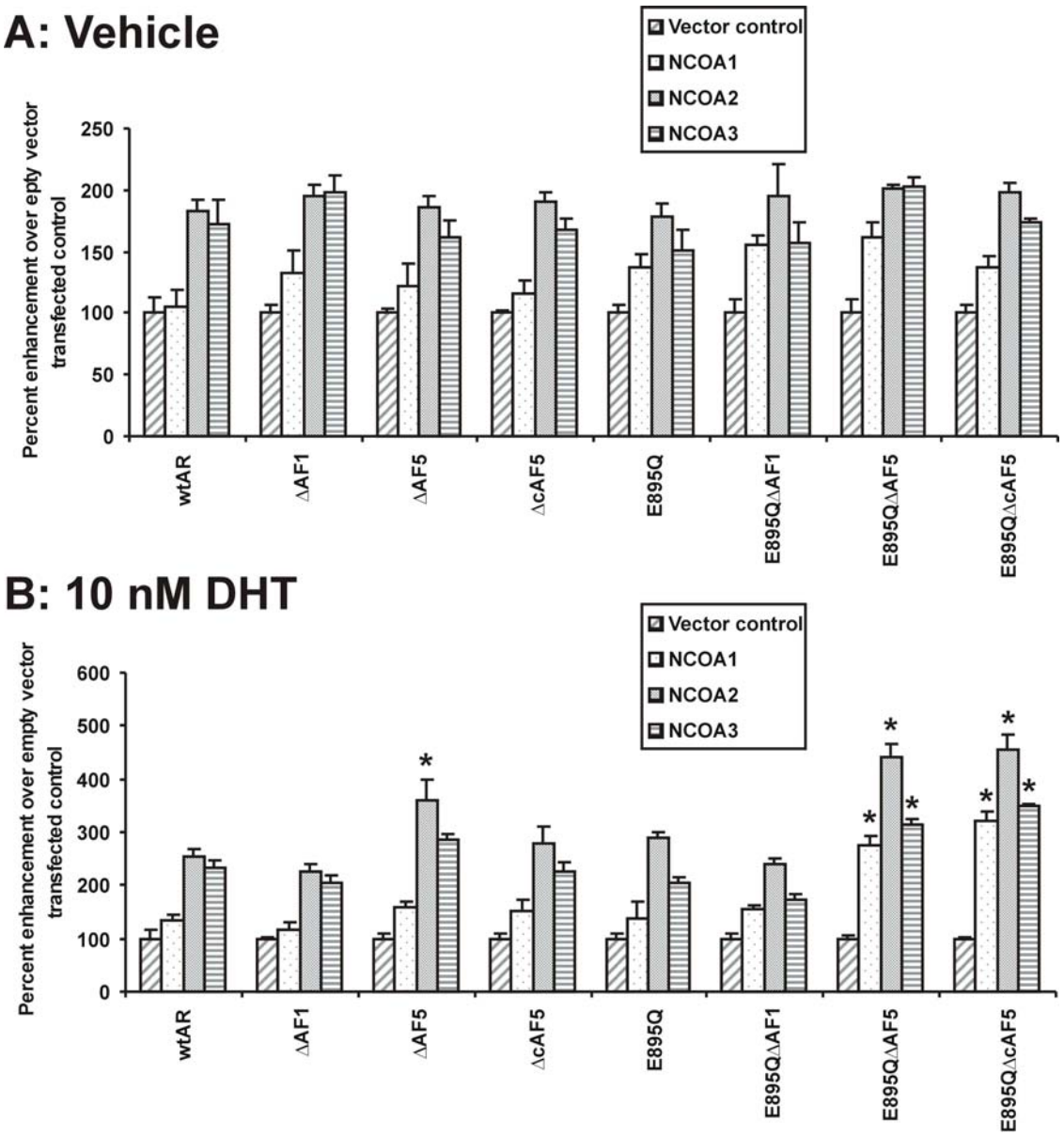


Figure 4.11: Deletion variants ΔAF5, E895QΔAF5 and E895QΔcAF5 have increased DHT-induced enhancement in response to the overexpression of p160 coregulators in comparison to wtAR. A: $PC3^{AR+}$ cells (10,000 per well) were transfected with 100 ng ARR3-tk-LUC, 0.5 ng AR variant expression vector along with 25 ng of pSG5-SRC1 (NCOA1), pSG5-GRIP1 (NCOA2) or pSG5-AIB1 (NCOA3) or the equivalent molar concentration of the empty vector pSG5. Wells were treated with vehicle control (EtOH), assayed and immunoblotted as described in Figure 4.2A. Data represent the percent enhancement of activity in the coregulator transfected wells compared to the respective empty vector wells for each variant where 100 indicates a lack of enhancement over the empty vector transfected wells. Data represents mean enhancement ± SEM of four independently treansfected wells. **B:** $PC3^{AR+}$ cells were transfected as described in A, but treated with 10 nM DHT. Data is presented as described in A. Data is representative of 2 independently performed experiments. All variants were compared to similarly treated wtAR by Mann Whitney U analysis of medians and * = $p<0.05$ when compared to the enhancement of wtAR by the specific coregulator.

4.4 Discussion

The amino and carboxyl domains of the AR collectively define the transcriptional response to androgens by acting as signal input adaptors for corepressor and coactivator molecules. Whereas large deletions of the AR have been demonstrated to affect overall structure of some proteins, deletions within the AR NTD have been shown to have little consequence on the key receptor function of ligand binding or on overall predicted protein size (Chamberlain et al. 1996). This may be due to the relatively unstructured nature of this domain in comparison to the AR DBD or AR LBD.

Studies in this chapter demonstrate that AF1 is essential for AR transcriptional competency irrespective of cellular context, N/C interaction or p160 interactions. Hydrophobic residues within AF1 are required for binding to the essential transcription factor IIF and the transcription factor IIH is also reported to interact with this region (McEwan et al. 1997; Lee et al. 2000; Betney et al. 2003). Therefore, this region may be required for interaction with the general transcriptional machinery rather than interactions with cofactors that govern cellular specificity of transcriptional response, such as corepressors or the p160 coactivators. Importantly, deletion of AF1 resulted in decreased AR steady state protein levels irrespective of the structural context. However, no single region of AF1 could be identified as solely responsible for reduced transactivation or steady state protein levels. Consequently, the decline in transactivation and protein level may be linked, a process that may involve both transcription and ubiquitinylation (Dennis et al. 2005; Gaughan et al. 2005).

The only AF1 deleted variant to exhibit activity was in the background of the AR variant T707, which in contrast to wtAR, is constitutively nuclear and binds to DNA in the timeframe of seconds rather than minutes as observed for full length AR (Tepper et al. 2002; Farla et al. 2004). These results highlight the importance of AR protein level for AR transcriptional

activity and contradict previous studies utilising only a single DNA concentration which concluded that AF5 is required for transcriptional activation of the constitutive AR variant (Jenster et al. 1995). In contrast, the findings in this chapter demonstrate that AF1 and AF5 exert different transactivation strengths on a constitutive receptor truncated of the LBD. Compared to the wtAR protein, AF5 becomes predominant in the truncated AR which we show may be due to the interaction of this domain with the p160 coregulators and increased stability.

Whereas it appears that AF5 is not essential for AR transcriptional activation in the context of a full length receptor, this domain is critical for input from the cellular complement of coregulators and the sensitivity of the transcriptional response to ligand. Importantly, the altrered sensitivity of the AF5 deleted AR variants is not dependent on promoter or cellular context. This indicates that this effect is an inherent property of the variant receptor. In support of the requirement for AF5 in governing receptor sensitivity to ligand, the N/C interaction is required for the stability of the ligand bound AR (He et al. 2000), which could conceivably impact on the sensitivity of the receptor to ligand. Furthermore, over expression of the p160 coregulators has also been shown to increase transactivation by the AR in response to ligand (Alen et al. 1999; Bevan et al. 1999; Christiaens et al. 2002), so conversely, deletion of a domain that is responsible for this interaction may also lead to decreased receptor sensitivity to ligand. Therefore, these effects combined may result in the observed phenotype of the ΔAF5 and ΔcAF5 variants.

The residue E895 of the AR forms one end of the charge clamp of the AF2 pocket that accommodates both FQNLF and LxxLL peptides associated with N/C interaction and p160 binding, respectively. It has therefore previously been assumed that the substitution of E895Q would disrupt the charge clamp and disable both N/C interaction and p160 coregulator binding. However, recent crystal structures of peptide bound AR AF2 surfaces has revealed

that E895 residue is critical for binding the FQNLF peptide but does not make direct contact with LxxLL peptides due to a shift in its position in the AF2 pocket (Hur et al. 2004; He et al. 2006). Replacement of the negatively charged glutamic acid with a positively charged residue (that is, lysine) completely disrupts the charge clamp and results in the loss of both FQNLF and LxxLL binding (He et al. 2006). The effect of a neutral residue at this position (that is, glutamine) on LxxLL peptide binding and receptor structure is unknown. However, given that both E895QΔAF5 and E895QΔcAF5 AR variants respond to enhancement by p160 coregulators better than wtAR despite the apparent loss of both signal input domains suggests that the E895Q mutation is specific only for the disruption of the N/C interaction.

With recently published experiments conducted in live cells in real time the results presented in this chapter allow us to develop an integrated model of activation domain involvement in AR N/C and p160 interactions. In this model, binding of ligand results in a rapid intramolecular interaction between the amino and carboxyl termini of the AR that depends on the integrity of FQNLF and the AF2 pocket. This interaction predominates until the receptor translocates to the nucleus and binds to DNA (Schaufele et al. 2005; van Royen et al. 2007). Upon DNA binding, the receptor undergoes a conformational change in which the ^{23}FQNLF^{27} motif is displaced and a new N/C interaction is mediated by a single p160 coregulator molecule that binds to both AF5 and AF2. This bridging N/C (Shen et al. 2005) stabilises the receptor and fosters interaction with secondary coregulators and effective transcriptional activation. This model explains why the AF5 domain appears necessary for the N/C interaction as assessed by the mammalian 2-hybrid interaction assay; both the initial ^{23}FQNLF^{27}-AF2 association and the association with the p160 coregulators are required. In support of this hypothesis, over expression of the p160 coregulator NCOA2 can rescue the N/C interaction of the N/C deficient ^{23}FQNLF^{27} to ^{23}AQNAA^{27} mutant (Shen et al. 2005). Furthermore, increased binding of NCOA1 to the AR NTD has been demonstrated for an AR variant mutated at amino acid 21 which impairs the N/C interaction (Callewaert et al. 2003).

Critically, these two interactions are exclusive and occur in sequence, with ^{23}FQNLF^{27}-AF2 interaction necessary to intiate AR transcriptional activation *in vivo* while the p160 coactivator bridging provides sensitivity of AR signalling in response to androgens. Nonetheless, the AF2 domain appears capable of recruiting p160 coregulators in the absence of AF5. In favour of the above model, deletion of any portion of AF5 in combination with the E895Q mutation resulted in receptors exhibiting a response to p160 coactivator over expression greater than wtAR, presumably since i) the absence of an N/C interaction provides a more permissive environment for p160 coactivator recruitment to AF2 and ii) the reduced steric requirement for an AF2 bound p160 to also bind AF5.

Further evidence for a domain bridging model comes from the other steroid receptors. ERα undergoes a direct measurable N/C-like interaction only in the presence of p160 coregulators (Benecke et al. 2000). Whereas coregulator binding is not necessary for interaction between amino and carboxyl terminal domains of the MR, coregulator binding may enhance the interaction (Rogerson et al. 2003). Moreover, GR can interact with NCOA2 via both its AF1 and AF2 domains, and PR forms an N/C interaction that can be enhanced by p160 coregulators (Tetel et al. 1999; Charmandari et al. 2006). Together, these data suggest that p160 coregulators may act similarly on all steroid receptors to mediate or stabilise bridging of their amino and carboxyl terminal domains.The ability to adopt an N/C interaction in the presence of ligand but not p160 coregulators is confined to the oxy-steroid receptors (i.e. the AR, PR, GR and MR), with the AR N/C being the most comprehensively characterised ligand-mediated interaction.

In summary, this chapter has demonstrated that the AF1 domain is essential for the transcriptional activity of the AR regardless of cellular context. Conversely, the AF5 domain is important in mediating sensitivity of AR mediated transcription in response to androgens via coregulator interactions. Furthermore, our data highlights the importance of p160

interactions to the output from the mammalian 2-hybrid N/C interaction assay, and demonstrate that N/C and p160 interactions, while mutually exclusive, result in maximum transcriptional competency of the receptor in response to androgens. Future studies will define whether this is a generalised mechanism for all of the oxy-steroid receptors.