# MOLECULAR MECHANISMS OF ANDROGEN AND ANTIANDROGEN ACTION

MOLECULAIRE WERKINGSMECHANISMEN VAN
ANDROGENEN EN ANTIANDROGENEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. P.W.C. AKKERMANS, M.A.
EN VOLGENS HET BESLUIT VAN HET COLLEGE VOOR
PROMOTIES.

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 29 JANUARI 1997 OM 13.45 UUR.

DOOR

CORNELIS WILLIBRORDUS KUIL

GEBOREN TE HEEMSKERK

**PROMOTIE-COMMISSIE:**

| | |
|---|---|
| PROMOTOR: | PROF. DR. J.A. GROOTEGOED |
| OVERIGE LEDEN: | PROF. DR. E.R. DE KLOET<br>PROF. DR. F.H. SCHRöDER<br>PROF. DR. IR. T.J. VISSER |
| CO-PROMOTOR: | DR. E. MULDER |

Dit proefschrift werd bewerkt binnen de Vakgroep Endocrinologie & Voortplanting.
Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam.

In dankbare herinnering aan mijn ouders

# CONTENTS

page
Abbreviations 7

Chapter 1: AIM AND SCOPE OF THIS THESIS 9
1.1 Steroid hormone action 10
1.2 Steroid hormone antagonists 10
1.3 Aim and scope of this thesis 11

Chapter 2: INTRODUCTION 13
2.1 Structural and functional domains of steroid hormone receptors 14
2.1.1 Evolution of the gene family 14
2.1.2 DNA binding domain 15
2.1.3 Ligand binding domain 17
2.1.4 Amino-terminal domain 19
2.2 Molecular mechanisms of antiandrogen action 20
2.2.1 Introduction 20
2.2.2 Antagonist-receptor interactions 21
2.2.3 Role of dissociation rate in antagonist action 21
2.2.4 Subcellular localisation of antagonist-bound receptors 22
2.2.5 Dissociation of the heat-shock protein-receptor complex 23
2.2.6 Receptor dimerization and DNA binding 25
2.2.7 Transcription regulation 27
2.2.7.1 Transactivation 27
2.2.7.2 Transrepression 29

Chapter 3: MECHANISM OF ANTIANDROGEN ACTION: CONFORMATIONAL CHANGES OF THE RECEPTOR 31

Chapter 4: LIGAND-INDUCED CONFORMATIONAL ALTERATIONS OF THE ANDROGEN RECEPTOR ANALYZED BY LIMITED TRYPSINIZATION 39

Chapter 5: DNA-BINDING ABILITY OF ANDROGEN RECEPTORS IN WHOLE CELLS: IMPLICATIONS FOR THE ACTION OF ANDROGENS AND ANTIANDROGENS 55

Chapter 6: FUNCTIONAL IN VIVO INTERACTION BETWEEN THE AMINO-TERMINAL, TRANSACTIVATING DOMAIN AND THE LIGAND BINDING DOMAIN OF THE ANDROGEN RECEPTOR 69

Chapter 7: GENERAL DISCUSSION 89
7.1 Androgen receptor structure-activity relationships 90
7.1.1 Three-dimensional structure of the ligand binding domain 90
7.1.2 Similarities in the ligand binding pocket of nuclear hormone receptors 92
7.1.3 Antagonists and the structure of the ligand binding pocket 94

**Contents**

7.1.4 Models of the ligand binding pocket(s) 95
7.2 Cellular mechanisms of antiandrogen action 96
7.3 Interactions of the N- and C-terminal domains of the androgen receptor 98
7.4 Antiandrogen action *in vitro* versus *in vitro* 100
7.5 Future investigations 101

References 104

Summary 118

Samenvatting 121

List of publications related to this thesis 124

Curriculum vitae 125

Nawoord 126

## Abbreviations

| | |
|---|---|
| AF | activation function |
| AR | androgen receptor |
| ARE | androgen response element |
| ARL | androgen receptor in LNCaP cell |
| BS | Bluescript |
| cDNA | complementary deoxyribonucleic acid |
| CHO | chinese hamster ovary cell line |
| CMV | cytomegalo virus |
| CPA | cyproterone acetate |
| (k)Da | (kilo)Dalton, molecular mass |
| DHT | dihydrotestosterone |
| DNA | deoxyribonucleic acid |
| ER | estrogen receptor |
| ERE | estrogen response element |
| GR | glucocorticoid receptor |
| GRE | glucocorticoid response element |
| HeLa | human cervix carcinoma cell line |
| hsp | heat-shock protein |
| hsp70 | 70 kDa heat-shock protein |
| hsp90 | 90 kDa heat-shock protein |
| ICI334 | ICI 176.334 (bicalutamide) |
| $K_d$ | equilibrium dissociation constant |
| LBD | ligand binding domain |
| LNCaP | human lymph node carcinoma of the prostate (cell line) |
| LUC | luciferase |
| MMTV | mouse mammary tumor virus |
| MR | mineralocorticoid receptor |
| mRNA | messenger ribonucleic acid |
| OH-F | hydroxyflutamide |
| p(prefix) | plasmid |
| PAGE | polyacrylamide gel electrophoresis |
| PR | progesterone receptor |
| R1881 | methyltrienolone |
| RAR | retinoic acid receptor |
| RBA | relative binding affinity |
| RU 486 | RU 38486 (mifepristone) |
| RU 908 | RU 23908 (anandron) |
| RXR | retinoid acid receptor |
| S | Svedberg unit, sedimentation coëfficient |
| SDS | sodium dodecyl sulfate |
| SV | Simian virus |
| T | testosterone |
| TAD | transactivation domain |
| TAF | TBP-associated factor |

**Abbreviations**

| | |
|---|---|
| TAU | transcription activation unit |
| TBP | TATA binding protein |
| TF | transcription factor |
| TIF | transcription intermediairy factor |
| TR | thyroid hormone receptor |

# AIM AND SCOPE OF THIS THESIS

# Chapter 1

## 1.1 Steroid hormone action

The steroid hormones testosterone and 5α-dihydrotestosterone (androgens) control the development, differentiation and function of male reproductive and accessory sex tissues, such as seminal vesicle, epididymis and prostate. Changes in cell properties induced by androgens require the presence of a specific cellular receptor, the androgen receptor (AR). The AR is a member of the subfamily of intracellular receptors (Evans, 1988) which also include the progesterone receptor (PR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and estrogen receptor (ER). The cellular concentration of these receptors is extremely low, under normal physiological conditions rarely exceeding 0.01% of total cellular protein (McDonnell *et al.*, 1987). As a result, it was only with the cloning of the receptor cDNAs and subsequent genetic analysis that a clearer understanding of structure-function relationships of the receptor proteins has emerged. The cDNAs for all of the known steroid hormone receptors have been cloned and sequenced. A comparison of their deduced amino acid sequences reveals regions of extensive homology indicating that they belong to a family of closely related proteins. Functional mapping of the intracellular receptors indicates that the most highly conserved region comprises the DNA binding domain, whereas the carboxyl terminus contains sequences required for hormone binding, dimerization, nuclear localization, interaction with heat-shock proteins, and transcription regulation. The N-terminal domains of the receptor proteins are mainly involved in transcription regulation (Beato, 1989; Tsai and O'Malley, 1994; Beato *et al.*, 1995).
The steroid hormone receptors are latent transcription factors which are activated upon interaction with their specific ligands. The mechanism by which the AR and other steroid hormone receptors mediate their biological effects in target cells is comparable. In the absence of hormone, the latent receptor resides in a large macromolecular complex comprising heat shock protein 90 (hsp90), hsp70, the immunophilin p59 and other proteins (Smith and Toft, 1993). Binding of the respective ligand triggers a complex set of interactions of the receptor with chromatin and with a variety of other proteins, finally leading to modifications in the pattern of gene expression and cell fate (Fig. 1.1).

## 1.2 Steroid hormone antagonists

Inhibition of biological action of steroid hormones can be obtained by two major routes: first, by occupation of the receptor with an antagonist instead of an agonist (steroid receptor antagonists), or second, by prevention of formation of the agonist in the biological system. Formation of agonists can be prevented through excision of hormone-producing organs (e.g. castration) or by blockade of hormone biosynthesis. Although substances inhibiting biosynthetic pathways are also often called antagonists, this type of antagonists is not the subject of this thesis.
For all classes of steroid hormones, substances are known that antagonize biological action of agonists by competing for binding to the receptor (Wakeling, 1992). Although an antagonist does bind to the receptor, subsequent steps in the process of receptor activation do not proceed normally. The basis for this deficiency is not well understood, but it is assumed that the conformation of the receptor bound to an antagonist is different from that bound to an agonist. As a result, its participation in one or more critical events (shown in Fig. 1.1), such as disruption of the macromolecular complex, dimerization, phosphorylation, binding to DNA, or transactivation of target genes, cannot take place.
Hormone antagonists can be used for inhibition of steroid hormone action. As such, they find

their most extensive use in palliative treatment of hormone-related malignancies and in control of reproductive processes. In addition to their role in the clinic, steroid receptor antagonists are important tools to decipher molecular and cellular mechanisms of steroid hormone receptor function (Agarwal, 1994).

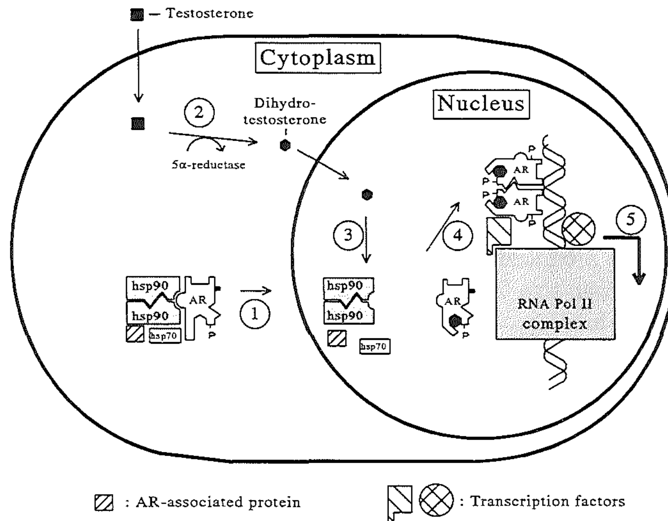



*Figure 1.1. Model of androgen receptor function. The key protein in androgen action is the androgen receptor (AR), which becomes phosphorylated and binds to heat shock proteins (hsp) after translation (1). The ligand testosterone (T) can be converted into dihydrotestosterone (DHT) by the enzyme 5α-reductase (2). Upon binding T or DHT, which may occur in the cytoplasm or the nucleus (3), the macromolecular complex dissociates and the AR is able to bind to DNA as a dimer (4). This triggers the regulation of transcription of specific target genes (5).*

## 1.3 Aim and scope of this thesis

The aim of the present investigations was to address the question of what makes the difference in the mechanisms of action between an agonist and an antagonist of the AR. The antihormones that will be considered are those which display competitive binding to the AR. The questions one may want to ask concern the type of competitive binding of the antagonist (at the same site or a different site); the existence of a 'hard shell' binding site for the antagonist (*"lock and key"* model) versus adaptable binding (*"induced-fit"* model); and the effect of the antagonist on the molecular dynamics or the conformation of the hormone-receptor complex. Fundamental to elucidation of the mechanisms of hormone antagonism is, of course, an understanding of agonist action itself. In order to define the frame of thought which underlie the present work it will be helpfull to summarize the present understanding

of the different steps in steroid hormone action. Therefore, in Chapter 2, the structure of the AR and its mechanism of action will be discussed in more detail. Subsequently, the effects of steroid hormone antagonists on the many molecular aspects of steroid hormone action will be considered.

The interactions between the AR and its ligand, either hormone or antihormone, would be best studied by comparing the dynamics of the three-dimensional structures of these complexes. However, at the beginning of the studies presented in this thesis neither the methods nor the data were available to perform analysis of three-dimensional structures. An indirect manner to obtain data on interactions between the AR and its ligands, is a limited proteolytic digestion of the receptor protein. Due to ligand binding, proteolytic sites can be more or less accessible for the enzyme, indicative for conformational changes within the receptor protein. In Chapters 3 and 4, results are presented of these studies on the conformational changes of ligand-receptor complexes. Differences in proteolysis resisting fragments of the AR, as induced by ligand binding, were evaluated with antibodies and receptor mutants. From these studies, performed with *in vitro* produced androgen receptors in a cell-free system, it was hypothesized that there are two mechanisms to accomplish antiandrogenic activity *in vivo*: I) interference of the antagonist with binding of the receptor to DNA, and II) reduction and/or blockade of the interaction of the DNA-bound receptor with the transcription initiation complex.

To address this hypothesis, the DNA-binding ability of the receptor was now studied in intact cells with a promoter interference assay, as described in Chapter 5. The promoter interference assay makes use of the principle that proteins, when bound to the DNA near a transcription initiation site of a gene, strongly repress the activity of that gene. After validation of the assay for the androgen receptor, various agonist/antagonist-receptor complexes were studied. The data were correlated with the ability of these complexes to activate transcription from an androgen-response reporter construct. The results obtained from these studies in whole cells (Chapter 5) support the hypothesis from Chapters 3 and 4 on the molecular mechanisms of antiandrogen action.

Most steroid hormone receptors have transcription activation regions in both the N-terminal domain and the ligand binding domain. In contrast, for the AR transcription activation regions can only be subscribed to the N-terminal domain. In analogy to other steroid hormone receptors, this activation function in the N-terminal domain of the AR was considered to be ligand-independent. However, in the studies described in Chapters 3-5 it was found that some androgen receptor antagonists induce DNA-binding of the receptor, but still do not fully activate the AR. We therefore hypothesized that androgens, but not antiandrogens, function by mediating an interaction of the N-terminal transcription activation domain with other transcription factors. As a consequence, the ligand binding domain has to transmit a signal to the N-terminal domain, or even directly interacts with the N-terminal domain. The interaction between the N-terminal domain and ligand binding domain of the AR was investigated as descibed in Chapter 6. This interaction was studied in cells expressing both the ligand binding domain and the N-terminal transactivating domain, not linked together by peptide bonds. In accordance with our hypothesis, an interaction was found between the separate N-terminal domain and the ligand binding domain in the presence of androgens, but not in the presence of antiandrogens.

Finally, Chapter 7 forms the general discussion of the results described in the preceeding chapters.

# INTRODUCTION

## Chapter 2

## 2.1 Structural and functional domains of steroid hormone receptors

### 2.1.1 Evolution of the gene family

Cloning of nuclear receptor proteins and determination of their primary structures have demonstrated a structural similarity of receptors for steroid and thyroid hormones and for retinoic acid, which have comparable domains for ligand binding, DNA binding, and transactivation (Fig. 2.1). Based on this knowledge, other proteins with similar structures are found for which no physiological ligands have been identified. The role of these so-called orphan receptors (O'Malley, 1989; O'Malley and Coonely, 1992) in physiology is not clear yet.

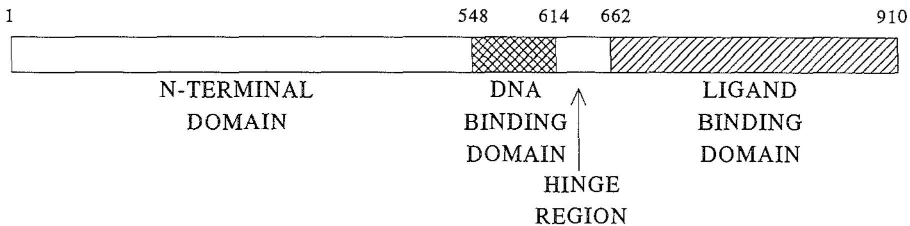



*Figure 2.1. Domain structure of the human AR. The human AR protein [910 amino acid residues (Faber et al., 1989)], as well as other members of the nuclear receptor family, consists of: an N-terminal transcription regulation domain (amino acids 1-548), a central DNA binding domain (amino acids 548-614) and a C-terminally located ligand binding domain (amino acids 662-910). In between the DNA binding domain and ligand binding domain, a hinge region is located (amino acids 614-662).*

It is believed that the different members of the nuclear receptor superfamily have evolved from an ancestral multi-domain gene (O'Malley, 1989; Dorit *et al.*, 1990; Amero *et al.*, 1992; Laudet *et al.*, 1992). Duplication and mutations of this precursor gene have resulted in the variety of receptors that is known to date. The family can be divided into groups on basis of homology in their DNA binding domain or ligand binding domain. The phylogenetic tree on the basis of similarity in the DNA binding domain can be divided into three subfamilies: (I) the thyroid hormone/retinoic acid receptor subfamily; (II) the orphan receptor subfamily; and (III) the steroid hormone receptor subfamily. The last subfamily can be divided into the GR group and an ER group, including the estrogen-related receptor 1 and 2 (Forman and Samuels, 1990; Laudet *et al.*, 1992; Amero *et al.*, 1992). The tree constructed on basis of conservation of the central part of the ligand binding domain shows a similar distribution of the different receptors in three subfamilies as found on basis of the DNA binding region. For a few receptors, a different position is found, suggesting that in some cases the DNA binding domain and ligand binding domain have evolved independently, most likely due to domain swapping between various receptors (Laudet *et al.*, 1992). The N-terminal domain of the receptors is hypervariable, and it has not been possible to group the receptors on basis of homology in this region.

### 2.1.2 DNA binding domain

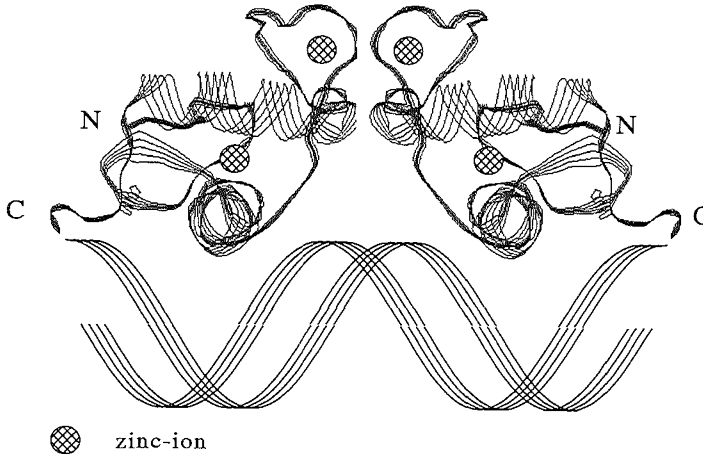
The specific functions of the members of the nuclear receptor superfamily depend upon the genes that they regulate. Part of the specificity is due to recognition of DNA sequences, called response elements, with which a receptor interacts. Responsible for this recognition is the best conserved domain of the nuclear receptor family, the DNA binding domain (Fig. 2.1). This domain is rich in cysteine, arginine and lysine residues, and contains two zinc ions which are tetrahedrally coordinated by cysteine residues (Freedman *et al.*, 1988). Zinc coordination is important for the structural integrity and the DNA binding function of this region (Freedman *et al.*, 1988; Pan *et al.*, 1990; Zilliacus *et al.*, 1992; Predki and Sarkar, 1992).

The three-dimensional structures of the DNA binding domains of the GR, ER, and retinoic acid receptors RAR and RXR have been determined by nuclear magnetic resonance (NMR), and the structures of the DNA bound dimeric complexes of GR and ER DNA binding domains have been studied by X-ray crystallography (Hard *et al.*, 1990; Schwabe *et al.*, 1990; Luisi *et al.*, 1991; Schwabe *et al.*, 1993a,b; Knegtel *et al.*, 1993; Baumann *et al.*, 1993; Lee *et al.*, 1993). The overall structure of the different DNA binding domains is similar, which made it possible to model the AR DNA binding domain (Fig. 2.2A). The DNA binding domain has two subdomains, each composed of the motif (zinc domain-helix-extended region). The zinc domain contains the zinc ion, which is coordinated by four cysteines. The α-helices start between the third and fourth cysteines in the zinc clusters and are followed by extended regions. The two subdomains are folded into one compact domain. The two α-helices that follow the zinc domain are packed perpendicularly to each other, and hydrophobic residues in the α-helices form the hydrophobic core of the DNA binding domain.

The steroid hormone receptors can be divided into two groups depending upon the recognition of their response element, which are identified as DNA sequences that are bound by the receptors. All steroid hormone receptors bind to (imperfect) palindromic response elements with a 3-base pair spacer between the half-sites. The GR group of receptors, also including AR, PR and MR, recognizes the GRE consensus half-site TGTTCT (Roche *et al.*, 1992; Truss and Beato, 1993). The ER, as well as most non-steroid receptor members of the nuclear receptor superfamily, bind to a consensus half-site TGACCT (Fig. 2.3), called an ERE. Conservation among the response elements suggests that the receptor amino acid residues responsible for DNA binding must be conserved, so that only minor change(s) in the amino acid sequence of the DNA binding domain might change the specificity. Indeed, the three amino acid residues glycine, serine and valine in the so-called P-box (GSckV; one letter symbol for amino acids; capitals indicate consensus amino acid residues) of the GR group of receptors are essential for GRE recognition (Fig. 2.2B), whereas the essential amino acid residues in the P-box of the ER are EGckA (Mader *et al.*, 1989). A GR can be generated that bind functionally to an ERE by changing the three P-box amino acid residues (GSV) towards the ER amino acid residues (EGA) (Danielsen *et al.*, 1989; Umesono and Evans, 1989).

Steroid hormone receptors, in contrast to several other nuclear receptors (Rastinejed *et al.*, 1995), bind their response elements only as homodimers. Contacts of the two receptor DNA binding domains span the minor groove of the 3-base pair spacer between the half-sites (Truss and Beato, 1993; Zilliacus *et al.*, 1995). The symmetric arrangement brings identical regions of each protein to the dimerization interface. The amino acid residues mediating the dimerization contacts are clustered in the D-box, located between the first and second cysteine residue of the second subdomain (Fig. 2.2B).

A. Model of the AR DNA binding domain bound, as a dimer, to DNA




B. Sequence and functional motifs of the AR DNA binding domain

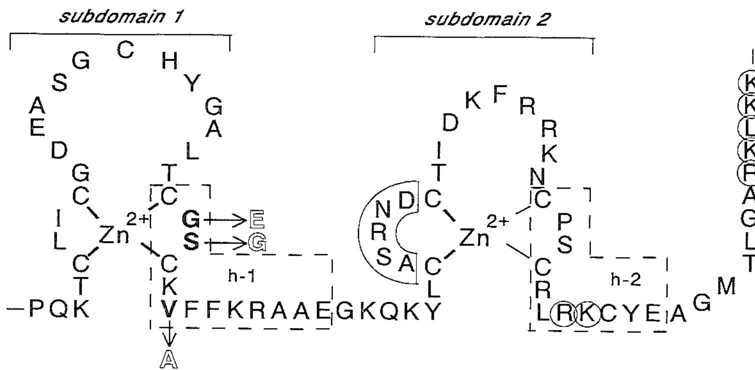



***Figure 2.2.*** *A AR DNA binding domain bound to DNA. A schematic view of an AR DNA binding domain dimer bound to its response element is shown. The recognition helices are positioned in the major groove of the DNA binding domain, and the C-terminal zinc domains form a dimer interface.* ***B*** *AR DNA binding domain showing the Cys-Zn coordination and α-helices. The putative α-helices are boxed in with dashed lines (h-1 and h-2). The DNA binding domain is composed of two subdomains, which are indicated by brackets. The amino acid residues in the P-box (responsible for GRE recognition) are indicated in bold, whereas the open letters are the comparable amino acid residues in the ER DNA binding domain. The D-box of amino acid residues, involved in dimerization, is shown by a solid box. Finally, amino acid residues involved in nuclear localisation are encircled.*

No major differences in the recognition of its response element has been detected for the members of the GR group of receptors, GR, PR, AR and MR, although minor differences cannot be excluded. Thus, specificity of gene regulation within this group of receptors is obtained at another level than DNA binding, and several mechanisms can be postulated including differential expression of the receptors, different capacities of the receptors to modulate chromatin structure (Archer *et al.*, 1994; Mymryk and Archer, 1995; Truss *et al.*, 1995), receptor-specific interactions with other transcription factors and auxiliary DNA elements (Adler *et al.*, 1992; de Vos *et al.*, 1994; Pearce and Yamamoto, 1993).

```
                   -6 -5 -4 -3 -2 -1           1  2  3  4  5  6
Consensus GRE       G  G  T  A  C  A  N  N  N  T  G  T  T  C  T
                    c  t  c  t     c                    c
                    t     g

Consensus ERE       A  G  G  T  C  A  N  N  N  T  G  A  C  C  T
                       a                                      c
```

***Figure 2.3.*** *Consensus GRE and ERE. The consensus sequences are based on base pair conservation in functional response elements. The base at each position that is present in the majority of the response elements is shown by a large letter, and bases that are less common are shown by small letters (Zilliacus et al., 1995). The half-sites are separated by three random base-pairs, indicated with NNN.*

### 2.1.3 Ligand binding domain

The ligand binding domain of the steroid hormone receptors consists of approximately 250 amino acid residues and is functionally complex. In general, it contains regions important for heat-shock protein association (Dalman *et al.*, 1989; Cadepond *et al.*, 1991), dimerization (Guiochon-Mantel *et al.*, 1989; Fawell *et al.*, 1990), transactivation (Hollenberg *et al.*, 1988; Lees *et al.*, 1989; Danielian *et al.*, 1992; Sartorius *et al.*, 1994) and, most importantly, ligand binding (Giguere *et al.*, 1986; Rusconi and Yamamoto, 1987; Dobson *et al.*, 1989; Jenster *et al.*, 1991). Although most of these functions require only small stretches of the amino acid sequence, ligand binding appears to involve a majority of this domain, since most of the mutations identified in the ligand binding domain compromise the ability of the altered receptor to bind hormones. Single point mutations could decrease ligand affinity (McPhaul *et al.*, 1992; Quigley *et al.*, 1995), change hormone- or antihormone specificity (Veldscholte *et al.*, 1990; Culig *et al.*, 1993), or transactivation capacity (Bevan *et al.*, 1996) of the AR. The ligand binding domain is linked to the DNA binding domain by a flexible hinge region. This hinge region contains the nuclear localisation signal, which is recognised by the nuclear pore complex and is involved in nuclear import of the GR (Picard and Yamamoto, 1987), PR (Guiochon-Mantel *et al.*, 1989), AR (Jenster *et al.*, 1993; Zhou *et al.*, 1994) and ER (Ylikomi *et al.*, 1992). This bipartite signal consists of two basic amino acid residues (Arg, Lys) and an arginine-lysine stretch, spaced by 10 amino acid residues (for the AR: RKcyeagmtlgaRKLKK; Fig. 2.2B), and shows homology with the nucleoplasmin nuclear localisation signal (Robbins *et al.*, 1991).

Several attempts were made to model the three-dimensional structure of the ligand binding domain of steroid hormone receptors, based on sequence homology with known protein structures (Lemesle-Varloot *et al.*, 1992; Goldstein *et al.*, 1993). Recently, the crystal structure of the ligand binding domain of the retinoid-X receptor (RXRα; Bourguet *et al.*, 1995), the retinoic acid receptor (RARγ; Renaud et al, 1995), and the thyroid hormone receptor (TRα; Wagner *et al.*, 1995) were elucidated. The ligand binding domains form antiparallel α-helical "sandwiches", composed of 11 (RARγ) or 12 (RARα, TRα) topologically conserved α-helices (H1-H12) and two (RXRα, RARγ) or four (TRα) short ß-strands (schematically shown in Fig. 2.4). The crystal structures of the ligand binding domains bound with agonist reveals that the ligand is completely buried within the hydrophobic ligand binding pocket. As a result of a conformational change within the ligand binding domain after binding of the ligand, the entry site of this domain is closed by H12. Due to the altered conformation, the C-terminally located AF-2 (activation function 2) is activated. Although the RXRα, RARγ and TRα belong to another subfamily of the nuclear receptors (Laudet *et al.*, 1992), the homology in the ligand binding domain with the steroid hormone receptors is high. Therefore, it can be speculated that the three-dimensional structures of the ligand binding domains of steroid hormone receptors show homology with that of the retinoic acid/thyroid hormone subfamily. However, as will be discussed below, a direct activation of transcription by an AF-2 region has not been observed for the AR (Jenster *et al.*, 1995)

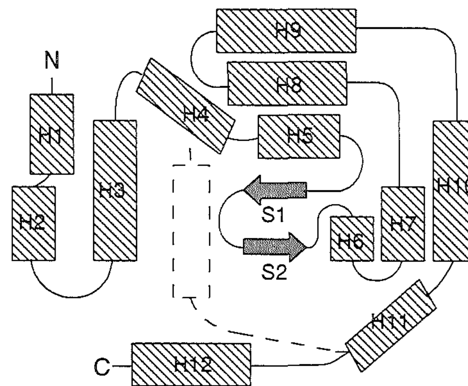



***Figure 2.4.*** *Generalised model of the ligand binding domain, with the secondary structural elements α-helices (H1-H12) and ß-strands (s1-s2) (adapted from Renaud et al., 1995). The α-helices H1-H11 are organised in a three layer structure with H4, H5, H6, H8 and H9 sandwiched between H1-H3 on one side, and H7, H10 and H11 on the other. H12 protrudes from the unliganded ligand binding domain, whereas it folds back towards the ligand binding domain in its liganded state (shown by a dashed box).*

### 2.1.4 Amino-terminal domain

The amino-terminal region of the steroid hormone receptors is the least conserved domain, both in size and sequence. This domain is capable to modulate transcriptional activation of

a target gene by making protein-protein contacts with both basal (McEwan *et al.*, 1993 and 1994) and specific transcription factors (Kupfer *et al.*, 1993, Adler *et al.*, 1992; Pearce and Yamamoto, 1993). Deletion mapping of this domain in the different steroid hormone receptors delineates several amino acid sequences essential for the N-terminal activation function AF-1 (Imakado *et al.*, 1991; Meyer *et al.*, 1992; Dahlman-Wright *et al.*, 1994; Jenster *et al.*, 1995). Deletion mapping in the wild type AR showed that almost the entire N-terminal domain (amino acid residues 1-485) was necessary for full transcriptional activity (Jenster *et al.*, 1995). In contrast, with a constitutively active AR, i.e. a receptor protein only containing the N-terminal and DNA binding domains, amino acid residues 360-528 were sufficient to obtain full transcriptional activity within the same cell-type as used for the ligand-dependent wild type receptor. This demonstrates the capacity of the AR to use different regions in the N-terminal domain for transcription activation. Furthermore, the involvement of different amino acid residues within the N-terminal domain of the wild type AR and the constitutively active AR suggest that the ligand binding domain influences interaction between the N-terminally located activation domain and other transcription factors.

The function of transcription factors is often regulated by reversible phosphorylation (Hunter and Karin, 1992). All steroid hormone receptors that have been examined are phosphoproteins, and show an increase in overall phosphorylation in response to binding hormone in intact cells (reviewed by Kuiper and Brinkmann, 1994). Most of the phosphorylation sites of AR (Kuiper *et al.*, 1993a; Zhou *et al.*, 1995), GR (Hoeck and Groner, 1990; Bodwell *et al.*, 1991), PR (Denner *et al.*, 1990; Poletti and Weigel, 1993), and ER (Ali *et al.*, 1993; LeGoff *et al.*, 1994) are located in the amino-terminal domain. In addition, phosphorylation sites were found in the ligand binding domain of the ER (Auricchio, 1989; Castoria *et al.*, 1993), and in the hinge region of the PR (Denner *et al.*, 1990). The fact that most sites are located in the amino-terminal domain suggests a role of phophorylation in regulating transcriptional activity. In support of a role in modulating transcriptional activity, mutation of single phosphorylation sites of the human ER reduced, although to a limited extent, the ER mediated gene transcription in transfection studies (Ali *et al.*, 1993; Le Goff *et al.*, 1994; Kato *et al.*, 1995). In addition, some reduction in transcriptional activity of mouse GRs mutated at multiple phosphorylation sites was found (Mason and Housley, 1993). In general, phosphorylation does not appear to be an absolute requirement for the ability of several other steroid hormone receptors to induce gene activation from viral or natural promoters in transient co-transfection assays. However, phosphorylation could be involved in other receptor functions, such as DNA and steroid binding (Arnold *et al.*, 1995), intracellular trafficking (Orti *et al.*, 1993), modulating activity with other cellular proteins, and recycling of receptors (Bai *et al.*, 1994; Hu *et al.*, 1994; Kuiper and Brinkmann, 1994).

The amino-terminal domain of the human AR is very hydrophobic and contains glutamine, proline, and glycine stretches (Lubahn *et al.*, 1988; Chang *et al.* 1988; Faber *et al.*, 1989). The glutamine stretch is polymorphic in length and encompasses 11 to 31 amino acid residues (Sleddens *et al.*, 1992 and 1993). The function, if any, of the polyglutamine region has yet to be clearly established. A glutamine-rich region of the zinc-finger transcription factor Sp1 is necessary for its trans-activation function (Courey *et al.*, 1989). However, deletion of the glutamine-stretch in the AR did not modify trans-activating properties on a MMTV promoter (Simental *et al.*, 1991; Jenster *et al.*, 1994), and resulted in an increase in activity on the promoter region of the prostate specific antigen (PSA) gene. Expansion of the trinucleotide repeat CAG resulted in a decrease of the transactivation capacity of the receptor (Jenster *et*

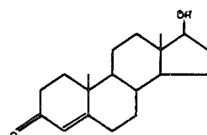
*al.*, 1994), and is associated with X-linked spinal and bulbar muscular atrophy, a rare motor neuron disorder [(Kennedy's disease), LaSpada *et al.*, 1991].

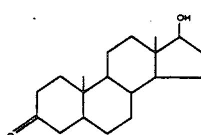
## 2.2 Molecular mechanisms of antiandrogen action

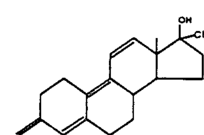
### 2.2.1 Introduction

Androgen receptor antagonists inhibit the biological effects of androgens, and are frequently used in the treatment of hormone-based dysfunctions such as prostate abnormalities, acne, female hirsutism, and hypersexuality. Furthermore, the synthetic antagonists are important tools to decipher the molecular mechanism of transactivation by androgens. In the next paragraphs, various aspects of antiandrogen action will be discussed at the molecular and cellular levels.

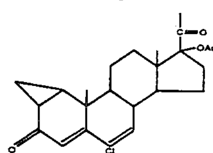


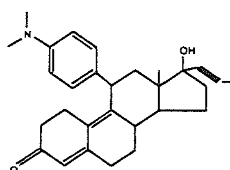


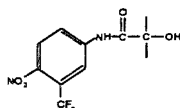



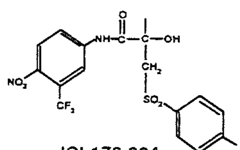




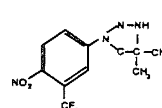




*Figure 2.5. Structures of androgen receptor agonists and antagonists.*

On a structural basis, androgen receptor antagonists can be divided into two groups: the steroidal antiandrogens and the nonsteroidal antiandrogens. The structures of the antagonists used in the various studies in this thesis (Chapters 3-6) are shown in Figure 2.5. The steroidal antiandrogens [e.g. cyproterone acetate and RU 38486 (mifepristone; although developed as an antiprogestagen, this compound also has antiandrogenic activity)] block androgen action, but in addition have progestational and glucocorticoidal activities and are therefore called non-

pure antiandrogens. Due to their progestational activity, GnRH and consequently serum levels of LH, testosterone (T) and dihydrotestosterone (DHT) are down-regulated *in vivo*. In contrast, the nonsteroidal antiandrogens [e.g. hydroxyflutamide, RU 23908 (nilutamide) and ICI 176.334 (bicalutamide)] show a variable degree of activity on the hypothalamus-pituitary-gonadal axis, which results in an increased LH level and a subsequent rise in serum testosterone and dihydrotestosterone (Neumann and Töpert, 1986; Raynaud and Ojasoo, 1986; Furr *et al.*, 1987). The nonsteroidal antagonists are also referred to as pure antiandrogens.

### 2.2.2 Antagonist-receptor interactions

Development of antiandrogens and other steroid receptor antagonists in general has largely depended on the study of chemical structure-activity correlations of agonists and antagonists. In so-called topographically mapping attempts of the ligand binding site of the receptors, many different ligands were superimposed in order to explore the space available for binding. A fundamental premise to generate a model of the ligand binding pocket of one of the steroid hormone receptors in this way is that all molecules interact with that receptor at a fixed binding pocket in the same (or similar) manner. Initially, it appeared that a hydrophobic subsite was present in the ligand binding pocket of the PR and GR, able to accommodate the large 11ß-substituents of antiprogestagens and antiglucocorticoids (e.g. RU 38486). This subsite seemed to be a common feature of all steroid hormone receptors, and in addition, the concept of this subsite had the potential to rationalize agonistic and antagonistic activity (Teutsch *et al.*, 1988; Gronemeyer *et al.*, 1992). However, the lack of a structure-affinity relationship of different ligands undermines the *"lock and key"* model, even if some conformational flexibility and adaptability of the ligand is taken into account (Teutsch *et al.*, 1995). Also, a number of experimental results on structure-activity of various compounds (Cook *et al.*, 1992; Benhamou *et al.*, 1992; Garcia *et al.*, 1992) cannot be explained any longer by this simple *"lock and key"* model of ligand docking to the steroid hormone receptors. This has led to adapt the more versatile, but also more elusive, *"induced fit"* model in which the receptor protein adapts its conformation, by induced fit, to the ligand, which results in a reduction of the conformational mobility and a tighter packing of the ligand binding domain (Jorgensen, 1991). This phenomenon could be compared, in some way, to a crystallization process in which the ligand plays the role of a nucleating agent, as demonstrated for the recently resolved three-dimensional structures of the ligand binding domains of the thyroid hormone/retinoic acid receptor subfamily (see section 2.1.3). In agreement with this *"induced fit"* model, several studies show that agonists and antagonists of steroid hormone receptors induce distinct conformations of the ligand binding domain (Moudgil *et al.*, 1989; Weigel *et al.*, 1992; Allan *et al.*, 1992a, b; Beekman *et al.*, 1993).

### 2.2.3 Role of dissociation rate in antagonist action

Until recently, most antiandrogens were characterized by a rather low relative binding affinity for the AR, ranging from less than 1% to 10% of that of the synthetic androgen R1881 (Kemppainen *et al.*, 1992; Veldscholte *et al.*, 1992a; Culig *et al.*, 1993). It has been suggested that a fast dissociation of the steroid receptor ligand would explain the antagonistic properties of this compound (Raynaud *et al.*, 1980). However, several antagonists (i.e. cyproterone acetate, hydroxyflutamide, and RU 23908) show agonistic properties for the mutant receptor that is present in LNCaP cells, a cell line derived from a Lymph Node Carcinoma of the Prostate. This mutant receptor ARL contains an amino acid substitution in the ligand binding

domain [amino acid residue 868: Thr to Ala (Veldscholte *et al.*, 1990)], which is responsible for the shift towards agonistic activities of the forementioned antihormones. As the relative binding affinities of cyproterone acetate, hydroxyflutamide, and RU 23908 for the ARL increase only marginally (Veldscholte *et al.*, 1992a), it seems unlikely that the dissociation rates were sufficiently changed to play a major role in the switch from antagonistic to agonistic properties.
Also on a theoretical basis one can predict that fast ligand dissociation per se does not lead to an antagonistic action. Transformation of the receptor to an activated form might proceed during a reversible dissociation process. If antagonist action indeed is elicited by the fast dissociation of the compound from the receptor, then at least one irreversible step is required. Otherwise, a high concentration of the compound would drive the receptor towards transcription activation, in which case this compound would be an agonist (Veldscholte *et al.*, 1994).
Finally, fast dissociation of the ligand is not a general characteristic of antagonists of steroid hormone action, and also not longer true for antiandrogens. Some recently developed nonsteroidal antiandrogens show increased binding affinities, even exceeding the affinity of testosterone (Battmann *et al.*, 1994; Teutsch *et al.*, 1994). So, although the dissociation rate of antagonists may play a role in the antagonistic action of some compounds, it is certainly not the only existing mechanism.

### 2.2.4 Subcellular localisation of antagonist-bound receptors

The subcellular localisation of steroid hormone receptors has been the subject of many studies for over 20 years. According to the *"two-step"* models established independently by Jensen and Gorski (Gorski *et al.*, 1968; Jensen *et al.*, 1968) the unoccupied receptors were supposed to reside in the cytoplasm and to translocate to the nucleus after hormone binding and transformation. In general, two methods are available to study the subcellular localization of receptors. First, steroid hormone receptors can be located in the cell by immunocytochemical methods, whereas the second method employs cell fractionation and salt extraction to determine cytosolic or nuclear localisation of the receptor.
In agreement with the *"two-step"* model, cytoplasmic staining after hormone depletion and nuclear staining after hormone-induced nuclear translocation have been detected with immunocytochemistry for the GR (Fuxe *et al.*, 1985; Wikström *et al.*, 1987) and the MR (Alnemri *et al.*, 1991). However, the unoccupied PR and ER were located immunocytochemically in the nuclei of target cells (King and Greene, 1984; Perrot-Applanat *et al.*, 1985). The subcellular localisation of the unliganded AR is still controversial. Immunocytochemistry demonstrated a nuclear localisation of the AR in prostate tissue of man who underwent androgen ablation therapy (van der Kwast *et al.*, 1991), in castrated rats (Sar *et al.*, 1990), and in transfected HeLa cells (Jenster *et al.*, 1993). In contrast, expression of the receptor in COS, CV-1, and CHO cells clearly show cytoplasmic staining of the AR (Simental, 1991; Kemppainen et al, 1992; Jenster *et al.*, 1993). A high degree of overexpression in these transfected cells, as shown for the GR (Martins *et al.*, 1991), may be an explanation for the cytoplasmic label. Also cell type differences complicate the distribution pattern observed for the unliganded AR: cytoplasmic staining was found in the ventral prostate, seminal vesicle and coagulating gland, but not in the epididymis of adult castrated rats (Paris *et al.*, 1994). This implicates differences in subcellular localisation between target cells, although artificial redistribution due to the fixation and staining procedures cannot be

completely excluded. However, in all the immunocytochemical studies the ligand-bound receptor resides in the nucleus of the cells, irrespective of the nature of the ligand: agonist or antagonist.
Unoccupied steroid hormone receptors are known to be easily extractable. This is the reason for their presence in the cytosol after cell fractionation, even if they are nuclear proteins in intact cells, as demonstrated with immunocytochemistry for the PR and ER (King and Greene, 1984; Perrot-Applanat *et al.*, 1985). Upon agonist binding, the AR is transformed to a tight nuclear binding state, i.e. only extractable from the isolated nuclei with high salt buffers (Barrack and Coffey, 1987; Mowszowicz *et al.*, 1988; Veldscholte *et al.*, 1992a; Berrevoets *et al.*, 1993). The results obtained with immunocytochemistry and cell fractionation favour a model which involves qualitative differences in nuclear binding: unliganded receptors, loosely bound within the nucleus, are detected in the cytosol upon cell-fractionation, but can remain nuclear in the cell fixation procedures used in immunocytochemistry. These qualitative differences in nuclear binding can readily be shown upon binding of antagonists to the AR. The androgen receptor antagonists hydroxyflutamide and ICI 176.334 do not induce transformation of the wild type AR into the tightly bound nuclear form in transient transfected COS cells, as determined by cell fractionation. In the presence of cyproterone acetate, which shows partial agonistic activity in these cells, there is some nuclear retention of the AR (Berrevoets *et al.*, 1993), indicative for at least a partial transformation into the tight nuclear-bound form. As mentioned in the previous paragraph, the antagonist-bound receptors were all nuclear in immunocytochemical studies (Kemppainen *et al.*, 1992; Jenster *et al.*, 1993). The mutant receptor ARL can be transformed to a tight nuclear binding state by agonists and the antagonists cyproterone acetate and hydroxyflutamide. This effect is shown both in LNCaP cells (Veldscholte *et al.*, 1992a), and in transient transfected COS cells (Berrevoets *et al.*, 1993). However, due to a mutation in the ligand binding domain, these forementioned antagonists act as full agonists and activate the mutant receptor ARL, as determined in both growth studies (Wilding *et al.*, 1989; Schuurmans *et al.*, 1990) and transactivation studies with an androgen-responsive reporter construct (Veldscholte *et al.*, 1990). In contrast, ICI 176.334 still acts as an antagonist, and does not induce tight nuclear binding of the mutant receptor ARL. So, it is tempting to speculate that tight nuclear binding correlates with the DNA binding state of the receptor protein and, as such, with the ability to activate transcription.

### 2.2.5 Dissociation of the heat-shock protein-receptor complex

In the absence of ligand, all steroid hormone receptors are recovered from cytosol in transcriptionally inactive 8-10S receptor complexes. Although the exact protein composition of these complexes is unknown, they are at least comprised of a receptor monomer, a dimer of a 90 kDa heat-shock protein and the immunophilin p59 [also referred to as the HSP56 or FKBP52 (Tai *et al.*, 1986; Smith *et al.*, 1990; Smith, 1993; Pratt, 1994)]. Studies with mutant receptors have revealed that these proteins are predominantly associated with the ligand binding domain (Pratt *et al.*, 1988; Carson-Jurica *et al.*, 1989), although HSP90 can also interact with a positively charged region at the C-terminal part of the DNA binding domain of the ER (Chambraud et al,., 1990). Several functions have been proposed for the heat-shock proteins, which are also reffered to as molecular chaperones (Bohen *et al.*, 1995); these include proper folding of the ligand binding domain to acquire high-affinity ligand binding, transport of the steroid hormone receptor through the cytosol, and the maintenance of the unliganded receptors in a transcriptionally inactive state (reviewed in Pratt, 1993;

Georgopoulos and Welch, 1993; Bohen *et al.*, 1995b). From these functions, a role of heat-shock proteins in proper protein folding and, as a result, acquirement of high ligand binding affinity is the most favoured one (Bresnick *et al.*, 1989; Bagchi *et al.*, 1990; Picard *et al.*, 1990; Bohen *et al.*, 1995).
For the AR, HSP90 and the immunophilin p59 have unambiguously been shown to be part of an 8S receptor complex (Sanchez, 1990; Marivoet *et al.*, 1992; Nemoto *et al.*, 1992; Veldscholte *et al.*, 1992b). A mutant AR lacking the ligand binding domain is constitutively active (Simental et al, 1991; Jenster *et al.*, 1991), and is not associated with HSP90. This supports the hypothesis that the ligand binding domain is the major domain for HSP90 association. These results also suggest that both the ligand binding domain and its associated proteins act as repressors of the activation function of the AR.

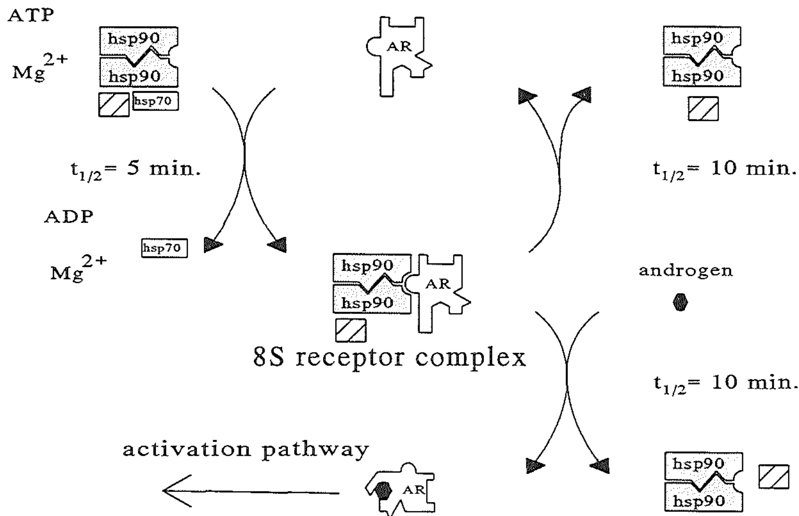



***Figure 2.6.*** *Model for the HSP90-AR assembly and hormone dependent dissociation of HSP90 [adapted from Smith (1993)]. The AR associates with a complex of proteins, including HSP90 and HSP70, in an ATP and $Mg^{2+}$ dependent manner (8S receptor complex). In a temperature-dependent manner, HSP90 dissociates from the AR (half-life of approximately 10 min at 25 °C). As re-association with HSP90 has a smaller half-life, predominantly AR-HSP90 complexes exist. If HSP90 dissociates from an androgen-bound AR, the AR establishes a new conformation that prevents its re-association with HSP90. The liganded AR enters the activation pathway, leading to interaction with the transcription machinery.*

In its unliganded state, the receptor complex is thought to be in dynamic equilibrium with a non-HSP90-bound receptor form (exemplified for the AR in Fig. 2.6), and this equilibrium is influenced greatly by receptor occupancy with ligand. HSP70 and other chaperones appear to reassemble HSP90 onto the unliganded receptors in an ATP- and $Mg^{2+}$-dependent

mechanism (Smith, 1993; Johnson and Toft, 1994; Bohen *et al.*, 1995b). As reassemblance of the unliganded receptor with heat-shock proteins is faster than disassemblance, most of the receptor is found associated with heat-shock proteins. Upon hormone binding *in vivo* and *in vitro*, the nonreceptor proteins dissociate from the complex in a temperature dependent way. Ligand binding per se may not increase the rate of dissociation of HSP90 but may simply preclude the reverse reaction, i.e. reassembly of heat-shock proteins to the receptor, by ligand induced conformational changes (Smith, 1993). The dissociation of HSP90 upon ligand binding is thought to allow receptors to dimerize, to bind to DNA, and to activate transcription, although recently some results indicate that a steroid hormone receptor can bind to DNA even in its associated state (Srinivasan *et al.*, 1994; Metzger *et al.*, 1995).
One of the suggested molecular mechanisms of antihormone action is the failure of antagonists to promote dissociation of associated proteins from the receptor complex and, as a consequence, the complex with bound antagonist remains in a latent state (Moudgil and Hurd, 1987; Baulieu, 1989; Segnitz and Gehring, 1991; Beck *et al.*, 1993). Recent studies with the LNCaP cell-line have shown that binding of agonists, including cyproterone acetate and hydroxyflutamide, results in dissociation of HSP90 and p59 from the receptor complex. As a result, transcriptionally competent complexes are formed (Veldscholte *et al.*, 1992b). Upon binding of the antagonist ICI 176.334 to the mutant receptor ARL, present in the LNCaP cells, HSP90 and p59 do not dissociate from the receptor complex, as both proteins could still be co-immunoprecipitated with an antibody recognizing the AR. This indicates that for the mutant ARL there is at least one antagonist which exert its action through inhibition of dissociation of the existing receptor complex.

### 2.2.6 Receptor dimerization and DNA binding

Important steps, essential for steroid hormone receptors to activate gene expression, are dimerization and specific DNA binding of the receptor. Many transcription factors, including the nuclear receptor superfamily, undergo dimer formation in acquiring high affinity DNA binding (Jones, 1990). Also the AR binds to its response element only as a dimer (Wong *et al.*, 1993a; Kallio *et al.*, 1994a), whereas changes in spacing between the two ARE half-sites, normally three base-pairs, abolishes the ability of the AR to form stable complexes with DNA (Kallio *et al.*, 1994a). It is not clear whether dimerization of the AR takes place before binding to the DNA, as suggested for the GR, PR, and ER (Fawell et ., 1990; Cairns *et al.*, 1991; De Marzo *et al.*, 1992), or is a consequence of binding of inactive receptor monomers to DNA. In the ER, the major dimerization interface is localized in the hormone binding domain (Fawell *et al.*, 1990), but also the second zinc finger in the DNA binding domain forms protein-protein contacts when the receptor is bound to DNA (Kumar and Chambon, 1988). As amino acid residues essential for interactions in the ligand binding domain have been shown to overlap with residues essential for hormone binding (Fawell *et al.*, 1990), it is conceivable that hormone binding may result in stabilized dimerization. As a consequence, binding of antihormones to the receptor could inhibit receptor activation either by destabilization of dimerization or by direct inhibition of DNA binding.
Depending on their proposed mechanisms of action in relation to DNA binding, progesterone and estrogen receptor antagonists have been tentatively divided into two classes (Klein-Hitpass *et al.*, 1993; Green, 1990). In this classification, the so-called "type I antagonists" interfere with binding of the receptor to DNA. The other class of antagonists [type II (Klein-Hitpass *et al.*, 1993)] induce stable, high affinity DNA binding of the receptor but block the

interaction of the receptor with the transcription initiation complex. Partial agonistic activity can be observed for the type II antagonists, and this activity is proposed to originate from the ligand-independent activation function in the N-terminal domain (Berry *et al.*, 1990). This partial agonistic activity is a cell and promoter context specific effect (Berry *et al.*, 1990; Meyer *et al.*, 1990), and can be influenced by protein kinase activators and inhibitors (Beck *et al.*, 1992; Moyer *et al.*, 1993; Nordeen *et al.*, 1993; Sartorius *et al.*, 1993; Fujimoto and Katzenellenbogen, 1994). In contrast, the type I antagonists are full antagonists, most likely due to their inability to induce DNA binding of the steroid hormone receptors.
The role of antagonists in DNA binding of steroid hormone receptors is probably the most investigated and discussed mechanism of antihormone action. For example, RU 38486 was found to induce both dimerization and DNA-binding of the PR as determined in gel shift assays, which are developed to study protein-DNA interaction *in vitro* (Klein-Hitpass *et al.*, 1991; De Marzo *et al.*, 1992). Evidence for DNA binding of the RU 38486-bound receptor was also obtained from three types of transfection assays. First, RU 38486 act as a partial agonist and is able to stimulate transcription from a reporter plasmid (Meyer *et al.*, 1990). Second, competition assays have demonstrated that the receptor is able to compete for binding to target DNA even when it is complexed with the antagonist (Delabre *et al.*, 1993). Third, genomic footprinting on the MMTV promoter in stably transfected cells indicated that RU 38486 maintained the hypersensitive chromatin state, as found in the presence of agonists (Mymryck and Archer, 1995). In contrast, another study with genomic footprinting stated that RU 38486 did not promote binding of the PR to a chromosomally integrated MMTV-promoter. This effect was also found at concentrations of RU 38486 that completely inhibited the agonistic activity of progestagens, indicative that the effect was not due to a difference in ligand binding affinity (Truss *et al.*, 1994). Furthermore, RU 38486-bound PR was able to activate transcription without binding to a progesterone response element, indicative that DNA binding is not a prerequisite of the antagonist-bound receptor to activate transcription (Tung *et al.*, 1993). So, although most studies provide evidence for induction of DNA binding of PR by RU 38486, inhibition of DNA binding by RU 38486 cannot be completely excluded, especially not when the antagonist-receptor complex interacts with intact chromatin instead of naked DNA.
The ability of the AR to bind to its response element in the promoter region of the regulated gene is believed to be hormone dependent *in vivo*. However, the results obtained with gel shift assays by various investigators show a controversy in hormone dependency of AR binding to DNA. Several studies reveal hormone independent binding of the AR to its response element (Kallio *et al.*, 1994a; Xie *et al.*, 1992; Kuiper *et al.*, 1993), whereas the receptor required intracellular hormone exposure to obtain DNA binding in other studies (Wong *et al.*, 1993a; Kaspar *et al.*, 1993). Although the hormone dependency of DNA binding was absent in several studies, in these studies androgen binding to the AR-DNA complexes still had an effect on the electrophoretic mobility of this complexes. The mobility of the agonist-bound AR-DNA complexes in the gel shift assays increased (Kaspar *et al.*, 1993; Kallio *et al.*, 1994b), most likely due to androgen-induced conformational changes within the receptor protein. In cases where binding of the receptor to DNA was hormone independent, neither cyproterone acetate nor ICI 176.334 could dissociate the DNA-receptor complex (Kallio *et al.*, 1994b). Furthermore, these antagonists did not increase the mobility of the DNA-receptor complex as was observed for the agonist-bound receptor, indicative for conformational differences between agonist- and antagonist-bound receptor-DNA complexes (Kallio *et al.*,

1994b). In a study, where binding of the AR to DNA was found to be hormone-dependent (Wong *et al.*, 1993a), the ability of a variety of ligands to induce DNA binding paralleled their ability to induce transcription activation. The antihormones cyproterone acetate and RU 38486, which show partial agonistic activity in transactivation assays (Kemppainen *et al.*, 1992; Culig *et al.*, 1993), promote DNA binding of the AR, whereas the full antagonist hydroxyflutamide inhibit AR DNA binding *in vitro* (Wong *et al.*, 1993a). Based on these last results, both cyproterone acetate and RU 38486 may be tentatively indicated as type II antagonists which induce DNA binding of the receptor, whereas hydroxyflutamide inhibits DNA binding and, as such, represents a type I antagonist. Whether DNA binding of an antagonist-bound AR is also inhibited in the intact cell is the subject of our studies in Chapter 5.

The amino acid residues involved in dimerization of the AR have not yet been identified. In contrast to other steroid hormone receptors, results of some studies suggest a role for the amino-terminal domain in dimerization and DNA binding of the AR (Wong *et al.*, 1993a; Kallio *et al.*, 1994a). Dimerization and DNA binding of AR, produced in recombinant baculovirus-infected insect cells, required incubation in the presence of androgens, but only when one or both components of the proposed dimer contain the amino-terminal domain (Wong *et al.*, 1993a). Otherwise, DNA binding of the mutant receptors without N-terminal domain was hormone independent. Furthermore, analysis of DNA binding capacity of amino-terminal deletion mutants, in combination with the wild type AR, revealed that hetero-dimerization between the mutant and wild type receptor was favored over homo-dimerization (Kallio *et al.*, 1994a). This phenomenon of favoured hetero-dimerization may also explain the dominant negative effect of N-terminally truncated receptors on trans-activation of a reporter construct by the full-length AR (Palvimo *et al.*, 1993). So, the amino-terminal domain of the AR may modulate receptor dimerization and DNA binding.

### 2.2.7 Transcription regulation

#### 2.2.7.1 Transactivation

Effects of steroid hormones are mediated, via their respective receptor, by either positive or negative modifications in the transcription rate of responsive genes. Transcription initiation by RNA polymerase II involves a complex hierarchy of both protein-protein and protein-DNA interactions. The process of transcription activation starts with the regulated and ordered assembly of basal transcription factors [e.g. TFIIA, TFIIB, TATA-binding protein (TBP), TFIIE, TFIIF] into a pre-initiation complex on the promoter region of genes (Zawel and Reinberg, 1993; Buratowski, 1994; Struhl, 1996). The first step is binding of the TFIID complex to the TATA-box; the TFIID complex is composed of the DNA-binding component TBP and a variety of other subunits called TBP-associated factors (TAFs). The basal TFIID complex seems to be the central part in communication between the RNA polymerase II and other activators, such as the steroid hormone receptors. These last transcriptional regulators bind to their DNA consensus elements, located in the promoter region of the regulated gene, and communicate with the TFIID complex, either directly or via so-called co-activators (Tsai and O'Malley, 1994). Recently, one TFIID subunit has been demonstrated to be a target for steroid hormone receptors; $TAF_{II}30$ was shown to interact specifically to the AF-2 region in the ER (Jacq *et al.*, 1994).

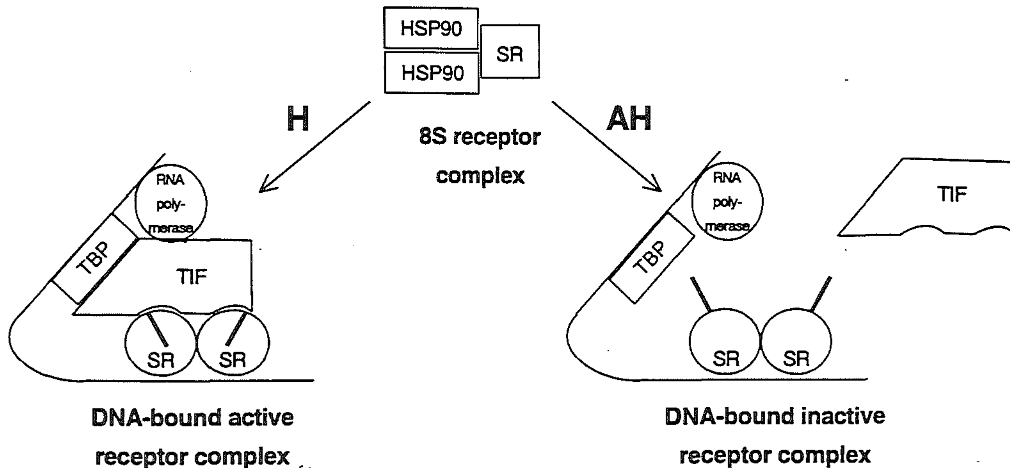



***Figure 2.7.*** *Model of the role of hormone and antihormone in action of steroid hormone receptors (adapted from Allan et al., 1992a). Binding of ligand to the 8S receptor complex induces a conformational change which leads to complex dissociation, receptor dimerization, and DNA binding. Both agonist (H) and antagonist (AH) stimulate DNA binding of the receptor (SR) via a conformational change within the ligand binding domain. However, the agonist renders the ligand binding domain in a compact structure, whereas the antagonist leaves the C-terminus exposed to the solution. This structural distinction may underlie the inability of the antihormone-receptor complex to interact with transcription intermediary factors (TIF), and to stimulate transcription. TBP: TATA binding protein.*

Several antihormone-receptor complexes are proposed to bind *in vivo* to the responsive enhancer elements of genes to be regulated (Reese and Katzenellenboogen, 1992; Metzger *et al.*, 1995), but these complexes elicit only a low level of transcription activation. In addition, both agonist- and several antagonist-bound receptors can maintain an open chromatin structure of a responsive promoter, a prerequisite for transcription activation (Pham *et al.*, 1991; Mymryk and Archer, 1995). Taken together, these results indicate that steroid hormone receptor binding to DNA and the resulting chromatin hypersensitivity is functionally separate from transcriptional activation *in vivo*. Furthermore, they suggest that binding of an agonist to a steroid hormone receptor is necessary to regulate transcription activation subsequent to the DNA binding step, presumably by regulating interaction of the receptor with the basal transcription machinery (Allan *et al.*, 1992b). In this regard, antihormones can function by establishing receptor-DNA complexes that are transcriptionally non-productive.
In a model explaining the mechanism of antagonist action, described recently by O'Malley and collaborators (Fig. 2.7), in the process towards a transcriptionally active receptor protein the importance of a conformational change within the ligand binding domain is emphasized (Allan *et al.*, 1992a; Beekman *et al.*, 1993; Baniahmed and Tsai, 1993; Tsai and O'Malley, 1994). The model argues that antagonists bind to regions of the ligand binding domain, which results in dimerization and DNA binding, but leaves the C-terminus of the ligand binding

domain in a form still available for protease (Allan *et al.*, 1992a) and antibody recognition (Vegeto *et al.*, 1992; Weigel *et al.*, 1992). As a result, a repressor function is not removed and the receptor is not able to induce transcription. In accordance with a repressor function of the C-terminus, a mutant PR deleted of the last 42 amino acid residues, acquired full agonistic activity upon binding of one of the 11ß-substituted antagonists RU 38486, ORG 31806, or ORG 31376 (Vegeto *et al.*, 1992). Therefore, it was hypothesized that the ligand-induced alterations in the conformation of the ligand binding domain might be sensed by cellular factors that mediate the activation function in the ligand binding domain.
Indeed, multiple factors have been demonstrated to associate *in vitro* with the ligand binding domain of steroid hormone receptors in a ligand-dependent manner. These include a mouse nuclear protein TIF1 (Le Douarin *et al.*, 1995), the human and mouse homolog of the yeast adaptor Sug1: TRIP1 (Lee *et al.*, 1995) and mSUG1 (Baur *et al.*, 1996), the yeast protein SPT6 (Baniahmed *et al.*, 1995), the ER-associated proteins ERAP160 (Halachmie *et al.*, 1994), RIP160, RIP140 and RIP80 (Cavailles *et al.*, 1994 and 1995), and the steroid receptor coactivator SRC-1 (Oñate *et al.*, 1995). Interaction of ERAP160, RIP140, TIF1, and SRC-1 with the receptor protein was inhibited by the addition of antihormones (Halachmi *et al.*, 1994; Le Douarin *et al.*, 1995; Oñate *et al.*, 1995; Baur *et al.*, 1996). However, with the exception of SRC-1, none of these receptor-associated proteins have been shown to enhance receptor-mediated transcriptional activity in transfection studies. In addition, none of these proteins have been shown to interact with the AR. So, it remains to be determined whether these and/or other proteins are involved in AR-mediated transcription regulation. As transcription activation regions of the AR can only be subscribed to the N-terminal domain of the AR and not to the ligand binding domain (Jenster *et al.*, 1995), it can even be doubted whether the ligand binding domain is directly involved in interaction with transcription intermediary factors.

#### 2.2.7.2 Transrepression

So far, only those effects of hormones and antihormones have been discussed which result in up-regulation of transcription activation, by binding of the liganded receptor to the promoter region of genes to be activated. However, important biological responses conveyed by steroid hormone receptors also include down-regulation of gene activity, called transrepression. The anti-inflammatory action of glucocorticoids, by repression of cytokine and cell-adhesion genes, is probably the best known example in which transrepression of gene-activity plays a major role (Barnes and Adcock, 1993; Bauerle and Henkel, 1994). Also several genes down-regulated by androgens have been identified, which include genes encoding the α- and ß-subunits of luteinizing hormone (LH) (Clay *et al.*, 1993), the low-affinity neurotrophin receptor (Persson *et al.*, 1990), syndecan (Leppa *et al.*, 1992), TRPM-2 or clusterin (Wong *et al.*, 1993b), and the AR itself (Lindzey *et al.*, 1993).
The proposed mechanisms behind transcriptional repression are diverse. In some cases, the presence of so-called negative GREs has been suggested. These nGREs show sequence homology to the regular GREs, but it was proposed that the GR binds as a trimer, instead of a dimer, to this response element (Drouin *et al.*, 1993). However, whether these elements are negative per se or simply overlap with DNA-binding sites of other proteins is still unclear (Drouin, 1993; Cairns *et al.*, 1993). The repressive effect of androgens on the expression of genes encoding α- and ß-subunits of LH seems to involve direct interaction of the receptor with DNA (Clay *et al.*, 1993), thereby inhibiting binding of a stimulating regulatory protein

[JRE transcription factor (Andersen *et al.*, 1990)]. Recently, it has become evident that additional regulatory mechanisms for transcriptional inhibition by nuclear receptors exist, which do not involve DNA binding of the receptor (Pfahl *et al.*, 1994; Ray *et al.*, 1994; Scheinmann *et al.*, 1995a; Caldenhoven *et al.*, 1995). As a consequence of the different mechanisms in transrepression and transactivation, antihormones which are unable to activate transcription by their responsive receptor, may still have repressive activity.

One important class of target genes that are potentially repressed by steroid hormone receptors are those under the positive control by members of the AP-1 transcription factor superfamily. Several groups have reported mutual antagonism between steroid hormone receptors and the components of AP-1, the Fos and Jun proteins (reviewed in Pfahl *et al.*, 1994; Herrlich and Ponta, 1994). Depending on the promoter, the cell line, and the receptor type, both positive and negative regulatory interactions have been described between the AP-1 complex and steroid hormone receptors; and this is likely to involve direct protein-protein interactions between AP-1 and steroid hormone receptor. Deletion mutagenesis and domain-swapping experiments of the GR indicated that different domains of the receptor are involved in transactivation and transrepression, and these entities can be fully separated (Heck *et al.*, 1994; Helmberg *et al.*, 1995). In addition, specific ligands can be designed which can dissociate the transactivation and transrepression abilities of nuclear receptors (Fanjul *et al.*, 1994; Chen *et al.*, 1995). These results open possibilities for differential regulation of gene activity *in vivo*. Moreover, it is known that the nuclear factor κB (NF-κB) can be antagonized by the ER (Ray *et al.*, 1994), GR (Ray and Prefontaine, 1994; Scheinmann *et al.*, 1995a; Caldenhoven *et al.*, 1995) and PR (Kalkhoven *et al.*, 1996). Most likely, inhibition of NF-κB-regulated genes involves direct protein-protein interactions between the steroid hormone receptor and NF-κB (Ray *et al.*, 1994; Ray and Prefontaine, 1994; Scheinmann *et al.*, 1995a), although an indirect inhibition of the transactivation capacity of NF-κB is not excluded (Scheinmann *et al.*, 1995b; Auphan *et al.*, 1995).

As mentioned above, several studies report transrepression activity of the AR, either by direct binding to DNA (Clay *et al.*, 1993) or by interaction with other transcription factors, especially AP-1 (Kallio *et al.*, 1995). However, the physiological relevance and importance of transrepression *in vivo* mediated by the AR remain to be clearly established. In addition, activity of several other transacting factors, known [e.g. cAMP-response element binding protein CREB (Lindzey *et al.*, 1993) and octamer transcription factor-1 (Brüggemeier *et al.*, 1991; Kutoh *et al.*, 1992)] or (still) unknown, may be influenced by steroid hormone receptors. Furthermore, the influence of androgen receptor antagonists on transrepression activity of the AR remains to be determined.

# MECHANISM OF ANTIANDROGEN ACTION: CONFORMATIONAL CHANGES OF THE RECEPTOR

C.W. Kuil and E. Mulder

Published in
Molecular and Cellular Endocrinology
102 (1994), R1-R5

## Chapter 3

**Summary**
Androgen receptor mRNA was translated *in vitro*, and androgen- and antiandrogen-bound receptor complexes were studied using limited proteolytic digestion by trypsin. Partial proteolysis of androgen-bound receptor protein resulted in a 29 kDa proteolysis resisting fragment, whereas antiandrogen binding stabilised a 35 kDa fragment. Both fragments contain the entire ligand binding domain, and the 35 kDa fragment extended into the hinge region of the receptor. Several antiandrogens show agonistic properties for a mutated androgen receptor (LNCaP cell variant); trypsin digestion of antiandrogen-bound mutated receptor also resulted in a 29 kDa fragment. Our results point to an important difference between antiandrogens and antagonists of other steroid hormone receptors. Antiandrogens result in protection of both the hinge region and C-terminus of the androgen receptor against proteolytic attack, whereas other studies showed that antiestrogens and antiprogestagens expose the C-terminal end of the ligand binding domain of their respective receptors to protease. Differences in conformation of the hinge region distinguish androgen-bound from antiandrogen-bound receptor complexes, which represents an important feature of antiandrogen action.

### Introduction
The study of the mechanism of antiandrogen action is of great interest, not only because of the therapeutic potential of antiandrogens, but also because these compounds are important tools to elucidate the molecular mechanism of action of androgens. Androgens initiate effects in target cells through a ligand-activated transcription factor, the androgen receptor (AR). Like all members of the steroid hormone receptor family, the AR binds to its response element after a ligand-dependent activation process and interacts with the transcription complex to regulate gene transcription (Carson-Jurica *et al.*, 1990; Smith and Toft, 1993).
Antiandrogens compete with androgens for binding to the AR, but binding of antagonists does not result in full transformation of the receptor to a transcriptionally active form. Several mechanisms for the resulting inhibitory effect have been postulated, and recently a subdivision of antagonists in two distinct classes has been made (Reese and Katzenellenbogen, 1991; Klein-Hitpass *et al.*, 1991; Gronemeyer *et al.*, 1992). One class of antagonists does not, or with decreased efficiency, promote DNA binding of the receptor, whereas the other class of antagonists promotes DNA binding but induces an abnormal conformation of the ligand-binding domain. The latter class of antagonists may give rise to a partial agonistic effect, through a transcription activation function in the N-terminal domain of the receptor.
For the progesterone receptor, it was recently shown that binding of progestagens and antiprogestagens results in different susceptibility of the receptor to proteolytic digestion. Antagonists induced protection of a smaller progesterone receptor fragment than agonists did (Allan *et al.*, 1992a, b), and studies with antibodies indicated that a short region at the C-terminal end of the progesterone receptor is involved in this difference (Allan *et al.*, 1992a; Weigel *et al.*, 1992).
The presented data concern differences in susceptibility of androgen- and antiandrogen-bound receptor complexes to proteolytic digestion.

### Materials and methods
*Materials*: RNA transcription kit and pBluescript II KS- were obtained from Stratagene (La Jolla, USA). Nuclease-treated rabbit reticulocyte lysate was purchased from Promega

(Madison, USA). L-[$^{35}$S]methionine (s.a.>1000 mCi/mM) was obtained from Amersham (Buckinghamshire, UK). Trypsin (type III), soybean trypsin inhibitor (type I-S), goat-anti-mouse agarose and goat-anti-rabbit agarose were obtained from Sigma (St. Louis, USA). R1881 (methyltrienolone) was purchased from NEN (Boston, USA). Cyproterone acetate was a gift from Schering AG (Berlin, FRG), hydroxyflutamide from Schering USA (Bloomfield, USA) and ICI 176.334 ("Casodex") from ICI Pharmaceuticals (Macclesfield, UK). Dihydrotestosterone and testosterone were obtained from Steraloids (Wilton, USA).

*Plasmid construction*: The coding sequence for the wild type human AR (AR0) was excised from the expression vector pSVAR0 (Brinkmann *et al.*, 1989) and subcloned in the *Sal*I site of pBluescript to obtain pBSAR0. The recombinant pBSAR0 615-910 was obtained from pBSAR0 after digestion with *Sac*I and religation. The recombinants were linearized with *Xho*I for transcription. The coding sequence for the mutant LNCaP AR (ARL) was subcloned between the *Sal*I and *Bam*HI sites of pBluescript (pBSARL). For linearization, the recombinant was digested with *Xba*I.

*In vitro transcription and translation*: Both *in vitro* transcription and *in vitro* translation in the presence of L-[$^{35}$S]methionine were performed according to manufacturer's instruction. For *in vitro* transcription of pBSAR0 and pBSAR0 615-910, $T_7$ RNA polymerase was used to produce sense mRNA, whereas pBSARL was transcribed with $T_3$ RNA polymerase.

*Limited proteolytic digestion of in vitro produced receptors*: Two µl of labeled translation mix was pre-incubated for 1 h at room temperature with 3 µl of ligand solution diluted in water. For limited proteolytic digestion, 5 µl trypsin (40 µg/ml, dissolved in water) was added to the pre-incubation mix, followed by an incubation for 15 min at room temperature. After incubation, 20 µl SDS sample buffer was added. Samples were boiled for 3 min, and 15 µl was loaded on 0.1% (w/v) sodium dodecyl sulfate-12.5% (w/v) polyacrylamide gels. After electrophoresis (Laemmli, 1970), the gels were vacuum-dried at 80 °C for 45 min and autoradiography was performed overnight.

*Immunoprecipitation*: Labeled translation mix (20 µl) was hormone-treated and digested with trypsin as indicated above. After digestion, soybean trypsin inhibitor was supplemented to a final concentration of 200 µg/ml. Goat-anti-rabbit or goat-anti-mouse agarose (100 µl, diluted 1:4 in PBS (phosphate-buffered saline)) was incubated for 2 h at 4 °C with 1 µl of the indicated polyclonal rabbit or monoclonal mouse antiserum. The polyclonal antiserum SP066 (epitope amino acids 892-910) and monoclonal antibody F52 (epitope amino acids 593-612) were described previously (Zegers *et al.*, 1991; Veldscholte *et al.*, 1992). Following this incubation, the resin was washed three times with 1 ml PBS and added to the limited proteolytic digest of the receptor. After incubation for 2 h at 4 °C, the resin was washed three times with 1 ml PBS, and 25 µl of sample buffer was added. Electrophoresis was performed as described above.

## Results and Discussion

Limited proteolytic digestion of [$^{35}$S]methionine labeled, *in vitro* produced AR was used to detect conformational changes upon androgen or antiandrogen binding to the receptor. The *in vitro* produced AR showed steroid binding properties similar to those observed for AR isolated from mammalian cells (Kuiper *et al.*, 1993). After incubation of the AR with androgen or antiandrogen, a limited amount of trypsin was added and the digestion products were analyzed by denaturing gel electrophoresis. Either in the absence of ligand (Fig. 3.1A)

or in the presence of a steroid with no affinity for the AR (dexamethasone; result not shown), the AR was completely digested to fragments that were undetectable with electrophoresis. Proteolytic digestion of AR incubated either with the synthetic androgen R1881 or the natural ligands dihydrotestosterone and testosterone, resulted in a 29 kDa proteolysis resisting fragment (Fig. 3.1A; result for testosterone not shown). Incubation of AR with the antiandrogens cyproterone acetate, hydroxyflutamide, or ICI 176.334 before tryptic digestion resulted in stabilisation of a 35 kDa fragment (Fig. 3.1A). The concentrations of the different ligands used varied according to their differences in relative binding affinities of the ligands for the AR (Veldscholte *et al.*, 1992).

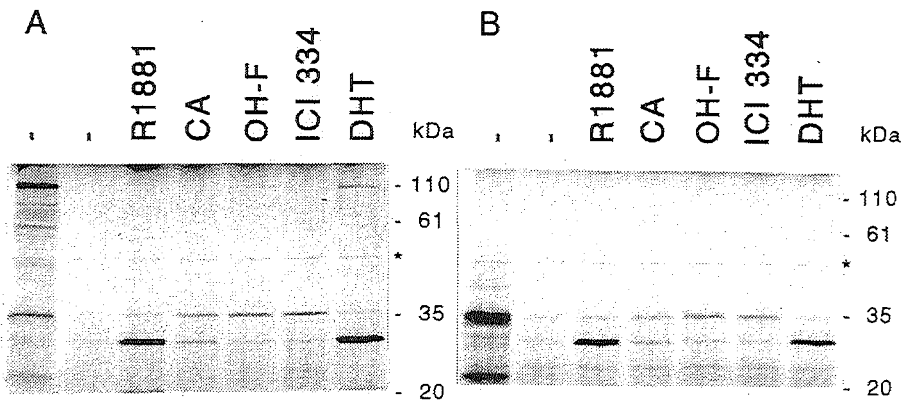



*Figure 3.1. Limited proteolytic digestion of in vitro produced AR (A) and AR0 615-910 (B) bound with different androgens and antiandrogens. Lane 1: no trypsin added. Lane 2: control digestion without steroid (-). Lanes 3-7: 10 nM R1881, 1 μM cyproterone acetate (CA), 10 μM hydroxyflutamide (OH-F), 10 μM ICI 176.334 (ICI 334), and 10 nM dihydrotestosterone (DHT), respectively. Molecular mass markers are indicated at the right. * indicates a non-specific band.*

Formation of a 29 kDa fragment was the result of binding of an agonist, whereas stabilisation of a 35 kDa fragment indicated binding of an antagonist. When increasing concentrations of the antiandrogen ICI 176.334 were added together with a constant level of R1881, digestion with trypsin resulted in less an amount of the 29 kDa fragment and an increased amount of the 35 kDa fragment (Fig. 3.2). As predicted from the relative binding affinities of the ligands, 50 % binding inhibition of 1 nM R1881 occurred at 1 μM ICI 176.334. When the concentration of R1881 was increased, the 29 kDa fragment reappeared (Fig. 3.2).
Several *in vitro* produced AR fragments, including a 35 kDa fragment, were already present before the start of the proteolytic digestion, probably due to alternative translation initiation. Therefore, the effect of trypsin on the 35 kDa fragment was studied in detail in separate experiments. A mutant AR cDNA, deleted of the N-terminal and DNA-binding domains (AR0 615-910), was translated *in vitro* into a predominant 35 kDa protein (Fig. 3.1B). Also for this truncated receptor protein, binding of either R1881 or dihydrotestosterone resulted in

formation of a 29 kDa fragment upon proteolytic digestion, whereas the antiandrogens predominantly stabilised the 35 kDa fragment. These results show that formation of the 29 kDa fragment is not dependent on interaction of the ligand binding domain with the N-terminal and DNA-binding domains.

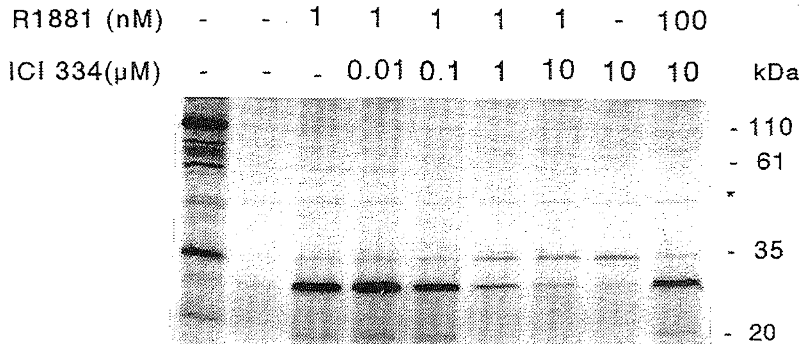



*Figure 3.2. Competition of 1 nM R1881 with increasing levels of ICI 176.334 (ICI 334). Ligands were bound to the in vitro produced AR before digestion with trypsin. Lane 1: no trypsin added. Lane 2: control digestion without steroid. Lanes 3-9: indicated levels of R1881 and/or ICI 176.334. Molecular mass markers are indicated at the right. * indicates a non-specific band.*

From these experiments it can be concluded that trypsin treatment of androgen- or antiandrogen-bound AR results in different proteolysis resisting fragments which suggest a different structural conformation. Comparable, but not similar, observations have been made for the progesterone and estrogen receptors (Vegeto *et al.*, 1992; Allan *et al.*, 1992a; Beekman *et al.*, 1993). In contrast with the results found for the AR, antagonist binding to these receptors resulted in smaller proteolysis resisting fragments than obtained after agonist binding.

Immunoprecipitation was performed to determine which part of the AR was removed upon conversion of the 35 kDa fragment into the 29 kDa fragment. After incubation of the full length AR with ligand, followed by limited proteolytic digestion with trypsin, the proteolysis resisting fragments were immunoprecipitated with different antisera (Fig. 3.3). Neither the 35 kDa fragment nor the 29 kDa fragment could be immunoprecipitated with the antiserum F52 which recognizes an epitope in the DNA-binding domain (Zegers *et al.*, 1991). Therefore, this epitope appears not to be present in both fragments. The antiserum SP066, raised against a peptide at the C-terminus of the AR, recognizes both fragments, which indicates that the difference in size of the 29 kDa and 35 kDa fragments is not located at the C-terminus of the AR.

These results indicate that, in the AR, a part of the hinge region is protected against degradation by trypsin in the presence of antiandrogens. This is in contrast with the results obtained for the progesterone receptor, where the C-terminus was only retained during proteolytic digestion in the presence of an agonist (Allan *et al.*, 1992a; Weigel *et al.*, 1992).

Another study (Vegeto *et al.*, 1992) showed that a truncated progesterone receptor, with a C-terminal deletion of 42 amino acid residues, could not bind a progestagen but still bound the antiprogestagen RU486. In addition, RU486 was able to act as an agonist in a transcription activation assay.

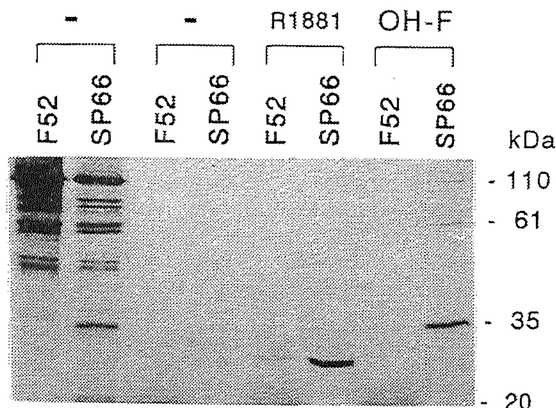



***Figure 3.3.*** *Immunoprecipitation of AR fragments with different antisera. After treatment with vehicle (-; lanes 3-4), 100 nM R1881 (lanes 5-6) or 100 nM hydroxyflutamide (OH-F; lanes 7-8), the AR was digested with trypsin. Lanes 1 and 2 were controls without trypsin. After digestion, immunoprecipitation was performed with the monoclonal antiserum F52 (lanes 1, 3, 5 and 7) or the polyclonal antiserum SP066 (lanes 2, 4, 6 and 8). Molecular mass markers are indicated at the right.*

LNCaP prostate tumor cells contain an AR (ARL) with a single amino acid change in the steroid binding domain (codon 868; Thr to Ala (Veldscholte *et al.*, 1990)). Both cyproterone acetate and hydroxyflutamide act as agonists for the ARL but ICI 176.334 still behaves as an antiandrogen with the ARL (Veldscholte *et al.*, 1992). ARL is therefore a useful tool to study the mechanism of action of antagonists. The *in vitro* produced ARL was treated with the same androgens and antiandrogens as described above for the wild type AR (Fig. 3.4). For R1881, dihydrotestosterone and ICI 176.334 the results for ARL were comparable with those obtained with the wild type AR. However, the antiandrogens cyproterone acetate and hydroxyflutamide, which gave rise to a 35 kDa fragment with the wild type AR, both induced formation of a 29 kDa fragment with the ARL. As these antiandrogens act as agonists for the ARL, it can be assumed that the single amino acid change in the ARL made it possible for both antiandrogens to induce a comparable conformation as formed after the interaction of an androgen with the wild type AR.

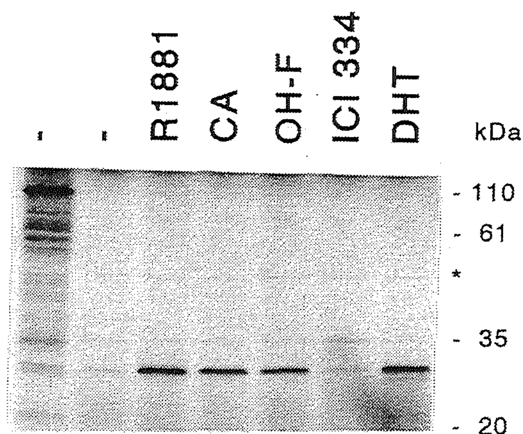



*Figure 3.4. Limited proteolytic digestion of in vitro produced mutant ARL bound with different androgens and antiandrogens. Lane 1: no trypsin added. Lane 2: control digestion without steroid (-). Lanes 3-7: 10 nM R1881, 1 μM cyproterone acetate (CA), 10 μM hydroxyflutamide (OH-F), 10 μM ICI 176.334 (ICI 334), and 10 nM dihydrotestosterone (DHT), respectively. Molecular mass markers are indicated at the right. * indicates a non-specific band.*

In conclusion: androgens and antiandrogens induce a different change in the conformation of the AR as detected by proteolytic digestion. This appears to involve the hinge region of the receptor, which is in contrast with studies on other steroid hormone receptors. Allan *et al.* (1992a)
proposed a general action of steroid receptor antagonists in preventing the formation of a transcriptionally competent conformation of the C-terminal end of the ligand binding domain. Conformational changes in the C-terminal end of the AR were not detected by trypsin treatment although several consensus sites for trypsin digestion are present. The results therefore suggest a difference in mechanism of antiandrogen action compared to other steroid hormone receptor antagonists.

**Acknowledgements**
We thank Dr. J.A. Grootegoed and Dr. J. Veldscholte for helpful discussions and critical reading of the manuscript.

# LIGAND-INDUCED CONFORMATIONAL ALTERATIONS OF THE ANDROGEN RECEPTOR ANALYZED BY LIMITED TRYPSINIZATION

Studies on the mechanism of antiandrogen action

C.W. Kuil, C.A. Berrevoets and E. Mulder

Published in
Journal of Biological Chemistry
270 (1995), 27569-27576

## Chapter 4


**Summary**

Limited proteolysis of *in vitro* produced human androgen receptor was used to probe the different conformations of the receptor after binding of androgens and several antiandrogens. The results provide evidence for 5 different conformations of the receptor, as detected by the formation of proteolysis resisting fragments: 1) an initial conformation of the unoccupied receptor not resisting proteolytic attack; and receptor conformations characterized by 2) a 35 kD proteolysis resisting fragment spanning the ligand binding domain and part of the hinge region, obtained with most antagonists, and in an initial step after agonist binding; 3) a 29 kD proteolysis resisting fragment spanning the ligand binding domain, obtained in the presence of agonists after an activation process; 4 and 5) 30 and 25 kD fragments, derived from 2 and 3, but missing part of the C-terminus, obtained with RU 486 (RU 486 has antiandrogenic properties, besides its effects as an antiprogestagen/antiglucocorticoid). Concomitantly with the change from 2 to 3 (and of 4 to 5 for RU486) dissociation of the 8S-complex of receptor with associated proteins occurred. With a mutant receptor (LNCaP cell mutation in C-terminal region), some antagonists activated transcription analogous to agonists, and induced the activated receptor conformation 3. A mutant lacking the C-terminal 12 amino acids bound RU486 but not androgens, and formed with RU486 also conformation 5. These data imply that, after the initial rapid binding of ligand, androgens induce a conformational change of the receptor, a process that also involves release of associated proteins. RU486 induces an inappropriate conformation of the C-terminal end, similar as found for its effect on the progesterone receptor. In contrast, the other antiandrogens act at a different step in the mechanism of action: they do not induce an abnormal conformation, but act earlier and prevent a conformation change by stabilizing a complex with associated proteins.


**Introduction**

The biological effects of androgens and other steroid hormones are mediated through intracellular receptors, belonging to the steroid and thyroid hormone receptor superfamily (Evans, 1988). Upon activation by the hormone, steroid receptors interact with specific DNA sequences, located in the flanking regions of target genes, resulting in modulation of the expression of these genes (Beato, 1989; Gronemeyer, 1991; Tsai and O'Malley, 1994). Steroid hormone receptor antagonists inhibit the biological effects of agonists, and are frequently used in the treatment of hormone-based dysfunctions in human. Furthermore, the synthetic antagonists are important tools to define the molecular mechanism of transactivation by steroid hormones (Baniahmed and Tsai, 1993; Agarwal, 1994).

Agonists and antagonists may change the spatial structure of the receptor in distinct ways, as was first indicated by gel retardation experiments: antagonist- and agonist-receptor-DNA complexes showed slightly different mobilities (El-Ashry *et al.*, 1989; Lees *et al.*, 1990; Sabbah *et al.*, 1991; Kallio *et al.*, 1994). Recently, limited proteolysis of progesterone, estrogen, and glucocorticoid receptors pinpointed the distinction in conformation between hormone- and antihormone-bound receptor at the very C-terminal end of the receptor. Hormone treatment induced a pronounced conformational change in receptor structure, resulting in a prominent proteolysis resisting fragment. Several antagonists, including those which inhibit binding of the receptor to a hormone response element *in vitro*, induced an equally dramatic but distinct change in the receptor conformation; the proteolysis resisting fragments induced by binding of the different antagonists to their receptors were smaller than

that induced by hormone binding (Allan *et al.*, 1992a; Allan *et al.*, 1992b; Beekman *et al.*, 1993). In addition, the monoclonal antibody C-262, raised against the last 14 amino acids of the progesterone receptor, could discriminate agonist- and antagonist-bound progesterone receptors. The antibody bound to the full-length receptor only in the presence of antagonist, whereas progesterone prevented the recognition of the receptor by C-262 (Vegeto *et al.*, 1992; Weigel *et al.*, 1992). Deletion of 42 amino acids abolished the binding of progesterone, but had no effect on binding of the antagonist RU486. Functional characterization showed that the mutant receptor induced transcription upon addition of RU486 (Vegeto *et al.*, 1992). These results led to the hypothesis that the activity of antagonists is based on the induction of a non-functional conformation at the C-terminus of steroid hormone receptors (Tsai and O'Malley, 1994; Baniahmed and Tsai, 1993; Agarwal, 1994; Allan *et al.*, 1992a).

Androgen binding to the androgen receptor (AR) changed the receptor structure in such a way that the entire ligand binding domain resisted proteolytic degradation (Kallio *et al.*, 1994; Kuil and Mulder, 1994; Zeng *et al.*, 1994). However, for the antiandrogen-bound receptor the outcome of preliminary studies on resistance against proteolytic degradation varied in different investigations. An unaltered conformation of the ligand binding domain, similar as seen in the absence of ligand, was reported by Kallio *et al.* (1994). A structural difference between agonist- and antagonist-receptor complexes in the area around the hinge region of the receptor was deduced from studies in our laboratory. In the presence of an antiandrogen, a part of the hinge region in addition to the ligand binding domain resisted proteolysis (Kuil and Mulder, 1994). Finally, Zeng *et al.* (1994) observed no difference in the size of the proteolysis resisting fragments in the presence of either the agonist dihydrotestosterone or the antagonists cyproterone acetate and hydroxyflutamide. Despite these differences in results, none of these three studies provided evidence for the involvement of the C-terminus of the androgen receptor in the mechanism of antiandrogen action. Therefore, the differences in structural alteration of the homologous ligand binding domains of steroid hormone receptors by antiandrogens and by other steroid receptor antagonists may reflect a difference in the mechanisms of antagonist action.

In this paper we extend the studies on androgen receptor conformation by inclusion of conditions that affect the transformation process of receptors. In addition, RU486 was included. Due to its broad steroid specificity, this compound also binds to the androgen receptor, although with a much lower affinity than for the progesterone and glucocorticoid receptor (Baulieu, 1989; Philibert *et al.*, 1991). Furthermore, we used the mutant receptor ARL (LNCaP-mutant) (Veldscholte *et al.*, 1990) to assess a correlation between the ligand-induced conformational alteration of the receptor and the ability to activate transcription in transfection studies. The results indicate a similarity in conformational alterations for the different steroid hormone receptors, but a distinction in mechanism of action between most antiandrogens and antagonists for other steroid hormone receptors.

## Experimental procedures

*Materials.* [$^3$H]R1881 (87 Ci/mmol) and unlabeled R1881 (methyltrienolone) were purchased from NEN (Boston, USA). [$^3$H]RU486 (38.4 Ci/mmol), unlabeled RU486 (RU 38486, mifepristone) and nilutamide (RU 23908) were gifts from Roussel Uclaf (Romainville, France), cyproterone acetate from Schering AG (Berlin, Germany), hydroxyflutamide from Schering (Bloomfield, USA), and ICI 176.334 ("Casodex") from ICI Pharmaceuticals

(Macclesfield, UK). L-[$^{35}$S]methionine (> 1000 mCi/mM) was obtained from Amersham (Buckinghamshire, UK), RNA transcription kit and pBluescript KS- from Stratagene (La Jolla, USA), rabbit reticulocyte lysate and recombinant RNasin from Promega (Madison, USA), trypsin (type III), goat-anti-mouse agarose and goat-anti-rabbit agarose from Sigma (St. Louis, USA), and BM chemiluminescence western blotting kit from Boehringer Mannheim (Mannheim, Germany). The mouse monoclonal antibody AC88 (recognizing HSP90) was generously provided by Dr. D.O. Toft (Mayo Clinic, Rochester, USA).

*Plasmid construction.* The expression vectors pSVAR (encoding a human AR of 910 amino acids) (Brinkmann *et al.*, 1989) and pSVARL (LNCaP mutation; codon 868, Thr to Ala of an AR of 910 amino acids) (Veldscholte *et al.*, 1990) were used for subcloning in pBluescript to obtain pBSAR and pBSARL respectively (Kuil and Mulder, 1994). The plasmid pBSAR 1-898, encoding a receptor with a C-terminal deletion of 12 amino acids, was constructed from pSVAR 1-898 (Jenster *et al.*, 1991). The mouse mammary tumor virus-luciferase (MMTV-LUC) reporter plasmid was kindly provided by Organon (Oss, The Netherlands).

*Cell culture and transfection.* HeLa cells were cultured and transfected, with some modifications, as described before (Velscholte *et al.*, 1992a). For transcription regulation studies, 1.5 x $10^5$ HeLa cells/well (10 $cm^2$) were transfected with either 0.5 μg of pSVAR or 0.5 μg pSVARL, and 0.5 μg MMTV-LUC, per well. Carrier DNA (pTZ) was added to a total of 5 μg per well. After 1 day, a glycerol shock [15% (w/v) glycerol in serum-free MEM, 1.5 min] was performed, whereafter the cells were washed and experimental media were added. Two days after transfection, cells were harvested for the luciferase assay.

*Luciferase assay.* For the luciferase (LUC) assay, 200 μl lysis-buffer [25 mM Tris/phosphate (pH 7.8), 15% glycerol, 1% Triton X-100, 8 mM $MgCl_2$, 1 mM dithiothreitol] was added to the phosphate-buffered saline (PBS)-washed cells. After an incubation for 10 min, supernatants were collected, 100 μl luciferine solution (1 mM luciferine, 1 mM ATP in lysis buffer) added, and luciferase activity was measured with a LUMAC Biocounter M2500. In the absence of hormone, LUC activity was less then 10% of the highest levels of LUC activity (at $10^{-9}$-$10^{-8}$ nM R1881). Experiments were performed in triplicate.

*In vitro transcription and translation.* Both *in vitro* transcription and translation, in the presence of unlabeled or [$^{35}$S]methionine, were performed according to manufacturer's instruction. Sense mRNA produced from pBSAR, pBSARL and pBSAR 1-898 was used for translation.

*Limited trypsinization assay.* Two μl of labeled translation mix was incubated at 25 °C with 3 μl of ligand solution diluted in water for the indicated time. Limited trypsinization was performed by addition of 5 μl trypsin solution (dissolved in water) either at 25 °C (15 min; 40 μg/ml trypsin) or at 0 °C (1h; 100 μg/ml trypsin). After incubation, 20 μl 1.5x SDS sample buffer was added. Samples were electrophoresed (Laemmli, 1970) and autoradiography was performed overnight. In some experiments, immuno-adsorbed receptor protein was used. Of the resuspended resin with adsorbed receptor protein, 80 μl was incubated with 10 μl of ligand solution for 1h at 25 °C, whereafter 10 μl trypsin solution (50 μg/ml) was added to the incubation-mix. After this incubation 25 μl 5x SDS sample buffer was added, samples were boiled, the resin removed by centrifugation, and 25 μl was loaded on the gel.

*Immunoprecipitation of receptor fragments and intact receptor.* Soybean trypsin inhibitor was added to the digestion mixture to a final concentration of 200 μg/ml. Goat-anti-rabbit or goat-anti-mouse agarose (100 μl, diluted 1:4 in PBS) was incubated for 2h at 4 °C with 1 μl of polyclonal rabbit antiserum SP066 (epitope amino acids 892-910)(Zegers *et al.*, 1991) or

mouse monoclonal antibody F52 (epitope amino acids 593-612)(Kuiper *et al.*, 1993). Following this incubation, the resin was washed with PBS and added to the limited proteolytic digest of the receptor. After incubation for 2h at 4 °C, the resin was washed with PBS, and 25 µl of sample buffer was added. Electrophoresis was performed as described above (Laemmli, 1970).

Immuno-adsorbed intact receptor was prepared with polyclonal rabbit antiserum SP197 (epitope amino acids 1-20)(Kuiper *et al.*, 1993) from non-digested translation mixture. After an incubation for 2h at 4 °C, the resin was washed with 0.5 M NaCl in buffer A [40 mM Tris/HCl (pH 7.4), 10 mM EDTA, 10% (w/v) glycerol], and resuspended in 1 ml buffer A, containing 1 mg/ml BSA, for proteolytic digestion experiments.

*Western immunoblot analysis.* Immunoaffinity purification and Western immunoblot analysis of androgen receptor complexes from LNCaP cells was performed as described previously (Veldscholte *et al.*, 1992a). In short, the monoclonal antibody F39.4.1, specific for the androgen receptor (Zegers *et al.*, 1991), was chemically cross-linked to protein A-sepharose and incubated with cytosol for 2 h at 4°C under rotation. Subsequently, the resin was washed in buffer A with or without 0.5 M NaCl, whereafter electrophoresis and Western blotting was performed. The monoclonal antibodies AC88, specific for HSP90 (Riehl *et al.*, 1985), and F39.4.1 were used as primary antibodies in a chemiluminescense protein detection method, performed as described by the manucfacturer (Boehringer Mannheim).

*Sucrose density gradients.* Sucrose density gradients [5-20% (w/v) sucrose] were prepared in buffer B [40 mM Tris/HCl (pH 7.4), 10% (w/v) glycerol, 10 mM dithiothreitol, 10 mM $Na_2MoO_4$, 50 mM NaCl]. In experiments with labeled ligands, reticulocyte lysate containing unlabeled translation products (25 µl) was incubated with either 10 nM [$^3$H]R1881 or 100 nM [$^3$H]RU486 to label the receptor. For determination of non-specific binding, 100-fold unlabeled steroid was added. Labeling occurred either for 2h at 4 °C or 1h at 25 °C, before addition of 25 µl of buffer B. Thereafter, free steroid was removed by incubation with 25 µl of dextran-coated charcoal suspension [0.1% (w/v) dextran, 1% (w/v) charcoal in 40 mM Tris/HCl (pH 7.4)] and centrifugation. Samples of 65 µl were loaded on to the gradients and run for 2h at 60,000 rev./min in a VTi 65 rotor (Beckman) at 4 °C. Fractions were collected from the bottom and assayed for radioactivity. Hemoglobine (4.6S), alkaline phosphatase (6.2S) and $^{14}$C-labeled aldolase (7.9S) were used as markers.

When no labeled ligand (antiandrogens) was available, [$^{35}$S]methionine was used to label translation products, and incubation occurred in the presence of unlabeled ligands. After sucrose density gradient centrifugation, fractions were denatured and electrophoresed as described under limited trypsinization assay. Autoradiograms were scanned, and density was calculated from recorded data.

*Hormone binding assay.* The hormone binding assays were performed with reticulocyte lysate containing unlabeled translation products. Lysate (10 µl) was incubated overnight at 4 °C with increasing concentrations of [$^3$H]RU486 (0.3-100 nM). In parallel incubations, 100-fold unlabeled RU486 was included to assess non-specific binding. Separation of bound and free steroid was done with protamine precipitation as described previously (Veldscholte *et al.*, 1990). Scatchard plot analysis was performed to determine binding constants.

## Chapter 4

## Results

*Transcription activation*

To compare the antiandrogenic properties of several antiandrogens, transcription activation was studied in a transient transfection system with either the wild type AR or a mutant androgen receptor (ARL). The AR and ARL expression vectors were transfected into HeLa cells, together with the androgen receptor sensitive reporter plasmid MMTV-LUC. The non-metabolizable, synthetic androgen R1881 induced LUC activity in a dose-dependent manner (Fig. 4.1). Cyproterone acetate and RU486 showed a limited partial agonistic effect with AR at concentrations up to 100 nM, whereas partial agonistic activity was not observed for hydroxyflutamide and ICI 176.334. In contrast, in cells with the mutant receptor ARL, cyproterone acetate and hydroxyflutamide induced LUC activity to the same levels as that observed with R1881. A partial agonistic activity of RU486 on ARL was found, whereas ICI 176.334 did not activate ARL. HeLa cells contain a limited amount of glucocorticoid receptor and RU486 is able to bind to these receptors. However, effects of RU486 on transcription activation were not due to this receptor system; in the absence of AR or ARL expression vectors, RU486 failed to induce LUC activity from the transfected MMTV-LUC reporter plasmid (not shown). Thus, with respect to its antiandrogenic properties, RU486 acted as an antagonist in the transient transfection system with AR and ARL, and did not show a higher agonistic activity for ARL than for AR.

*Protease resistance of androgen- and antiandrogen-receptor complexes*

For study of the ligand-induced conformational changes of the AR, *in vitro* produced AR was incubated in the presence of ligand for different periods of time at 25 °C, whereafter limited trypsinization was performed at 0 °C and proteolysis resisting fragments were analyzed (Fig. 4.2A). When both the androgens R1881 (10 nM), testosterone (100 nM), or dihydrotestosterone (10 nM) and the trypsin where added simultaneously (i.e. no pre-incubation of receptor and steroid at 25 °C), a 35 kDa proteolysis resisting fragment of the AR was formed (result shown for R1881: Fig. 4.2A, Lane 4). In the absence of ligand the receptor is completely degraded (Fig. 4.2A, lane 2). Addition of hormone therefore immediately induces a change in the conformation of the AR in such a way that the receptor was no longer completely degraded. With increasing incubation times at 25 °C, a shift in abundance of the 35 kDa proteolysis resisting fragment to a 29 kDa proteolysis resisting fragment of the AR occurred (Fig. 4.2A, Lanes 4-7). This shift in size of the proteolysis resisting fragment indicates a second conformational change of the AR induced by androgens. The antiandrogens hydroxyflutamide (10 μM), cyproterone acetate (1 μM), ICI 176.334 (10 μM), and RU 23908 (1 μM) also immediately induced resistance of a 35 kDa fragment of AR against trypsinization, but no conformational alteration was observed within an incubation period of 60 min at 25 °C (result shown for hydroxyflutamide: Fig. 4.2A, Lanes 8-11). In contrast to these antiandrogens, the presence of RU486 (1 μM) resulted in formation of 35 and 30 kDa proteolysis resisting fragments of the AR, and in a time-dependent shift towards shorter 29 and 25 kDa fragments (Fig. 4.2A, Lanes 12-15). Next, antisera were used to study the differences in composition of the proteolysis resisting fragments of the AR, incubated for 60 min at 25 °C before trypsinization. The C-terminus of the ligand binding domain of the RU486-bound AR did not resist trypsinization, as the 25 kDa fragment could not be immuno-precipitated with the antiserum SP066 (epitope at C-terminus).

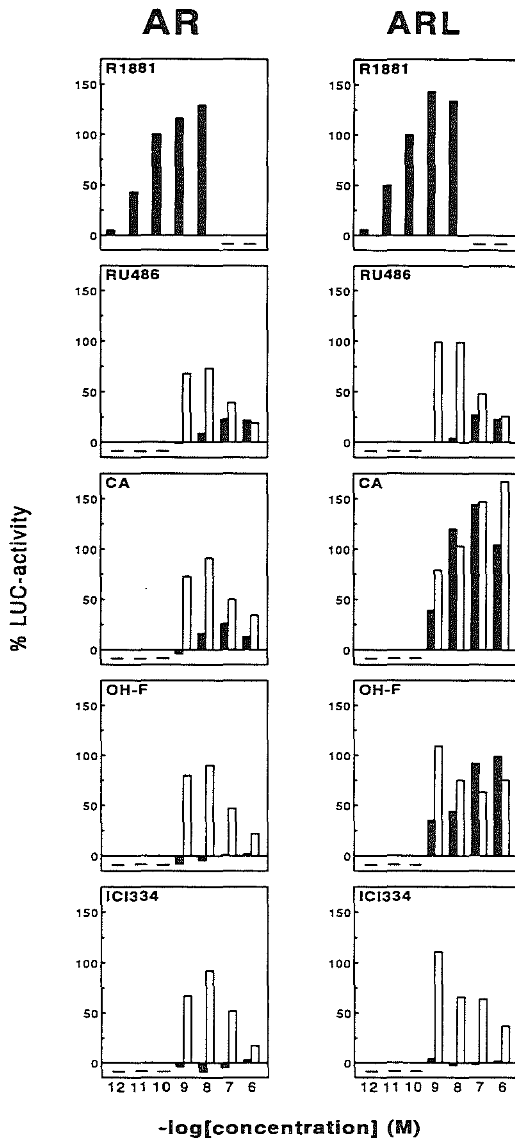



*__Figure 4.1.__ Transcriptional activity of the androgen receptor (AR) and LNCaP androgen receptor (ARL) in the presence of androgen, antiandrogens, and RU486. LUC activity was determined in Hela cells co-transfected with pSVAR or pSVARL and MMTV-LUC. After transfection, cells were incubated with R1881, hydroxyflutamide (OH-F), cyproterone acetate (CA), ICI 176.334 (ICI334) or RU486. LUC activity is indicated as percentage of the activity induced by 0.1 nM R1881 (=100%). LUC activities, induced by the indicated ligands are represented by closed bars. Open bars represent competition of 0.1 nM R1881 with the other ligands. The mean values of at least two experiments are shown. A LUC activity of <5% of that induced by 0.1 nM R1881 was observed with 10 nM R1881 in the absence of pSVAR or pSVARL, or in the absence of hormone.*

In contrast, both the 35 kDa and 29 kDa fragments could be precipitated with this antiserum (Fig. 4.2B, Lanes 16-21). Because both these last two fragments contain the C-terminus of the ligand binding domain, the differences in size are due to differences in extension into the hinge region, a region located at approximately 250 amino acids from the C-terminus. The antiserum F52 recognizes an epitope in the DNA binding domain, adjacent to the hinge region. None of the proteolysis resisting fragments could be immuno-precipitated with the antiserum F52 (not shown), indicating the absence of the DNA binding domain in all fragments.

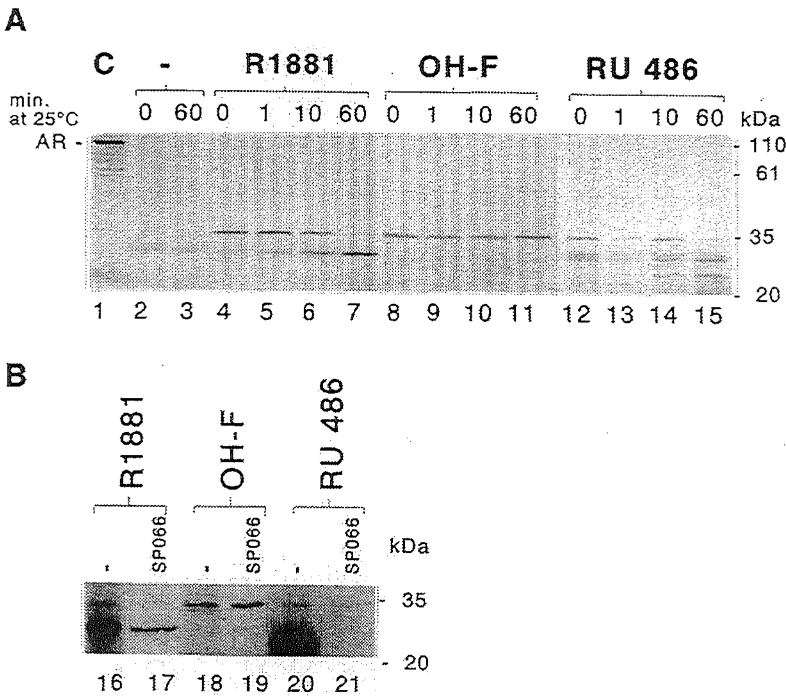



*__Figure 4.2.__ Limited trypsinization of androgen- and antiandrogen-bound AR. A. In vitro produced [$^{35}$S]AR was incubated for the indicated time (min) at 25 °C in the presence of vehicle (-), R1881 (10 nM), hydroxyflutamide (OH-F, 10 μM) or RU486 (1 μM) before limited tryptic (100 μg/ml) digestion for 1 h at 0 °C. The trypsin-treated samples were denatured and electrophoresed on a 12.5% acrylamide gel. Autoradiography was performed overnight. __B.__ In vitro produced [$^{35}$S]AR was incubated for 1 h at 25 °C in the presence of R1881 (Lanes 16-17), hydroxyflutamide (OH-F, Lanes 18-19) or RU486 (Lanes 20-21) and subjected to limited trypsinization (20 μg trypsin/ml, 15 min at 25 °C); thereafter samples were either left on ice (-; Lanes 16, 18 and 20) or immuno-precipitated with the polyclonal antiserum SP066 (SP066; Lane 17, 19 and 21).*

In conclusion: these results show differences in interaction with the receptor between antiandrogens cyproterone acetate, hydroxyflutamide, ICI 176.334 and RU 23908 and the antiandrogen RU486. Furthermore, they emphasize that receptors complexed with antiandrogens (except RU486) do not contain a trypsin-sensitive site available for the cleavage of the C-terminal 30-40 amino acids.

*Effect of molybdate and immuno-adsorption on proteolysis resisting conformation*
Transformation by hormones of steroid hormone receptor complexes into an activated form is thought to be accompanied by the release of several associated proteins, mainly belonging to the family of heat-shock proteins (HSPs; reviewed in Pratt *et al.*, 1989; Smith and Toft, 1993). A 90 kDa heat-shock protein (HSP90) is the major component in this complex and has been shown unambiquously to be part of unactivated steroid receptor complexes, both *in vivo* and *in vitro*. The HSPs are predominantly bound to the ligand binding domain of steroid hormone receptors. Release of the HSPs may be involved in the time-dependent conformational changes in the ligand binding domain that are detected with the limited trypsinization procedure in androgen- and RU486-bound receptors. Molybdate is known to stabilize steroid hormone receptor-HSP interactions (Pratt *et al.*, 1989). It was observed, that the time-dependent conformational change of AR induced by androgens and by RU486 was also delayed by the addition of molybdate (Fig. 4.3A). With antiandrogens, addition of molybdate increased the amount of the 35 kDa proteolysis resisting fragment (Fig. 4.3A, Lanes 6 and 7). In the absence of ligand (Figure 4.3A, Lanes 2 and 3) addition of molybdate had no effect; protected fragments were still not detectable. These results indicate that molybdate stabilized the first proteolysis resisting conformation of the receptor that is initially formed after binding of the ligand, and suggest a role for associated (heat-shock) proteins in the process of the time-dependent conformation change.
The effect of removal of associated proteins on the protease resistance of ligand-bound AR was examined with an immune-adsorbed receptor. *In vitro* produced receptor was adsorbed to an agarose matrix with an immobilized antibody that recognizes the N-terminus of the AR. Subsequently, the resin was washed with salt, which was reported to remove most of the associated proteins from glucocorticoid and progesterone receptors (Smith *et al.*, 1990; Smith, 1993; Pratt, 1993). The direct demonstration of the release of the major heat-shock protein HSP90 from the *in vitro* produced AR was not feasible, due to the low amount of AR in the reticulocyte lysate and the relatively high non-specific binding of HSP90 to the affinity resin. In an experiment with a larger amount of unlabeled androgen receptors, obtained from LNCaP cells, it was shown that the interaction of HSP90 with an immunopurified AR is disturbed upon a salt-wash, whereas ligand binding remained (dissociation of HSP90 shown in Fig. 4.3, lanes 15-18). Strikingly, when the *in vitro* produced, $^{35}S$-labeled AR on the affinity matrix was liganded with an antiandrogen, trypsinization now resulted in the formation of a 29 kDa fragment (Fig. 4.3, Lane 13), a fragment with the same size as formed with agonists (Fig. 4.3, Lane 12). Trypsinization of unliganded, and of androgen- or RU486-bound, immune-adsorbed AR showed results similar as seen with the non-immune-absorbed receptor (compare Fig. 4.3, Lanes 11, 12 and 14 with Fig. 4.2A, Lanes 7, 11 and 15). These results suggest that, after removal of associated proteins, antiandrogens, except RU486, are able to stabilize the same protease resisting conformation as agonists.

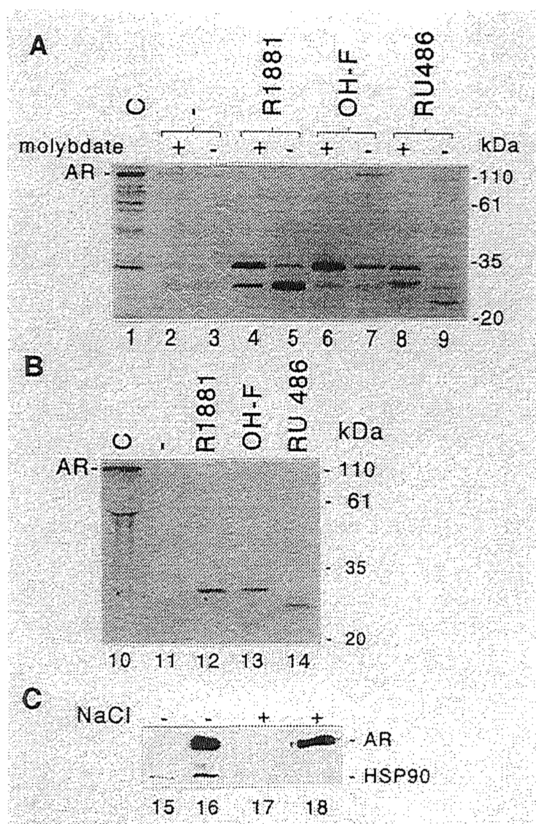



***Figure 4.3.*** *Effect of molybdate and removal of associated proteins on limited trypsinization of AR.* ***A.*** *In vitro produced [$^{35}$S]AR was incubated (1 h at 25 °C) in the presence of vehicle (-), R1881 (10 nM), hydroxyflutamide (OH-F; 10 μM), or RU486 (1 μM), with (+) or without (-) molybdate (20 mM). Before limited trypsinization (20 μg trypsin/ml, 15 min at 25 °C), molybdate was added to 20 mM in all samples.* ***B.*** *[$^{35}$S]AR was immuno-precipitated with the antiserum SP197 and salt-washed (0.5 M NaCl), before incubation with ligands as above. Limited digestion was performed with trypsin (5 μg/ml, 15 min at 25 °C), and samples were analysed as described in the legend of figure 4.2.* ***C.*** *HSP90 interaction with the AR. AR in cytosol obtained from LNCaP cells was immuno-precipitated with the antibody F39.4.1, washed without (-; Lane 16) or with (+; Lane 18) NaCl (0.5 M), and after electrophoresis visualized on a Western blot. Lane 15 and 17 show non-specific binding of HSP90 to the affinity resin without antibody F39.4.1. AR and HSP90 were stained with the specific antibodies F39.4.1 and AC88, respectively.*

*Limited trypsinization of mutated androgen receptors*

As described in the section on transcription activation, hydroxyflutamide and cyproterone acetate could activate the mutant receptor ARL, whereas ICI 176.334 and RU486 were antagonists also for ARL. Binding of hydroxyflutamide and cyproterone acetate to ARL induced an increase of the resistance of a 29 kDa fragment to trypsinization, indicating that both antiandrogens could induce a proteolysis resisting conformation of the receptor normally only seen with androgen receptor agonists (Fig. 4.4, result shown for R1881 and hydroxyflutamide: Lanes 3 and 4). For ICI 176.334 only a minor increase in intensity of the 35 kDa fragment is seen, and no formation of a 29 kDa fragment (Fig. 4, Lane 6). RU486 protected a fragment of 25 kDa to trypsinization, similar as seen for the wild type receptor

(Fig. 4.4, Lane 5). So, it appears that the mutation in ARL did not affect the conformational changes of ARL induced by these last two antagonists.
Deletion of the last 12 amino acid residues of the ligand binding domain of the AR completely abolished $^{3}$H-R1881 binding (Jenster *et al.*, 1991). In studies with the wild type androgen receptor described above, we observed protection by RU486 of a fragment of the receptor lacking the C-terminal amino acids. To study the effect of a deletion of 12 amino acid residues at the C-terminus in a limited proteolytic digestion assay, AR 1-898 was produced *in vitro* and incubated with different ligands. Limited proteolytic digestion resulted for both agonists and antagonists, except RU486, in a complete digestion of AR 1-898 into fragments not detectable after electrophoresis (Fig. 4.4, Lanes 9 and 10), similar as observed in the absence of ligand (Fig. 4.4, Lane 8). The complete digestion of AR 1-898 indicates the necessity of ligand binding for the resistance against trypsinization. RU486 protected a 25 kDa fragment of AR 1-898 against proteolytic degradation, indicating that the C-terminal amino acids are not required for RU486 binding (Fig. 4.4, Lane 11). The efficiency of protection of the 25 kDa fragment, however, is lower in comparison with wild type AR. The binding affinity of [$^{3}$H]RU486 was determined by Scatchard plot analysis (results not shown). The observed lower binding affinity of RU486 for AR 1-898 ($K_d$ 5.4 nM) as compared to AR ($K_d$ 1.6 nM) may explain the lower efficiency of protection of AR 1-898 by RU486. The study with the mutant AR 1-898 provides additional evidence for a different mechanism of action of the antagonist RU486.

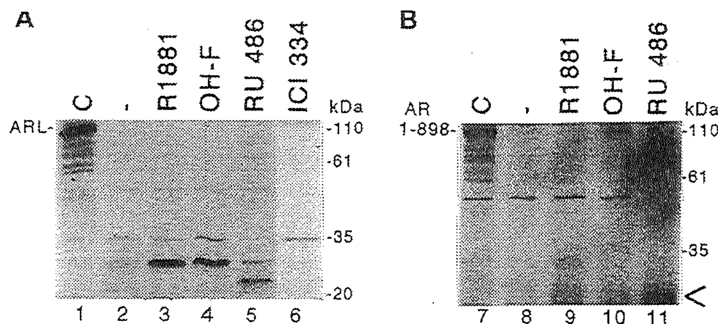



***Figure 4.4.*** *Limited trypsinization of mutated androgen receptors. Both in vitro produced ARL (A) and AR 1-898 (**B**) were incubated (1 h at 25 °C) in the presence of vehicle (-), R1881 (10 nM), hydroxyflutamide (OH-F, 10 μM) or RU486 (1 μM), before limited trypsinization (20 μg/ml, 15 min at 25 °C). Lanes 1 and 6 are controls (C) without trypsin added. In Lane 11 the position of the 25 kDa fragment is indicated (<). The band below 61 kDa is non-specific.*

*Sucrose density gradient centrifugation*
The experiments with molybdate-stabilized and salt-treated receptor complexes provided indirect evidence for a process, in which a change in proteolysis resisting conformation occurred simultaneously with a change in interaction with other proteins. Additional indications for such a process were obtained from the estimation of sedimentation values of

ligand-receptor complexes on sucrose density gradients. *In vitro* produced AR was complexed with [$^3$H]R1881 or [$^3$H]RU486, or labeled with [$^{35}$S]methionine and complexed with unlabeled ligands. Incubation was performed at 0 °C or at 25 °C, to mimic the incubation conditions as used for trypsinization of the receptor. AR liganded at 0 °C, with either the androgen [$^3$H]R1881 or the antiandrogen [$^3$H]RU486, sedimented as an 8S complex (Fig. 4.5A-B).

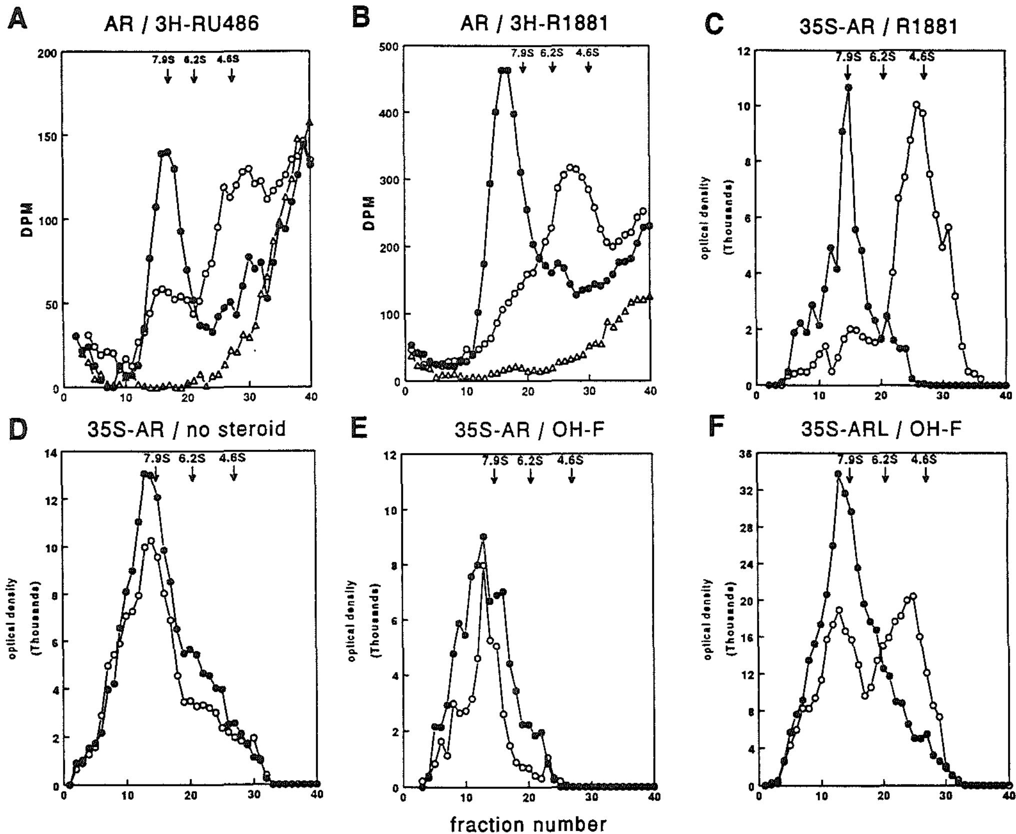



***Figure 4.5.*** *Sucrose gradient centrifugation of androgen receptors. Sucrose density gradient (5-20%) centrifugation of radioactive labeled receptor, incubated for 2 h at 0 °C (●) or 1 h at 25 °C (○).* ***A-B.*** *Labeling of AR occurred with [$^3$H]-steroid. Excess of unbound [$^3$H]-steroid was removed by dextran coated charcoal; non-specific binding was determined by incubation in the presence of 100-fold excess of unlabeled steroid for 2h at 0 °C (△).* ***C-F.*** *[$^{35}$S]-labeled receptor was incubated in the absence or presence of ligand as above, prior to sucrose density gradient centrifugation. In panel F results for mutant receptor ARL are shown. Fractions were subjected to electrophoresis, followed by autoradiography. Autoradiograms were scanned to determine optical density (arbitrary unit). Aldolase (7.9S), alkaline phosphatase (6.2S), and hemoglobin (4.6S) were used as standards. OH-F: hydroxyflutamide.*

Incubation at 25 °C resulted in formation of a 5S complex with R1881 and a 4S complex with RU486. Therefore, both ligands, which induced a second conformational change of the AR at 25 °C as detected by limited trypsinization, also transformed an initially present 8S receptor complex into a smaller complex on warming. Incubation of [$^{35}$S]AR complexed with non-radioactive R1881 showed similar 8S and 5S receptor forms as with [$^{3}$H]R1881-labeled receptor (compare Fig. 4.5B and C). In contrast, only 8S complexes were present after an incubation at 25 °C of the AR in the absence of ligand or in the presence of the antagonists cyproterone acetate, hydroxyflutamide, ICI 176.334 and RU 23908 (Fig. 4.5D-E).
The antagonists hydroxyflutamide and cyproterone acetate could activate transcription with the mutant ARL and protected a 29 kDa fragment of ARL against trypsinization, indicative for the release of associated proteins. The effect of binding of these ligands to ARL on the sedimentation value of the receptor complex was also studied. Indeed, both ligands partly shifted the initial 8S complex to a 5S complex after an incubation at 25 °C (Fig. 4.5F, result shown for hydroxyflutamide). A shift in sedimentation value of ARL from 8S to 5S in the presence of agonists, or to 4S in the presence of RU486, was seen on warming, equivalent to the results obtained with the wild type AR. Unliganded and ICI 176.334-bound ARL sedimented as an 8S complex also at 25 °C, a result similar as found for the wild type AR (results not shown).
In summary, the sucrose gradient centrifugation studies show that an 8S receptor complex was present under conditions when a 35 kDa fragment could be demonstrated by limited trypsinization. A 5S complex correlated with a proteolysis resisting fragment of 29 kDa, and was characteristic for the receptor form able to induce transcription. Binding of RU486 to the AR resulted in formation of a complex different from the agonist-receptor complex in both proteolysis resisting conformation and sedimentation characteristics (4S) (results are summarized in Fig. 4.6).

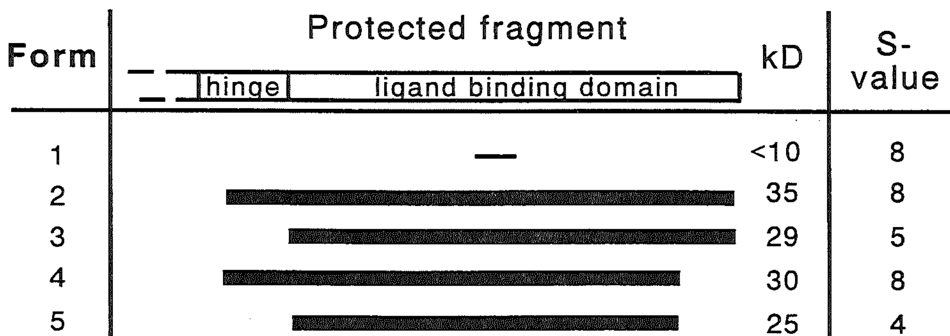



*Figure 4.6. Different forms of the androgen receptor. Five different forms of the androgen receptor can be detected with limited trypsinization and sucrose density gradient centrifugation. Form 1 represents the unoccupied receptor. Form 2 is obtained in the presence of either one of the various antagonists, except RU486, and also after the initial binding of agonists at 4 °C. Upon incubation of the androgen receptor with the agonist at 25 °C, Form 3 is found. Forms 4 and 5, derived of Form 2 and 3, are found in the presence of RU486. Part of the domain structure of the androgen receptor is shown schematically on top of the figure.*

## Chapter 4

### Discussion

Proteolytic analysis has proven to be a powerful method to analyse agonist- and antagonist-induced conformational changes of progesterone (Allan *et al.*, 1992a; Allan *et al.*, 1992b), estrogen (Beekman *et al.*, 1993), glucocorticoid (Allan *et al.*, 1992a), androgen (Kallio *et al.*, 1994; Kuil and Mulder, 1994; Zeng *et al.*, 1994) and retinoic acid receptors (Keidel *et al.*, 1994). In the present study, a detailed analysis of protease resisting fragments permitted the identification of two conformational changes after the binding of androgens. Immediately upon binding of an agonist to the wild type AR, the ligand binding domain (amino acids 662-910) and part of the hinge region (amino acids 612-662) resisted trypsinization (form 2 in Fig. 4.6) indicative for a more compact structure of the liganded protein than in the absence of ligand. After this rapid initial structural alteration, a second conformational change occurred as detected with limited trypsinization, concomitantly with a shift of a receptor complex sedimenting at 8S to a complex sedimenting at 5S (form 3 in Fig. 4.6). In analogy to other steroid hormone receptors (reviewed in Riehl *et al.*, 1985), the high molecular weight form of the AR presumably represents a complex of several proteins, including the 90 kDa heat-shock protein (Veldscholte *et al.*, 1992, Marivoet *et al.*, 1992; Nemeto *et al.*, 1992). The second conformational change likely results in dissociation of this complex and preceeds the formation of a transcriptionally active complex. The antiandrogens cyproterone acetate and hydroxyflutamide, which are agonists for the mutant ARL, induced in this mutant the two conformational changes, and released associated proteins. Previous studies with ARL already indicated that for the formation of the transcriptionally active form the release of HSPs is essential (Veldscholte *et al.*, 1992b). It can be assumed that the single amino acid change in the ARL made it possible for cyproterone acetate and hydroxyflutamide to induce both the second conformational change and HSP-dissociation, which results in agonistic activity.

Two nonexclusive models have previously been postulated to explain the mechanism of action of steroid receptor antagonists. In the first model, two types of antagonists are proposed (Reese and Katzenellenbogen, 1991; Klein-Hitpass et al, 1990; Gronemeyer et al, 1992). Both types of compounds compete with agonists for binding to the receptor, but they differ in their effect on subsequent steps in the mechanism of receptor activation. The so called "pure antagonists" have no partial agonistic activity, probably due to either decreased dimerization (Klien-Hitpass *et al.*, 1990; Fawell *et al.*, 1990), decreased binding of the receptor complex to DNA (Berry *et al.*, 1990; Bocquel *et al.*, 1993) or increased turnover (Gibson *et al.*, 1991). The other type of antagonists ("non-pure antagonists" with partial agonistic activity) provokes the transformation of the receptor to the DNA binding form but fails to promote its transcriptional activity, probably through the induction of an inappropriate conformation of the C-terminally located transcription activation region (AF-2) (Meyer *et al.*, 1990; Danielian, 1992). The cell and gene specific partial agonistic effects of some estrogen and progesterone receptor antagonists have been explained by the action of the ligand-independent transcription activation region (AF-1) (Gronemeyer *et al.*, 1992; Berry *et al.*, 1990; Green, 1990). This division in two types of antagonists has been challenged. For the "pure antiestrogen" ICI 164.384 (Fawell *et al.*, 1990), DNA binding was observed in some studies (Sabbah *et al.*, 1991; Pham *et al.*, 1991). For the antiprogestagen ZK 98299 the failure of the receptor to interact with DNA (Klein-Hitpass *et al.*, 1990) was subscribed to the lower binding affinity of the ligand for the receptor (Delarbre *et al.*, 1993) and could be overcome by increasing the ligand concentration. However, ZK 98299 could prevent the agonistic activity of the ("non-pure") antiprogestagen RU486 after stimulation of the cAMP signalling pathways (Beck *et al.*,

1993). A not yet well understood difference in phosphorylation of the receptor could possibly play a role (Beck *et al.*, 1993, Takimoto, 1992).
In a second model explaining the mechanism of antagonists, described recently by O'Malley and collaborators, the importance of a conformational change of the ligand binding domain is emphasized (Tsai and O'Malley, 1994; Baniahmed and Tsai, 1993; Allan *et al.*, 1992a). The model argues that agonists and antagonists recognize distinct regions of the ligand binding domain. Antagonists induce an incomplete conformational change, that results in dimerization and DNA-binding, but leave the C-terminus of the ligand binding domain in a form still available for protease (Allan *et al.*, 1992a) and antibody recognition (Vegeto *et al.*, 1992; Weiegl *et al.*, 1992). As a result, a surface repressor function is not removed and the receptor is not able to induce transcription.

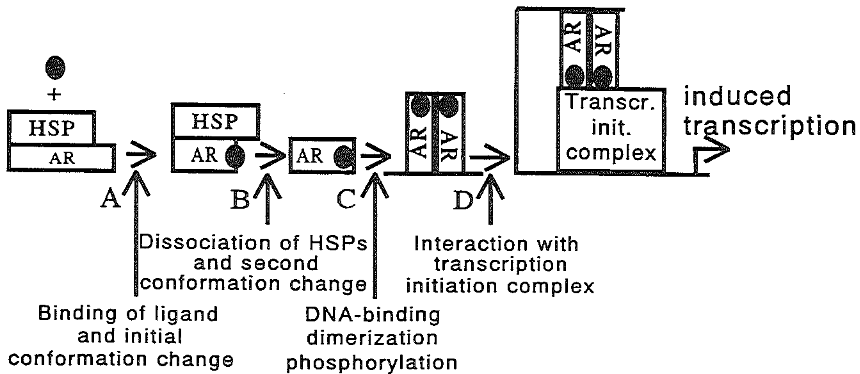



***Figure 4.7.*** *Model of the sequential steps in androgen receptor activation. Binding of an agonist to the inactive androgen receptor (AR), complexed with heat-shock proteins (HSP), induces an initial conformation change (A). Upon HSP-dissociation and a second conformational change (B), the receptor dimerizes, binds to DNA and phosphorylation occurs (C). The DNA-bound receptor interacts with the transcription initiation complex to regulate transcription (D). One, or more of the steps B, C, and D may be blocked after binding of an antagonist.*

We would propose to incorporate both models described above in one scheme, and add an additional step that might be inhibited by antagonists, to explain the results of our studies with the antiandrogens cyproterone acetate, hydroxyflutamide, ICI 176.334 and RU 23908 (Fig. 4.7). The key-point of this model is the step between a ligand-occupied complex of the receptor and associated proteins, and a receptor that has a changed conformation and has released these proteins. This step (step B in Fig. 4.7) is partly or completely blocked by the antiandrogens cyproterone acetate, hydroxyflutamide, ICI 176.334 and RU 23908. This step in the process of receptor activation has been previously proposed as a step that is impaired by antagonists (Moudgil and Hurd, 1987; Segnitz and Gehring, 1990; Renoir *et al.*, 1990), but recent observations of DNA-bound receptors occupied with antagonists do not favour this step

as critical for the action of most antiprogestagens and antiestrogens (Agarwal, 1994). We like to stress that more than one step may be involved in the mechanism of antagonist action, and that a strict division of antagonists in distinct categories might not be appropriate. The "pure" antagonists of the estrogen and progesterone receptor induce a partial conformational change, and might block the process of receptor activation at step C (DNA-binding, dimerization and/or phosphorylation by nuclear kinases) in Fig. 4.7. However, from our studies on antiandrogens, we have no new arguments to support an absolute distinction between antagonists blocking either step C or step D (interaction with the transcription initiation complex) in Fig. 4.7. RU486, when bound to the androgen receptor, induced an inappropriate conformational change of the receptor, but also showed partial agonistic properties apart from its antagonistic action. This might indicate inhibition of step D in Fig. 4.7, and emphasized that antiandrogenic activity may be exerted by different mechanisms.

Several data support the inhibition of step B (Fig. 4.7) in the above described model for the action of the androgen receptor antagonists cyproterone acetate, hydroxflutamide, ICI 176.334 and RU 23908. Binding of these antiandrogens first induced a rapid initial change within the AR, similar as seen in the presence of agonists and indicative for a comparable initial binding site on the receptor. However, in the presence of the above mentioned antiandrogens no indication for the induction of a second conformational change or release of associated proteins *in vitro* could be detected. Furthermore, no support was found for the involvement of the C-terminus of the AR in the mechanism of action of the four antiandrogens mentioned above. Despite the presence of several protease degradation sites, the C-terminus was always present in the proteolysis resisting fragment, obtained after limited proteolysis of these antiandrogen-receptor complexes. In addition, the mutant AR 1-898, with a C-terminal deletion of 12 amino acids, does not bind androgens (Jenster *et al.*, 1991) and could not induce a protease resisting conformation in the presence of androgens and the four antiandrogens. This suggests that the C-terminus is involved in binding of both androgens and the four antiandrogens, and consequently that these antiandrogens must interfere with another step in the process of receptor activation.

In conclusion: our data indicate that the antiandrogens cyproterone acetate, hydroxflutamide, ICI 176.334 and RU 23908 act at a different step in the mechanism of action of steroid hormones than several antiprogestagens and antiestrogens. These antiandrogens do not induce or stabilize an inappropriate conformation of the C-terminal end of the receptor, but act earlier and prevent a conformation change presumably by stabilizing a complex with different associated proteins. RU486 has a broad steroid specificity, and also binds to the androgen receptor. This latter compound showed that antiandrogenic activity might also be accomplished in a different way, by induction of a non-functional conformation of the ligand binding domain, similar as found for its effect on the progesterone receptor.

**Acknowledgements**

We wish to thank Dr. J.A. Grootegoed for helpful discussions and critical reading of the manuscript. We gratefully acknowledge the gifts of radioactive RU486 from Roussel Uclaf and the mouse monoclonal antibody AC88 from Dr. D.O. Toft.

# DNA-BINDING ABILITY OF ANDROGEN RECEPTORS IN WHOLE CELLS: IMPLICATIONS FOR THE ACTION OF ANDROGENS AND ANTIANDROGENS

C.W. Kuil and E. Mulder

Published in
Endocrinology
137 (1996), 1870-1877

## Chapter 5


**Summary**

In whole cells, the effect of several androgens and antiandrogens on the induction of DNA binding for the human wild type androgen receptor (AR) and a mutant receptor ARL (LNCaP mutation; codon 868, Thr to Ala) was examined, and related to the transcription activation ability of these receptors. To study DNA binding, an AR expression vector was cotransfected in CHO cells with a promoter interference plasmid CMV-$(ARE)_3$-LUC, containing androgen response element(s) between the TATA box of the cytomegalovirus promoter and the start site of luciferase gene transcription. Expression levels of the AR are upregulated by some agonists, but receptor expression levels are comparable for all antiandrogens studied. In the presence of androgens, the wild type AR is capable to reduce promoter activity of the CMV-$(ARE)_3$-LUC plasmid, indicating androgen-dependent DNA binding of the AR. The full antagonists hydroxyflutamide, ICI 176.334 and RU 23908 block AR binding to DNA. The antagonists cyproterone acetate and RU 38486 induce approximately 50% of the DNA binding found for androgens. In a transcription activation assay, the RU 38486-bound receptor was almost inactive and the receptor complexed with cyproterone acetate showed partial agonistic activity. Interaction of the antagonists cyproterone acetate, hydroxyflutamide and RU 23908 with the mutant receptor ARL resulted in both a DNA-bound and a transcriptionally active receptor. In conclusion: transformation of the AR to a DNA binding state in whole cells is blocked by several antiandrogens. Furthermore, studies with the antiandrogens cyproterone acetate and RU 38486 show that DNA binding alone is not sufficient to accomplish full transcriptional activity. Full activity requires additional changes, presumably in the protein structure of the receptor.


### Introduction

The androgen receptor (AR) belongs to a superfamily of ligand-inducable transregulators, which includes receptors for steroid and thyroid hormones, and vitamins (Laudet *et al.*, 1992). As for the other members of the superfamily, molecular genetic analysis has identified separable domains responsible for DNA binding, hormone binding and transactivation (Jenster *et al.*, 1991; Simental *et al.*, 1991). Upon androgen binding, the AR undergoes several sequential processes in order to interact with specialized regions on the DNA. These so-called androgen response elements (AREs) are commonly located in the regulating regions of target genes. Binding of the androgen-bound receptor to its response element results in the formation of a stable pre-initiation complex which allows efficient transcription initiation by RNA polymerase II (Evans, 1988; Beato, 1989).

Steroid receptor antagonists inhibit the biological effects of agonists, although the precise molecular mechanism(s) of these antagonists are unknown. In a model explaining the mechanism of antagonist action, described recently by O'Malley and collaborators, the importance of a conformational change of the ligand binding domain is emphasized (Allan *et al.*, 1992a; Baniahmed and Tsai, 1993; Tsai and O'Malley, 1994). The model argues that antagonists recognize regions of the ligand binding domain, which result in dimerization and DNA binding, but leave the C-terminus of the ligand binding domain in a form still available for protease (Allan *et al.*, 1992a) and antibody recognition (Vegeto *et al.*, 1992; Weigel *et al.*, 1992). As a result, a repressor function is not removed and the receptor is not able to induce transcription. In accordance with this model, binding of RU 38486 to an *in vitro* translated AR, resulted in both the protease digestion of the C-terminus of the ligand binding domain

(Zeng *et al.*, 1994; Kuil *et al.*, 1995) and dissociation of the heat-shock protein-receptor complex (Kuil *et al.*, 1995). Other results suggested that more steps could be involved in the mechanism of antiandrogen action: upon binding of the androgen receptor antagonists cyproterone acetate, hydroxyflutamide, ICI 176.334 or RU 23908, no indications were found for the involvement of the C-terminus of the androgen receptor in the mechanism of antiandrogen action (Kallio *et al.*, 1994; Kuil *et al.*, 1995). As an intact heat-shock protein-receptor complex is still present upon binding of these antiandrogens *in vitro* (Kuil *et al.*, 1995), we hypothesized that dissociation of the receptor from this complex, and subsequent binding of the receptor to DNA, would partly or completely be blocked by some antiandrogens within intact cells. Dissociation of the oligomeric receptor complex, and subsequent binding of the receptor to DNA, have previously been proposed to be impaired by antagonists (Moudgil and Hurd, 1987; Segnitz and Gehring, 1990, Truss *et al.*, 1994). However, recent observations of DNA-bound receptors do not favour these steps as critical for the action of antiprogestagens and antiestrogens (Reese and Katzenellenbogen, 1992; Delabre et a., 1993; Agarwal, 1994; Mymryk and Archer, 1995; Metzger *et al.*, 1995).
To find experimental support for the inhibition of DNA binding by several antiandrogens, we applied a promoter interference assay, described by Reese and Katzenellenbogen for the estrogen receptor (Reese and Katzenellenbogen, 1992). This assay makes it possible to examine the effect of ligands, both hormones and antihormones, on AR binding to DNA within mammalian cells. The promoter interference assay utilizes the principle of steric hindrance between the AR and basal transcription factors on a constitutive (cytomegalovirus [CMV]) promoter. Furthermore, the binding of the AR to DNA was related to the transactivation capacity of the various ligand-receptor complexes. The mutant receptor ARL (mutation in codon 868; Thr to Ala) was also investigated, as binding of several antiandrogens resulted in an active receptor complex for this mutant.

## Experimental procedures

*Materials:* R1881 (methyltrienolone) was purchased from NEN (Boston, USA). RU 38486 (mifepristone) and RU 23908 (nilutamide) were gifts from Roussel Uclaf (Paris, France), cyproterone acetate from Schering (Berlin, Germany), hydroxyflutamide from Schering, USA (Bloomfield, USA) and ICI 176.334 ("Casodex") from ICI Pharmaceuticals (Macclesfield, U.K.). All other steroids were purchased from Steraloids (Wilton, USA). BM chemiluminescence Western blotting kit was obtained from Boehringer Mannheim (Mannheim, Germany). The plasmids pcDNAI and pGL2 were obtained from Invitrogen (San Diego, USA) and Promega (Madison, USA), respectively. The mouse mammary tumor virus-luciferase (MMTV-LUC) reporter plasmid was kindly provided by Organon (Oss, The Netherlands).
*Preparation of AR and reporter plasmids:* Preparation of the MMTV-LUC reporter plasmid (de Ruiter *et al.*, 1995), and the expression plasmids for the wild type human AR [encoding an AR of 910 amino acids (Brinkmann *et al.*, 1989)], mutant ARL [LNCaP-mutation; codon 868, Thr to Ala (Velscholte *et al.*, 1990)] and mutant AR64 [codon 567, Cys to Ser and codon 570, Cys to Phe (Jenster *et al.*, 1993)] have been described previously. The CMV-LUC plasmid was constructed by cloning a *Sac*I-*Bam*HI fragment that spanned the luciferase transcription unit from pGL2 into pcDNAI (a plasmid containing the CMV-promoter), which had been digested with *Sac*I and *Bam*HI. CMV-$(ARE)_{1-3}$-LUC promoter interference plasmids (containing one to three androgen response elements) were prepared by ligation of double-

stranded oligonucleotides containing a consensus ARE [GGTACAgttTGTTCT (Roche *et al.*, 1992)], into the *Sac*I-site of the CMV-LUC plasmid.

*Cell culture and transfections:* Chinese Hamster Ovary (CHO) cells were maintained in Dulbecco's Modified Eagle's DME/F12 tissue culture medium, supplemented with 5% charcoal dextran-treated fetal calf serum. For promoter interference assay, CHO cells were plated at $1.5x10^5$ cells/well (10 $cm^2$), grown for 24 h and transfected by calcium phospate precipitation as described before (Veldscholte *et al.*, 1992a). Cells were transfected with 75 ng of expression plasmid, either encoding AR, ARL or AR64, and 7.5 ng of CMV-LUC or CMV-$(ARE)_{1-3}$-LUC plasmid. Carrier DNA (pTZ19) was added in each case to a total of 5 μg per well. For transcription regulation studies, CHO cells were plated at $0.6x10^5$ cells/well (4 $cm^2$) and transfected with 10 ng of AR or ARL expression plasmid, 200 ng MMTV-LUC reporter plasmid and 1.8 μg pTZ19 per well. After transfection, the cells were washed and experimental media were added. Upon an incubation period of 24 h, cells were harvested for the luciferase (LUC) assay, as described previously (Kuil *et al.*, 1995).

*Western immunoblot analysis:* Whole cell lysate was prepared by resuspending the cell pellet from a well (10 $cm^2$) in 200 μl of 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.08% (w/v) SDS, 0.6 mM phenylmethylsulfonyl fluoride and 0.5 mM bacitracin at 4 °C. The lysate was centrifuged (10 min, 1700g) and 20 μl of the supernatant was used for Western immunoblot analysis, essentially as described previously (Veldscholte *et al.*, 1992a). The polyclonal rabbit antiserum SP197, recognizing the AR [epitope: amino acids 1-20 (Kuiper *et al.*, 1993)] was used as the primary antibody in a chemiluminescence protein detection method, performed as described by the manufacturer (Boehringer Mannheim).

## Results

*Transcription activation studies of wild type AR and mutant ARL in CHO cells*

To compare DNA binding of the androgen receptor with effects on transcription activation both an *in vivo* DNA binding assay (described in the next section) and a transcription activation assay were used. Either wild type AR or mutant ARL expression plasmids were transiently transfected into CHO cells, together with the androgen receptor sensitive reporter plasmid MMTV-LUC. The mutant receptor ARL contains a mutation in the ligand binding domain: amino acid 868, Thr replaced by Ala, which leads to a decrease in steroid binding specificity (Veldscholte *et al.*, 1990). The ligands, both androgens and antiandrogens, differ in their binding affinities for the AR (Veldscholte *et al.*, 1990; Veldscholte etal., 1992a; Kemppainen *et al.*, 1992; Culig *et al.*, 1993); therefore, added concentrations of ligand were standarized accordingly. The non-metabolizable, synthetic androgen R1881 (1 nM) and the natural androgens dihydrotestosterone (1 nM) and testosterone (10 nM) induced LUC activity to the same level (Fig. 5.1A) and in a dose-dependent manner (results shown for R1881: Fig. 5.3). LUC activity induced by 100 nM of the steroidal antihormones cyproterone acetate and RU 38486 were approximately 55% and 15% of that observed with 1 nM R1881. [RU 38486 has antiandrogenic properties, besides its effect as an antiprogestagen/ antiglucocorticoid (Baulieu, 1989; Philibert *et al.*, 1990).] Partial agonistic activity was not observed for the non-steroidal antiandrogens hydroxyflutamide, ICI 176.334 and RU 23908 (used in concentrations up to 1 μM). All antiandrogens inhibited the LUC activity induced

by 0.1 nM R1881 (Fig. 5.1B).

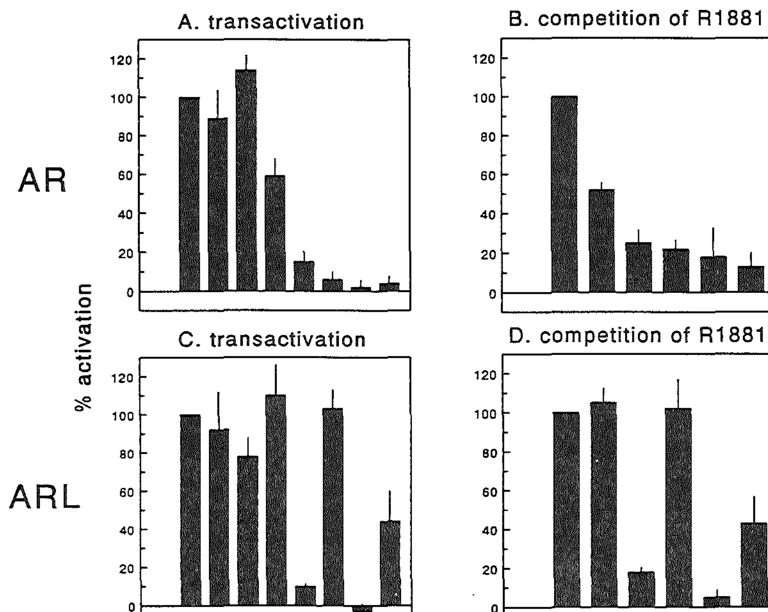



*__Figure 5.1.__ Transcriptional activity of the wild type androgen receptor (AR) and LNCaP androgen receptor (ARL) in the presence of androgens and antiandrogens. LUC expression was determined in CHO cells transient co-transfected with AR (A and __B__) or ARL (__C__ and __D__) expression plasmids and the reporter plasmid MMTV-LUC. A and __C__: After transfection, cells were incubated without hormone (-H) or with R1881 (1 nM), dihydrotestosterone (DHT; 1 nM), testosterone (T; 10 nM), cyproterone acetate (CPA; 100 nM), RU 38486 (RU486; 100 nM), hydroxyflutamide (OH-F; 1 μM), ICI 176.334 (ICI334; 1 μM) or RU 23908 (RU908; 1 μM) for 24h. LUC-activity in the presence of 1 nM R1881 was set at 100%. Values are the mean (± SEM) of three to four determinations, each determination was performed in triplicate. __B__ and __D__: For competition studies the various antiandrogens were added simultaneously with 0.1 nM R1881. The second bar shows the activity of 0.1 nM R1881 alone (set as 100%). Values are the mean (±SEM) of three determinations.*

In contrast, in cells with the mutant receptor ARL, cyproterone acetate and hydroxyflutamide induced LUC activity to the same level as that observed with R1881, dihydrotestosterone and testosterone (Fig. 5.1C). Some partial agonistic activity of RU 23908 on ARL was found, whereas RU 38486 and ICI 176.334 did not activate ARL. Lack of agonistic activity of RU 38486 and ICI 176.334 was not due to the absence of binding to the mutant ARL, as LUC-activity induced by 0.1 nM R1881 could be inhibited with both antihormones (Fig 5.1D). CHO cells contain a limited amount of glucocorticoid receptors and RU 38486

is able to bind to these receptors. However, the effect of RU 38486 on transcription activation was not due to this receptor system; in the absence of AR or ARL expression vectors, RU 38486 failed to induce LUC activity from the transfected MMTV-LUC reporter plasmid (not shown).

*Promoter activity of constructs containing multiple AREs*

DNA binding of the estrogen receptor in whole cells was studied previously by Reese and Katzenellenbogen (1992) with a promoter interference assay, based on the principle of steric hindrance between a transcription factor and basal transcription factors on a constitutively active promoter. In the present study, an AR-dependent promoter interference reporter plasmid was constructed by inserting consensus AREs (Roche *et al.*, 1992) into the *Sac*I site that lies between the TATA box of the constitutively active CMV-promoter and the start site of transcription of the mRNA, encoding the luciferase protein. Binding of AR at that position should hinder the assembly of the transcription initiation complex and hence reduce the expression of the LUC gene. The functionality of the CMV-$(ARE)_{0\text{-}3}$-LUC plasmids were verified by cotransfection of the construct with the expression vector for the wild type AR into CHO cells. The promoter activity was reduced in all ARE-containing promoter interference plasmids in the presence of R1881, whereas no influence on LUC expression was seen in cells cotransfected with the original CMV-LUC plasmid (Fig. 5.2A). The down-regulation of promoter activity was dependent on the number of inserts, being the largest with the CMV-$(ARE)_3$-LUC plasmid [remaining activity as compared to control: 47%]. In the absence of hormone, no differences in LUC-activity between the different reporter plasmids were observed.

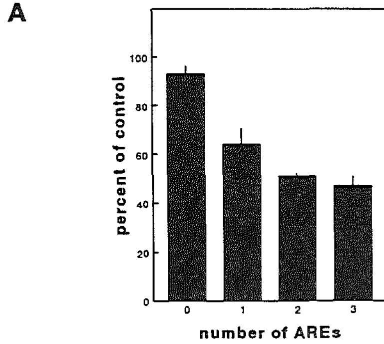
Overexpression of a transcription factor could sequester other factors necessary for the transcriptional activity of a promoter [i.e. squelching (Levine and Manley, 1989)]. As squelching does not require specific DNA binding or an intact DNA binding domain, the receptor mutant AR64 was studied to further verify that the reduction in promoter activity was actually due to DNA binding of the AR to the CMV-$(ARE)_3$-LUC plasmid. In this receptor mutant, the structure of the first zinc cluster is disrupted by the replacement of two of the four cysteine residues. Mutagenesis experiments have shown that these cysteine residues are essential for DNA-binding capacity of the receptor (Freedman, 1992). No effect on LUC expression was seen, when CMV-$(ARE)_3$-LUC plasmid was cotransfected with the AR64 expression plasmid in CHO cells in the presence of ligand (Fig. 5.2B). As expression levels of wild type AR and mutant AR64 were comparable, as analysed by Western blotting (Fig. 5.2C), a squelching phenomenon could not account for the repression of the promoter activity in the CMV-$(ARE)_3$-LUC plasmid. (The effect of ligand on AR expression level is discussed further in the section *AR expression levels and migration pattern.*)

To exclude an effect on promoter activity due to a limited amount of glucocorticoid receptor in CHO cells, we also examined the effect of R1881 and dexamethasone on cells transfected only with either the CMV-LUC or the CMV-$(ARE)_3$-LUC plasmid. In the absence of AR expression plasmid, no reduction of LUC expression from both plasmids could be demonstrated either in the presence of 1 nM R1881 or 100 nM dexamethasone (result shown for R1881; Fig. 5.2B).

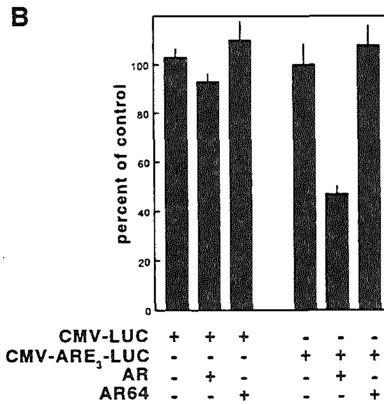
In summary, the results presented in Fig. 5.2, show that interference with promoter activity is dependent on the presence of AREs in the reporter plasmid, a functional AR and on the androgen R1881. This permits the conclusion that the LUC activity measured with this assay

system reflects DNA binding of the AR in whole cells.

A




B




C

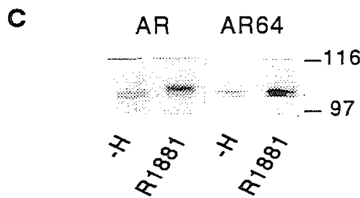



***Figure 5.2.*** *Effect of multiple AREs and different receptors on CMV promoter activity.* ***A.*** *CHO cells were cotransfected with AR expression plasmid and the CMV-LUC construct containing 0 to 3 AREs. After transfection, cells were treated with control vehicle or 1 nM R1881. Control values were determined as the LUC-activity in cells transfected with each CMV-(ARE)$_{0\text{-}3}$-LUC construct and AR expression vector in the absence of ligand and were set at 100%. Data represent the mean (± SEM) of four to six determinations, each determination was performed in triplicate.* ***B.*** *Promoter activity of CMV-LUC and CMV-(ARE)$_3$-LUC plasmid, cotransfected in CHO cells without or with the expression plasmids for wild type AR or mutant AR64 (mutations in DNA binding domain; codons 567 and 570, Cys to Ser) as indicated on the x axis. Cells were treated with control vehicle or 1 nM R1881, and LUC activity was measured. Control values were determined as under A, whereas the results are the means (± SEM) of five to six determinations.* ***C.*** *Western blot analysis of lysates from cells, transfected with wild type AR or the mutant AR64 and incubated with or without 1 nM R1881. Whole cell lysates were fractionated with gel electrophoresis, seperated proteins transferred to nitrocellulose and visualised with a chemiluminescence protein detection method, using the AR-specific polyclonal antiserum SP197 as primary antibody. Molecular mass markers (kDa) were run on a parallel lane and their positions are indicated on the right of the blot.*

*Comparison of transcription activation and DNA-binding ability of AR*
In the previous sections, we described that the complex of androgen and receptor both activated transcription from a MMTV-LUC plasmid and interfered with the constitutively active CMV-promoter. To investigate the dose-dependency of the ligand in both processes, we determined the dose-response curves of R1881 in both assays. Maximal effects of the AR on both transcription activation and promoter interference were reached at 1 nM R1881 and set at 100% activation or inhibition, respectively. The dose-response curve of R1881 on transcription activation matched the effect of R1881 on promoter interference (Fig. 5.3). Because promoter interference in this assay system reflects DNA binding of the AR, these results also indicate a direct correlation between percentage DNA binding and transcription activation.

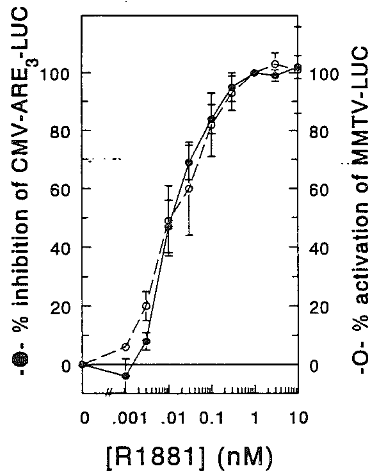



***Figure 5.3.*** *Effect of R1881 concentration on the promoter activity of the CMV-(ARE)$_3$-LUC plasmid. The CMV-(ARE)$_3$-LUC promoter interference plasmid was cotransfected with the AR expression plasmid in CHO cells, and increasing concentrations of R1881 were added (●). Inhibition of promoter activity in the presence of 1 nM R1881 was set at 100%. The effect of R1881 concentration on transcription activition of the MMTV-LUC reporter plasmid in tranfected CHO cells is also shown (○). Transcription activation in the presence of 1 nM R1881 was set at 100%. The data are the mean of two to three determinations (± SEM).*

*Influence of different hormones and antihormones on DNA binding ability of AR*
The promoter interference assay permits the study of DNA binding of the receptor in whole cells. Therefore, we examined the effect of several hormones and antihormones on their ability to reduce promoter activity of the CMV-(ARE)$_3$-LUC plasmid in the presence of either the wild type AR or the mutant ARL. Neither receptor interacted with the promoter interference construct in the absence of R1881, and treatment with R1881 (1 nM) resulted in a similar decrease (to 50%) of LUC activity. This implies a similar dependence on the ligand R1881 for DNA binding of both the AR and ARL to the AREs in the promoter interference construct. CHO cells exposed to the natural androgens dihydrotestosterone (1 nM) and testosterone (10 nM) displayed a comparable reduction in promoter activity of the CMV-(ARE)$_3$-LUC plasmid as found with 1 nM R1881 (defined as 100% inhibition, Fig. 5.4A and

B). Cells, expressing the wild type AR and incubated with the antagonists cyproterone acetate (100 nM) or RU 38486 (100 nM), also showed promoter interference, although less than in the presence of androgens (Fig. 5.4A). The complete antagonists hydroxyflutamide (1 μM), ICI 176.334 (1 μM), and RU 23908 (1 μM) hardly affected the promoter activity of the reporter plasmid (Fig 5.4A). For the mutant receptor ARL, binding of cyproterone acetate, hydroxyflutamide and RU 23908 resulted in a comparable reduction of LUC activity of the CMV-$(ARE)_3$-LUC plasmid as observed for the androgens (Fig. 5.4B). The full antagonist ICI 176.334 showed only a minor effect with the mutant ARL, resembling its effect on the wild type receptor. For the RU 38486-bound ARL, a sligthly smaller reduction in promoter activity was observed than for the wild type receptor.

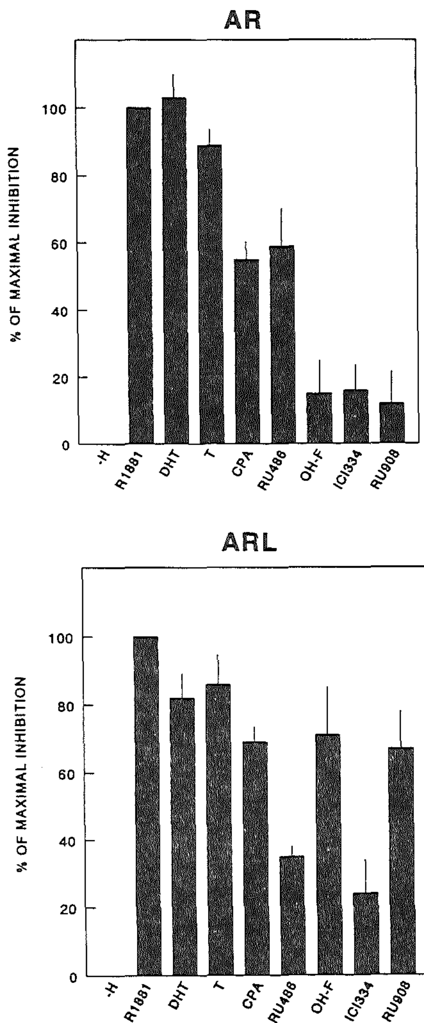



***Figure 5.4.*** *Promoter interference of the wild type AR and mutant receptor ARL in the presence of several androgens and antiandrogens. CHO cells were cotransfected with CMV-$(ARE)_3$-LUC promoter interference plasmid and wild type AR or mutant ARL expression plasmid, and incubated without hormone (-H), with 1 nM R1881, 1 nM dihydrotestosterone (DHT), 10 nM testosterone (T), 100 nM cyproterone acetate (CA), 100 nM RU 38486 (RU486), 1 μM hydroxyflutamide (OH-F), 1 μM ICI 176.334 (ICI334) or 1 μM RU 23908 (RU908). Inhibition of promoter activity in the presence of 1 nM R1881 was set at 100%. Values are the mean (± SEM) of four to five determinations.*

The results on transcription activation and promoter interference of both wild type AR and mutant ARL, complexed with different ligands, are summarized in Table 5.1. A correlation between DNA binding and transcription activation was observed for most compounds, with the exception of RU 38486.

*AR expression levels and migration pattern*
As hormone addition was shown to have effects on receptor stability (Kemppainen *et al.*, 1992) and phosphorylation status of the receptor (Jenster *et al.*, 1994), we wanted to exclude the possibility that the differences in the promoter interference assay were due to differences in receptor expression levels. Therefore, extracts were prepared from transfected cells after treatment with the various ligands and receptors were analysed by Western blotting. In the absence of hormone, the wild type receptor and the mutant receptor AR64 migrated as two protein bands [110-112 kDa (Fig. 5.2C)]. The difference in electrophoretic mobility represents differences in degree of phosphorylation of the receptor (Krongrad *et al.*, 1991; Kuiper *et al.*, 1991; Jenster *et al.*, 1995). An additional decrease in electrophoretic mobility of the receptor protein was observed, when CHO cells, expressing the wild type AR, were incubated in the presence of R1881 (Fig. 5.2C). These results are in agreement with observations described by others (Jenster *et al.*, 1994), and suggest the appearance of an additional protein form in the presence of R1881. The additional decrease in electrophoretic mobility was not observed for the mutant receptor AR64 in the presence of R1881 (Fig. 5.2C; and Jenster et al, 1994). The expression levels of wild type receptor (Fig. 5.5A) and mutant ARL (Fig. 5.5B) also increased in the presence of the natural androgens dihydrotestosterone and testosterone. In contrast, binding of antagonists to both receptors, even those antagonists which showed agonist activity with ARL, did not affect receptor expression levels (Fig. 5.5A and B). This indicates that for all antiandrogens studied, the receptor expression levels were comparable and that variations in promoter interference between the various antiandrogen-bound receptors could not be explained by differences in receptor expression levels.

**Discussion**
An essential step in androgen action is the transformation of the androgen receptor from a complex with heat-shock proteins (Veldscholte *et al.*, 1992a), to a DNA binding state. DNA binding of full-length AR has been studied previously by *in vitro* electrophoretic mobility shift assays with oligonucleotides containing a consensus ARE. In these studies, performed with wild type AR produced in a variety of systems (*in vitro* transcription/translation, transiently transfected COS-7 cells, or recombinant baculovirus-infected Sf9 cells), DNA binding of the receptor was either hormone-independent (Kallio *et al.*, 1994; Xie *et al.*, 1992; Kuiper *et al.*, 1993) or required intracellular hormone exposure (Wong *et al.*, 1993; Kaspar *et al.*, 1993). Demonstration of hormone-dependent DNA binding in the electrophoretic mobility shift assay is complicated by the isolation procedure of the AR protein, which may cause artificial receptor activation. In addition, results of *in vitro* studies generally do not account for receptor/ARE interactions under equilibrium conditions. In the present study, we have used a promoter interference assay that permits study of androgen receptor interaction with DNA in whole cells.
The promoter interference assay was originally described by Hu and Davidson (1987), who used it to show that *lac* repressor, bound to its operator near a transcription initiation site, strongly repressed the activity of a reporter gene. This same principle, also used to study

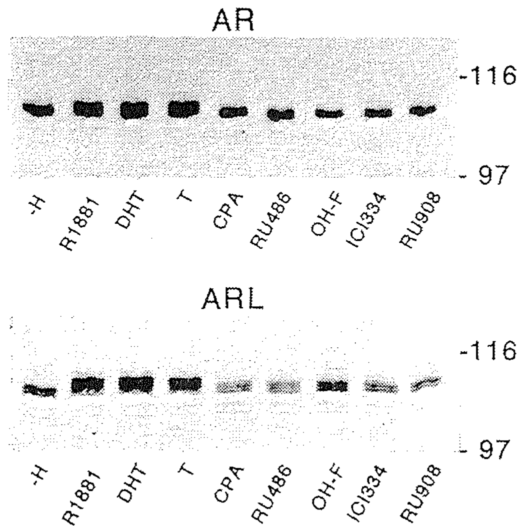



***Figure 5.5.*** *Effect of androgens and antiandrogens on expression levels of wild type AR and mutant ARL. Western blot analysis of lysates from CHO cells, transfected with either AR or ARL expression plasmid and incubated with the ligands as described in the legend of Fig. 5.4. Whole cell lysates were fractionated with gel electrophoresis, separated proteins transferred to nitrocellulose and visualised with a chemiluminescence detection method, using the AR-specific polyclonal antiserum SP197 as primary antibody. Molecular mass markers (kDa) were run on a parallel lane and their positions are indicated on the right of each blot.*

interaction of estrogen receptor with DNA (Reese and Katzenellenbogen, 1992), was applied by us to examine DNA binding of the AR in whole cells. In these studies, with a promoter interference CMV-$(ARE)_3$-LUC construct transfected into CHO cells, it was shown that reduced LUC activity resulted from inhibition of transcription due to androgen-dependent binding of the AR to its response elements. First, interference of CMV promoter activity was dependent on the presence of AREs in the reporter construct, second, on the expression of functional receptors, and, third, on the addition of androgens. Furthermore, we observed reduced LUC activity in transiently transfected CHO cells at AR concentrations that were functional within the cells: the amounts of AR expression plasmid needed to suppress LUC activity from the promoter interference plasmid were comparable to those necessary to activate transcription from a MMTV-LUC reporter plasmid. In addition, we could exclude that the decrease in LUC activity was due to squelching, a phenomenon which has been shown to occur at high expression levels of steroid hormone receptors (Meyer *et al.*, 1989).
The observation that the AR is unable, in the absence of ligand, to bind to AREs in the CMV-$(ARE)_3$-LUC reporter plasmid is in agreement with results from studies that showed that the unliganded forms of the progesterone (Truss *et al.*, 1994; Bagchi *et al.*, 1991), glucocorticoid (Dennis *et al.*, 1988), and androgen receptors (Wong *et al.*, 1993; Kaspar *et al.*, 1993) are unable to bind to their response elements on DNA. The androgen-dependent binding of the AR to DNA, as observed in the present study, supports a model in which binding of the

ligand to the receptor causes the dissociation of the heat-shock protein-receptor complex and exposes the major dimerization region of the receptor. This enables the liganded receptor to dimerize and bind with high affinity to its response element. These structural changes and related covalent modifications enable the bound receptor to function as a ligand-dependent transcription activator [reviewed by Tsai and O'Malley (1994)]. Results from other studies performed on progesterone (Mymryk and Archer, 1995) and estrogen receptors (Reese and Katzenellenbogen, 1992; Metzger *et al.*, 1995) do not support this model. These observations indicate that progesterone and estrogen receptors are capable of binding to DNA, within whole cells, in the absence of ligand and that the ligand is needed to enhance or stabilize the interaction of the receptor with response elements. We feel that the differences with respect to hormone dependency of DNA binding, between androgen and glucocorticoid receptors on the one hand and the estrogen receptor on the other, may warrant some caution of generalisation of steroid hormone receptor transformation from an inactive towards an active state.

Inability of androgen receptor antagonists to induce DNA binding of the receptor has been postulated as one of the molecular mechanisms of antiandrogen action (Veldscholte *et al.*, 1992a; Wong *et al.*, 1993). The full antagonists hydroxyflutamide, ICI 176.334 and RU 23908, which lack agonist activity (see Fig. 5.1; previously shown by Kuil *et al.*, 1995, Veldscholte *et al.*, 1990 and Kemppainen *et al.*, 1992), failed to induce DNA binding of the wild type AR in whole cells. The antagonistic activity of these compounds stems, therefore, from their inability to induce DNA binding of the receptor. Presumably, these compounds are unable to induce the necessary changes in the conformation of the AR to release the associated proteins (Kuil *et al.*, 1995). The antihormone-receptor complexes formed with cyproterone acetate and RU 38486 were capable of binding to DNA within the cell. RU 38486, which was transcriptionally almost inactive, reduced promoter activity in the promoter interference assay to the same extent as the partial agonist cyproterone acetate, indicating that DNA binding is a pre-requisite for trans-activation, but that DNA binding alone is not sufficient to ensure a transcriptionally active receptor. Androgen receptors, when associated with RU 38486, bind to their response elements in whole cells (see Fig. 4) and *in vitro* (Wong *et al.*, 1993). However, RU 38486 apparently induces an altered conformation of the C-terminus of the receptor as compared to the agonist-induced conformation, reflected by differences in proteolytic digestion pattern (Zeng *et al.*, 1994; Kuil *et al.*, 1995), migration on sucrose gradients (Kuil *et al.*, 1995), and by electrophoretic mobility shift assays (Wong *et al.*, 1990). Similar observations were made in studies with the RU 38486-bound progesterone receptor (Allan *et al.*, 1992a; Vegeto *et al.*, 1992; Weigel *et al.*, 199; Delabre *et al.*, 1993; Mymryk and Archer, 1995; Meyer *et al.*, 1990; DeMarzo *et al.*, 1992). Therefore, failure for the antagonist RU 38486 to transactivate is due to structural alterations in the ligand binding domain.

The progesterone and estrogen receptor antagonists have been tentatively divided into two classes depending on their level of action (Klein-Hitpass *et al.*, 1993; Green, 1990). The so-called "type I antagonists" interfere with binding of the receptor to DNA. The other class of antagonists [type II; including e.g. RU 38486 (Klein-Hitpass *et al.*, 1993)] induce stable, high affinity DNA binding of the receptor but block the interaction of the receptor with the transcription initiation complex. Recently, McDonnell *et al.* (1995) could distinguish the estrogen receptor agonists from partial agonists on the bases of molecular criteria. Additionally, a classification of the known estrogen receptor antagonists into three distinct

classes was proposed. These authors hypothesized that the estrogen receptor might exist in the cell in multiple conformations that represent the inactive state, the active state and several intermediate states. Antagonists exert their action by stabilizing a specific structure. These distinct conformations could result as a consequence of the ability of these compounds to keep the receptor in a specific conformation by blocking a progressive change from inactive to active. The effects of androgen receptor agonists and antagonists in our analysis suggests that the antiandrogens also promote stabilization of several different conformations. As a consequence the known AR antagonists can be classified into three distinct categories. When we adopt the convention established by Klein-Hitpass *et al.* (1993), the compounds hydroxyflutamide, ICI 176.334 and RU 23908 are type I antagonists, blocking the process that leads to DNA binding (see Table 5.1). We propose to add a further classification of the type II antiandrogens in two subtypes: the C-terminal end of the receptor is either involved (type IIa) or not involved (type IIb) in the mechanism of antagonist action. According to this classification, RU 38486 is a type IIa antagonist that induces an incorrect conformational change at the C-terminus of the ligand binding domain of the AR (Zeng *et al.*, 1994; Kuil *et al.*, 1995). Cyproterone acetate represents a type IIb antagonist which stabilizes the AR in a conformation that allows it to exhibit some transcriptional activity. Protease sensitivity studies did not reveal an abnormal conformation for the C-terminal part of the ligand binding domain after binding of this ligand (Kuil *et al.*, 1995).

***Table 5.1.*** *Summary of the effects of ligands on the wild type and mutated receptors.*

| | AR | | ARL | |
|---|---|---|---|---|
| | DNA binding | trans-activation | DNA binding | trans-activation |
| Androgen | ++ | ++ | ++ | ++ |
| CPA | + | + | ++ | ++ |
| RU486 | + | - | + | - |
| OH-F | - | - | ++ | ++ |
| ICI334 | - | - | - | - |
| RU908 | - | - | ++ | + |

-: no effect; +: limited effect; ++: full effect
CPA: cyproterone acetate; RU486: RU 38486; OH-F: hydroxyflutamide; ICI334: ICI 176.334; RU908: RU 23908

For the mutant receptor ARL some antiandrogens should be classified differently (see Table 5.1). RU 23908 induced DNA binding of ARL without the exhibition of full agonistic activity. As the C-terminus of the ligand binding domain is not involved in its antagonistic behavior (Kuil *et al.*, 1995), this compound represents a type IIb antagonist for ARL.

Hydroxyflutamide and cyproterone acetate are full agonists for ARL. It could be speculated that the single amino acid change in the mutant ARL contributed to ligand-induced changes in ARL conformation which potentiated transactivation (with cyproterone acetate), DNA binding (with RU 23908), or both (with hydroxyflutamide).
In conclusion: transformation of the wild type AR to a DNA binding state in whole cells is blocked by several antiandrogens (hydroxyflutamide, ICI 176.334 and RU 23908). Although DNA binding is a necessary step to accomplish transcriptional activity, studies with another antiandrogen (RU 38486) show that DNA binding alone is not sufficient: full transcriptional activity requires additional changes, presumably in the protein structure of the receptor. The classification of androgen receptor antagonists into several types, according to the site of action as discussed above, might be useful for the development of new compounds for clinical use.

**Acknowledgements**
The authors thank Drs J.A. Grootegoed and L. Blok for helpfull discussions and critical reading of the manuscript.

# FUNCTIONAL IN VIVO INTERACTION BETWEEN THE AMINO-TERMINAL, TRANSACTIVATING DOMAIN AND THE LIGAND BINDING DOMAIN OF THE ANDROGEN RECEPTOR

P. Doesburg[1,3], C.W. Kuil[2,3], C.A. Berrevoets[2], K. Steketee[1], E. Mulder[2], A.O. Brinkmann[2], and J. Trapman[1]

Departments of Pathology[1], and Endocrinology & Reproduction[2]

accepted for publication in Biochemistry

[3]P. Doesburg and C.W. Kuil contributed equally to this study

## Chapter 6


**Summary**

The ligand binding domain (LBD) and the amino-terminal, transactivation domain (TAD) of the androgen receptor (AR) were separately linked to the GAL4 DNA binding domain (DBD), and to the GAL4(TAD). Resulting constructs were tested in the yeast two-hybrid system for protein-protein interactions. In the presence of androgen [methyltrienolone (R1881) or dihydrotestosterone (DHT)] a transcriptionally active complex was formed, reflecting an association between the AR(LBD) and the AR(TAD). No interactions were found in the presence of low affinity ligands like estradiol (E2), promegestone (R5020) or progesterone (Pg). Use of the Thr-868-Ala mutated AR(LBD) in the assay resulted not only in a clear AR TAD-LBD interaction in the presence of R1881 and DHT, but also in the presence of E2, Pg and R5020, corresponding to the alteration in ligand specificity induced by the mutation. Co-expression of the fusion protein Gal4(DBD)AR(LBD) and the separate AR(TAD) also gave rise to the formation of a transcriptionally active complex. No interactions were found between two AR LBDs at low expression level of the two components. However, LBD-LBD interaction was detectable by application of a high expression vector for GAL4(TAD)AR(LBD), albeit at high ligand concentrations. To substantiate the observation of the AR LBD-TAD interaction, CHO cells were co-transfected with expression plasmids for a truncated AR, which lacks the TAD [AR(DBD)(LBD)], and the separate AR(TAD). This resulted in stimulation of a MMTV-LUC reporter gene in the presence of R1881, but not in the absence of hormone. This finding indicates that, like in the yeast system, in mammalian cells, TAD-LBD interactions are of importance for AR activation. In the mammalian system, a maximal AR TAD-LBD interaction was obtained at approx. 10-fold higher ligand concentrations than required for full-length AR activation. In the presence of low affinity ligands, the AR TAD-LBD interaction as measured by transcriptional activation was considerably weaker than the activity of the full-length AR. From the present results a concept of hormone dependent AR activation is proposed, which requires a functional, direct or indirect intramolecular interaction between the TAD and the LBD.


**Introduction**

The androgen receptor (AR) is a member of the steroid receptor superfamily of ligand dependent transcription factors (Tsai & O'Malley, 1994; Beato *et al.*, 1995; Mangelsdorf *et al.*, 1995). It mediates androgen induced physiological responses, which are essential for the development and maintenance of the male phenotype (Mooradian *et al.*, 1987). Consistent with the conserved structural and functional organization of the steroid receptor family members, several, apparently separate, functional domains have been characterized: an amino-terminal transactivation domain (TAD), a highly conserved DNA binding domain (DBD) and a moderately well conserved carboxyl-terminal, ligand binding domain (LBD) (Jenster *et al.*, 1991; Simental *et al.*, 1991).

Upon androgen binding, the AR can interact with its cognate androgen response elements (AREs), which are commonly located in the promoter regions of androgen target genes. Presumably, this AR(ligand)-ARE complex stabilizes the transcription preinitiation complex, in this way augmenting gene transcription by RNA polymerase II. Alternatively, it tethers an RNA polymerase II holoenzyme complex to the promoter (Tjian & Maniatis, 1994; Koleske & Young, 1995).

Steroid receptor binding sites commonly exhibit a palindromic structure, to which receptor homodimers can bind. One of the dimerization interphases is found in the D(distal)-box of the

DNA binding domain (DBD), as proven for glucocorticoid receptor (GR) and estrogen receptor (ER) homodimers by crystalographic analyses (Luisi *et al.*, 1991; Schwabe *et al.*, 1993). Because of the high level of DBD sequence conservation, an important function of the D-box in dimerization seems applicable to all steroid hormone receptors.

Analysis of ER dimer formation as demonstrated in gel retardation experiments with two receptor size variants, as well as the dissection of the nuclear uptake mechanism of the progesterone receptor (PR) indicated a ligand inducible dimerization interphase present within the LBD (Kumar & Chambon, 1988; Guiochon-Mantel *et al.*, 1989). These findings coincided with the identification of a twenty-two amino acid region within the ER LBD, required for receptor homodimer formation and high affinity DNA binding (Fawell *et al.*, 1990; Lees *et al.*, 1990). Sequence alignment among the steroid receptor family members revealed this region to be associated with a conserved heptad repeat of hydrophobic residues (Wurtz *et al.*, 1996).

Little is known about direct or indirect, intra- or intermolecular interactions between steroid receptor domains with different functions. Recently, we defined in transient transfection assays the presence of two separate TADs within the amino-terminal region of the AR (Jenster *et al.*, 1995). One of these TADs (TAU-1, amino acid residues 100 to 370) was found to be active in the presence of the ligand bound LBD, whereas in a truncated AR, which lacks the LBD, the region 360-485 (TAU-5) functions as a constitutively active transactivator of comparable strength. These observations suggested to us the occurrence of interactions between the TAD region and the LBD of the AR. This hypothesis was in accordance with androgen dissociation kinetics and *in vitro* AR degradation rates, which indicates that the AR amino-terminal region stabilizes the receptor by slowing the rate of ligand dissociation and AR degradation (Zhou *et al.*, 1995).

In this study we present an *in vivo* approach based upon yeast and mammalian protein-protein interaction assays, in which we investigated the occurrence of a functional interaction between the LBD and TAD of the human AR.

**Materials and methods**

*Materials*: Methyltrienolone (R1881) and promegestone (R5020) were purchased from NEN (Boston, MA). Cyproterone acetate was a gift from Schering (Berlin, Germany), hydroxyflutamide from Schering USA (Bloomfield, NJ) and ICI 176.334 (bicalutamide) from Zeneca Ltd. (Macclesfield, UK). All other steroids were purchased from Steraloids (Wilton, NH).

*Construction of plasmids*: Plasmids were constructed according to standard methods, and where denoted, blunt ended with Klenow DNA polymerase (Sambrook *et al.*, 1989). All GAL4 fusion constructs, and constructs including a PCR amplification step for preparation, were sequenced to verify the correct reading frame and the absence of random mutations.

The glyceraldehyde-3-phosphate dehydrogenase promoter-based yeast expression vector pG1 (Schena *et al.*, 1991) was kindly provided by Dr. Picard. We constructed the pG1 derivative pG1AR-I, containing the complete human AR coding region, by subcloning the 3.0 kb *Sal*I fragment from the mammalian hAR cDNA expression vector pSVAR0 (Brinkmann *et al.*, 1989) into the homologous pG1 site. A low expression level, corresponding to a low transactivating activity of pG1AR-I prompted us to subject the 5'-untranslated region to PCR-mediated mutagenesis (Higuchi *et al.*, 1988). Amplification was performed utilizing the forward primer 5'-CGGGATCCAAAAATGGAAGTGCAGTTAGGGCTGGGAAGGGTC-3'

(AR cDNA sequences are underlined), including an internal *Bam*HI site (double underlined), and the internal AR cDNA reverse primer 5'-GGAGCAGCTGCTTAAGCCGGGG-3' (*Afl*II site double underlined). The amplified, *Bam*HI-*Afl*II digested product was subsequently exchanged with the corresponding pG1AR-I fragment, yielding plasmid pG1AR-II, which now contained a consensus yeast translation initiation region preceded by a 30 bp leader sequence. pG1AR-L, containing the T868A-substitution, was constructed by transferring the 1.3 kb *Kpn*I-*Pst*I fragment of the AR(T868A) cDNA expression vector pSVAR-L (Veldscholte *et al.*, 1990) to the homologous sites in pBluescript II SK$^+$ (Stratagene, La Jolla, CA). The fragment was reexcised with *Kpn*I and *Sma*I, and exchanged with the corresponding *Kpn*I-*Sal*I (blunt ended) pG1AR-II fragment.

The expression vector pG1AR-5, encoding the constitutively active AR(TAD)(DBD), was constructed by exchanging the *Rsr*II-*Xba*I (blunt ended) fragment from the previously described pSVAR-5 (Jenster *et al.*, 1995) with the 1.7 kb *Rsr*II-*Sal*I (blunt ended) pG1AR-II fragment.

The androgen-inducible yeast expression vector pUCΔSS-26X containing a triple arranged 26 bp GRE(ARE) oligonucleotide, derived from the rat tyrosine aminotransferase gene, fused upstream to the yeast *CYC1* promoter region was provided by Dr. Picard (Schena *et al.*, 1991).

The GAL4($DBD_{1-147}$) two-hybrid cloning vector pGBT9 and the parental GAL4($TAD_{768-881}$) cloning vectors pGAD424, or the high level expression derivative pACT2 (all from Clontech, Palo Alto, CA), were used to generate GAL4-fusion protein constructs. The GAL4-AR($TAD_{3-494}$) fusion constructs were prepared by integration of the blunt ended 1.5 kb *Mlu*I-*Acc*651 fragment of the previously described pSVAR-3 (Jenster *et al.*, 1995) into the *Sma*I-site of pTZ19 (Pharmacia, Uppsala, Sweden). An additional *Bam*HI-site was introduced into the resulting vector pTZ19NAR by adding a *Bam*HI linker to the blunt ended *Eco*RI site in the polylinker. The AR(TAD) fragment was excised with *Bam*HI and cloned in frame into the corresponding sites in pGBT9 and pGAD424, yielding pGAL4(DBD)AR(TAD) and pGAL4(TAD)AR(TAD), respectively. This procedure resulted in the insertion of eight amino acid residues, intermediate to the chimeric substituents (PEFPGIPR and IEFPGIPR, respectively). GAL4-AR($LBD_{652-910}$) fusion constructs were generated by integration of the blunt ended 0.9 kb *Tth*III-*Sma*I fragment from pSVAR0 into the *Sma*I site of pTZ19. The AR(LBD) fragment was excised from the resulting plasmid pTZ19CAR, with *Bam*HI and cloned in frame into the homologous sites in pGBT9, pGAD424 and pACT2, yielding the plasmids pGAL4(DBD)AR(LBD), pGAL4(TAD)AR(LBD) and pACT-GAL4(TAD)AR(LBD), respectively. Additional amino acids, intermediate to the chimer substituents are PEFPGIP, IEFPGIP and ICMAYPYDVPDYASLGGHMAMEAPGIP, respectively. The latter includes the HA epitope (YPYDVPDYA) and flanking sequences present in pACT2.

The derivatives with the T868A substitution in the AR(LBD) were constructed by exchanging the 0.45 kb *Eco*RI fragment from pTZ19CAR with the corresponding pSVAR-L fragment. The fragment was reexcised and subcloned in frame into pGBT9 and pGAD424 via *Bam*HI and *Pst*I compatible ends, yielding the plasmids pGAL4(DBD)AR(LBD-L) and pGAL4(TAD)AR(LBD-L), respectively. pACT-GAL4(TAD)AR(LBD-L) was constructed by exchanging the internal 0.5 kb *Eco*RI fragment in pACT-GAL4(TAD)AR(LBD) with the corresponding fragment in pSVAR-L. Consequently, all GAL4-AR(LBD-L) chimeric derivatives exhibit identical amino acid additions between the GAL4 and AR parts, as compared to the wild type GAL4-AR(LBD) chimeras.

pAR($TAD_{3\text{-}494}$) was generated by exchanging the *Acc*651(blunt ended)-*Eco*RI fragment of pGAD424 with the 1.5 kb *Bam*HI(blunt ended)-*Eco*RI fragment of pTZ19NAR. The resulting plasmid pAR(TAD) now contained the AR(TAD) fragment cloned in frame to the SV40 large T antigen nuclear localization signal in pGAD424.
The mammalian expression plasmid pSVAR($TAD_{1\text{-}494}$) was obtained by modification of pSVAR-12 (Jenster *et al.*, 1993): the 1.3 Kb *Xba*I-*Eco*RI fragment, encoding the AR DBD and LBD, was deleted, and the remaining vector, containing the AR TAD was religated by a *Xba*I-*Eco*RI linker. For the expression plasmid pSVAR(DBD)(LBD-L), a 0.5 kb *Eco*RI-*Eco*RI fragment from pSVAR-L was exchanged with the same fragment in the previously described plasmid pSVAR(DBD)(LBD) (pSVAR-104; Jenster *et al.*, 1995).
*Yeast strains and growth*: AR transactivation activity was measured in the multiple protease deficient *Saccharomyces cerevisiae* strain BJ2168 (*MATa, pep4-3, pcr1-407, prb-1122, ura3-52, trp1, leu2*) (kindly provided by Dr. Picard). Two-hybrid protein-protein analysis was performed in strain Y190 (*MATa, ura3-52, his3-Δ200, ade2-101, trp1-901, leu2-3, leu2-112, GAL4Δ, GAL80Δ, URA3::GAL-lacZ, cyh$^r$, LYS2::GAL-HIS3*), which was purchased from Clontech. Yeast cells were grown either in standard YEPD medium (1% w/v yeast extract, 2% w/v peptone and 2% w/v dextrose, pH 5.8) or the appropriate selective minimal medium (0.67% w/v yeast nitrogen base without amino acids, 2% w/v dextrose, pH 5.8) supplemented to the nutritional requirements of the yeast transformants. All yeast transformations were carried out according to the lithium acetate method (Gietz *et al.*, 1992).
*ß-Galactosidase assay*: ß-galactosidase activity, indicative of AR transactivation activity was assessed by co-transfecting *S. cerevisiae* BJ2168 with the appropriate AR expression vector and the androgen inducible yeast reporter vector pUCΔSS-26X. ß-galactosidase activity of the GAL4-chimers expressed in strain Y190 involves the activation of an integrated $UAS_{GAL1}$-lacZ reporter gene, and depends on the *in vivo* functional reconstitution of GAL4 due to interactions between AR fragments fused to separate GAL4 TAD and DBD domains.
A saturated culture of yeast transformants of either strain, propagated in 1-5 ml of the appropriate selective medium, was diluted to an $A_{600}$ of 0.2 in the same selective medium supplemented with the respective AR ligands. Subsequently, the yeast cells were incubated to midlog phase ($A_{600}$ approximately 1.0; 6-8 h). To 1.0 ml Y190 culture (spun down and resuspended to a final volume of 0.1 ml in minimal medium), or 0.1 ml BJ2168 culture, 0.7 ml Z-buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 0.27% v/v ß-mercaptoethanol, pH 7.0) was added. The cells were permeabilized by adding 50 µl $CHCl_3$ and 50 µl 0.1% SDS, and vortexed for 30 sec. The reactions were started with the addition of 0.16 ml prewarmed ß-galactosidase substrate o-nitrophenyl-ß-galactoside (0.4% w/v in 0.1 M phosphate buffer, pH 7.0) at 30°C. The reactions were quenched after development of the yellow reaction product (1 h or less), by adding 0.4 ml 1 M $Na_2CO_3$. Absorbance at 420 nm ($A_{420}$) was read, and ß-galactosidase activity was calculated using the following equation:

$$\beta\text{-}galactosidase(U) = \frac{1000 * A_{420}}{t * V * A_{600}}$$

t = reaction time (min); V = volume of yeast culture added to Z-buffer (ml)
*Immunoblot analysis of proteins expressed in yeast*: AR and GAL4-AR chimer expression was assessed by immunoblot analysis of yeast extracts. For this, 50 ml YEPD medium was

inoculated with 5 ml of a saturated culture of yeast transformants, propagated in the appropriate selective medium containing 2% dextrose, and grown until an $A_{600}$ value of 0.4-0.6. The cultures were poured into 100 ml centrifuge tubes, which were halfway filled with ice, and harvested by centrifugation (1000xg) for 5 min at 4°C. The pellet was successively washed in 50 ml ice-cold $H_2O$, collected by centrifugation, and resuspended into 100 µl ice-cold S-buffer [20 mM Tris-HCl (pH 8.0), 50 mM ammonium acetate, 2 mM EDTA, 0.5 mM bacitracin, 0.5 mM leupeptin, 2 mM PMSF] per 7.5 $A_{600}$ units of cells. The cell suspension was transferred to a 1.5 ml microcentrifuge tube containing 100 µl glass beads [acid washed glass beads; 425-600 microns, (Sigma, St. Louis, MI)] and 100 µl ice-cold 20% TCA per 7.5 $A_{600}$ units of cells. The cells were disrupted by vigorous vortexing for 10 min at 4°C, after which the supernatant was withdrawn. The second aliquot was obtained after washing the glass beads with 500 µl of an ice-cold, 1:1 mixture of 20% TCA and S-buffer. The combined samples were pelleted by centrifugation at 14,000 rpm for 10 min at 4°C, and resuspended into 10 µl loading buffer [120 mM Tris-HCl (pH 6.8), 8 mM EDTA, 3.5% (w/v) SDS, 14% (v/v) glycerol, 1 mM PMSF and 5% (v/v) ß-mercaptoethanol] per $A_{600}$ units of cells. The samples were successively boiled for 10 min, centrifuged at 14,000 rpm for 10 min, after which 25 µl of the supernatant was used for SDS-PAGE and Western blotting. The blot was blocked with 5% non-fat dry milk and incubated with either the polyclonal antiserum SP197 (recognizing AR amino acid residues 1-20; Kuiper *et al.*, 1993), a monoclonal antibody against the GAL4 TAD (Clontech, Palo Alto, CA) or a monoclonal antibody against GAL4 DBD (Santa Cruz Biotechnology, Santa Cruz, Ca). Proteins were visualised by chemiluminescence using the "Renaissance" Western blotting kit (DuPont/NEN, Boston, MA)

*CHO cell culture, transfection and LUC assay*: Chinese Hamster Ovary (CHO) cells were maintained in Dulbecco's Modified Eagle's DME/F12 culture medium, supplemented with 5% dextran coated charcoal-treated fetal calf serum. For transfection experiments, CHO cells were plated at a density of $1.5x10^5$ cells/well (10 $cm^2$) and grown for 24 h. Transfections were performed according to the calcium phosphate coprecipitation method as described previously (Veldscholte *et al.*, 1992 a,b). Cells were transfected with AR expression plasmids pSVAR0 (25 ng), pSVAR-L (25 ng), pSVAR(TAD) (1 µg), pSVAR(DBD)(LBD) (60 ng), or pSVAR(DBD)(LBD-L) (60 ng), or a combination of these constructs, and 500 ng of the MMTV-LUC reporter plasmid (kindly provided by Dr. Dijkema, Organon, Oss, Netherlands). Carrier DNA (pTZ19) was added in each case to a total of 5 µg per well. After transfection, the cells were washed and AR ligands were added. Following 24 h incubation, cells were harvested for the luciferase (LUC) assay, as described previously (Kuil *et al.*, 1995).

*Immunoaffinity purification and immunoblot analysis of proteins expressed in CHO cells*: For analysis of protein products of the various AR expression plasmids, CHO cells were plated at $1.5\times10^6$ cells/175 $cm^2$ and transiently transfected, using the calcium phosphate precipitation method, with pSVAR0 (20 µg), pSVAR(TAD) (20 µg), pSVAR(DBD)(LBD) (80 µg) or a combination of pSVAR(TAD) and pSVAR(DBD)(LBD) (20 and 80 µg, respectively). After 48 h cytosol was prepared. For this, cells were washed once in icecold PBS, and harvested by scraping in 1 ml of icecold buffer A [10 mM sodium phosphate, 1.2 mM EDTA, 12 mM 1α-thioglycerol, 10 mM DTT, 10% (v/v) glycerol, 10 mM $Na_2MoO_4$, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin (pH 7.4)]. The cells were lysed by 3 cycles of freeze-thawing (freezing in liquid nitrogen, thawing at 10°C) and centrifuged for 10 min at 400,000xg. The supernatant was used for immunoprecipitation with either the monoclonal antibody F39.4.1 (directed against AR amino acid residues 301-320; Zegers *et al.*, 1991) or

F52.24.4 (directed against amino acid residues 593-612) as described previously (Veldscholte *et al.*, 1992a,b). For the immunoprecipitation of AR(DBD)(LBD) with F52.24.4, 300 μl cytosol was used, and NaCl was added to a final concentration of 0.5 M. Immunoprecipitation of the full-length AR and AR(TAD) were done with 300 μl and 100 μl cytosol samples, respectively. Subsequent to SDS-PAGE and Western blotting, the membrane was blocked with 5% non-fat dry milk and incubated with the polyclonal antisera SP197, or SP066 (directed against AR amino acid residues 892-910) (Zegers *et al.*, 1991). The proteins were visualized by the chemiluminescence method.

## Results

*Expression and transactivating activity of the wild type and mutated androgen receptors in S. cerevisiae*

To evaluate the transactivating properties of the wild type AR in yeast, the complete protein coding sequence was inserted into the yeast expression vector pG1, yielding the expression plasmid pG1AR-II (see Materials and Methods). Similar expression vectors were made for AR-L (pG1AR-L) and AR(TAD)(DBD) (pG1AR-5). AR-L contains the T868A substitution in the AR(LBD), which alters the ligand specificity of the receptor, as previously shown in ligand binding and transactivation experiments in mammalian cells (Veldscholte *et al.*, 1990). AR(TAD)(DBD) lacks the LBD and is constitutively active in mammalian cells. In mammalian cells, full-length AR and AR(TAD)(DBD) use different, largely separated TADs (TAU-1 and TAU-5, respectively), both located within the amino-terminal domain (Jenster *et al.*, 1995).

Protein expression was assessed by immunoblot analysis of extracts of strain BJ2168, transiently transfected with either pG1AR-II, pG1AR-L or pG1AR-5, using the AR specific polyclonal antibody SP197. The antibody detected immunoreactive proteins of the appropriate lengths [110 kDa for full-length AR and AR-L, and 80 kDa for AR(TAD)(DBD)] (Fig. 6.1A). The expression level of AR(TAD)(DBD) was slightly lower than that of the full-length proteins.

Next, the transactivating activities of the three different proteins were determined. As depicted in Fig. 6.1B, in the absence of ligand, neither wild type AR nor AR-L were active. In contrast, AR(TAD)(DBD) was very efficient in activation of the reporter gene. Addition of dihydrotestosterone (DHT; $10^{-6}$M), however, activated both full-length AR and AR-L. The level of activation was approximately 3-fold lower than AR(TAD)(DBD) activity, which, as expected, was essentially not affected by the addition of DHT to the culture medium. Our data indicated that both TAU-1 and TAU-5 were active in yeast, albeit that TAU-5 activity was more prominent under the conditions tested.

In a subsequent series of experiments, dose-response curves to different ligands were determined for full-length AR and AR-L. For most ligands, in the yeast system an approximately 10-fold higher concentration was required than in mammalian cells to obtain maximal activity (Fig. 6.1C). This might be due to limited permeability of the different ligands in yeast, or due to differences in active transport mechanisms (Kralli *et al.*, 1995). Progesterone (Pg) seemed relatively more active in yeast than the other steroids tested. The activation of full-length AR and AR-L by the androgenic compounds R1881 and DHT was comparable (see Fig. 6.1C). Approximately 100-fold more ligand was required for strong activation of the full-length, wild type AR by the progestins Pg and R5020 as compared to

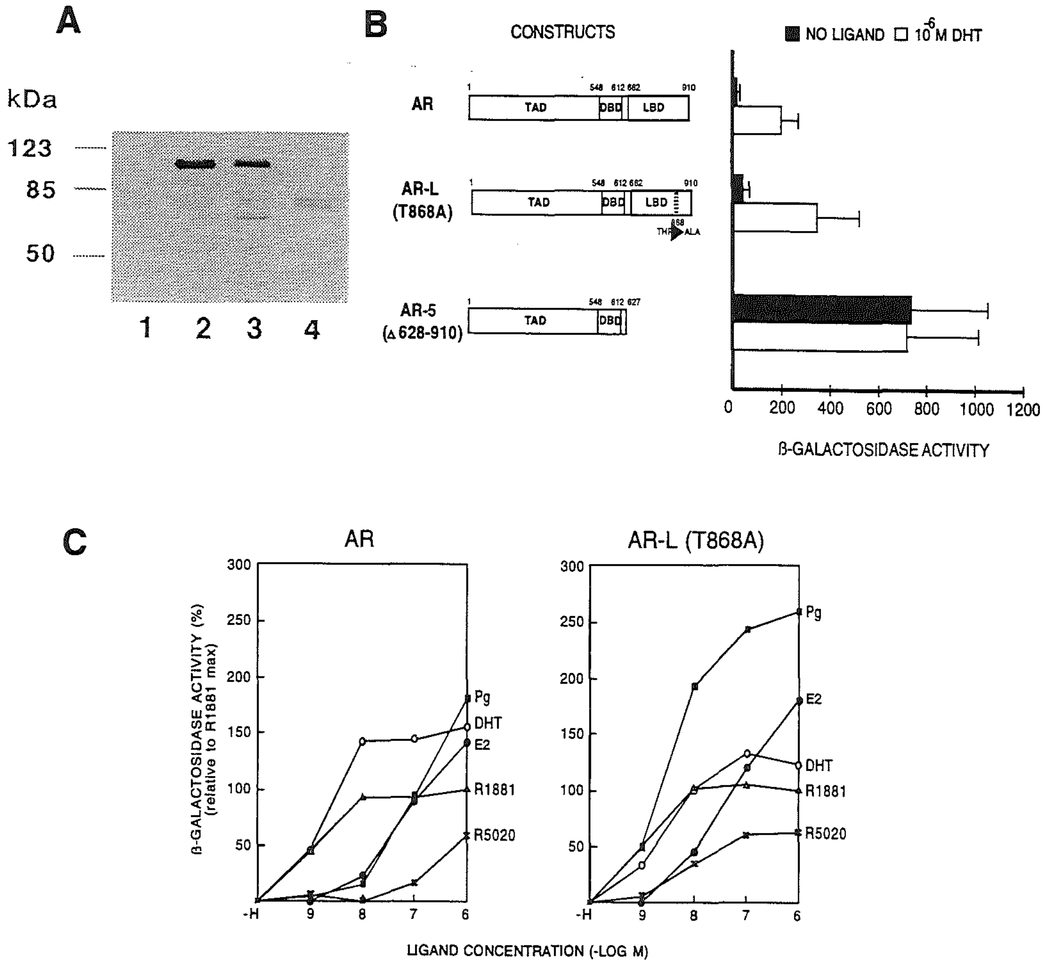



*Figure 6.1. Protein expression and transcriptional activity of wild type and mutant ARs in S. cerevisiae BJ2168. A. Expression levels of wild type AR (Lane 2), the mutant AR-L (Lane 3), and the mutant AR-5 (Δ627-910) (Lane 4) were tested in cell free extracts of yeast, transformed with the respective plasmids, by immunoblotting with the polyclonal antiserum SP197 (against amino acid residues 1-20 of the AR) as described in Materials and Methods. Lane 1 represents the mock transformed yeast. Molecular mass standards were run on a parallel lane (kDa). B. ß-Galactosidase activity was determined in yeast transformed with the indicated AR expression plasmid and the androgen-inducible reporter plasmid UCΔSS-26X, and incubated without hormone or in the presence of DHT ($10^{-6}$ M). ß-Galactosidase values represent the mean (± SEM) of three separate determinations. C. Dose-response curves of activation of the wild type AR and mutant AR-L in the presence of R1881, DHT, E2, Pg, or R5020. ß-Galactosidase activity in the presence of $10^{-6}$ M R1881 was set at 100%. Values represent the mean of three separate determinations.*

activation of AR-L. For estradiol (E2)-dependent activation of wild type AR and AR-L the difference was small. So, although the yeast system was slightly less selective, in general these findings were in agreement with those previously found in HeLa cells transiently transfected with a wild type AR or AR-L expression vector (Veldscholte *et al.*, 1990), and those observed in CHO cells, as presented below. In conclusion, the yeast system was found to be suitable for the study of AR functioning.

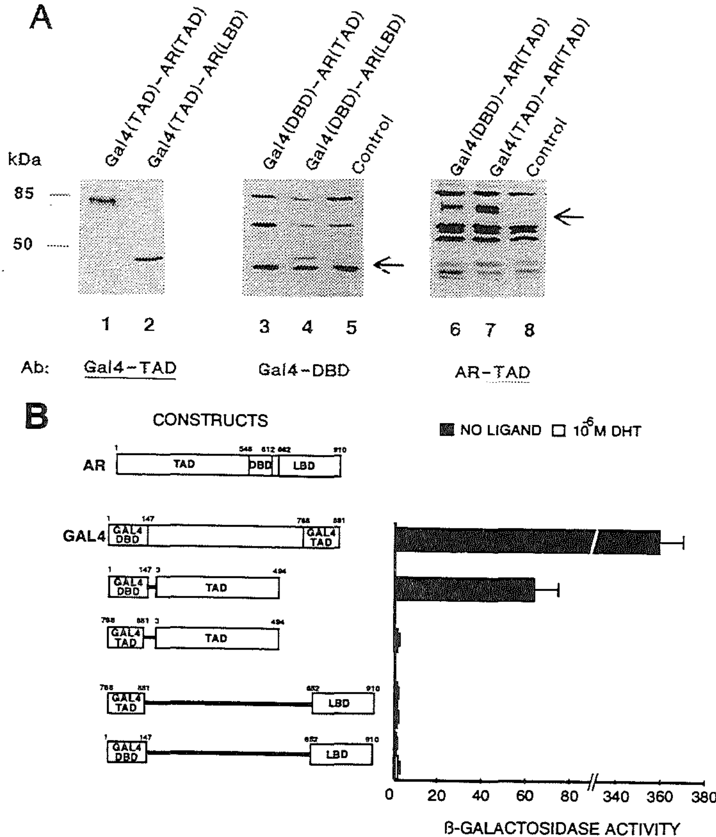



***Figure 6.2.*** *Protein expression and transcriptional activity of seperate GAL4 fusion proteins in S. cerevisiae Y190.* ***A.*** *Expression levels of the various GAL4 fusion proteins (indicated above the lanes) were determined in cell free extracts of yeast, transformed with the respective expression plasmids, by immunoblotting with antisera recognizing, respectively, the GAL4 transcription activation domain (TAD) (Lanes 1 and 2), the GAL4 DNA binding domain (DBD) (Lanes 3 to 5) or the AR-TAD (Lanes 6 to 8) as described in Materials and Methods. Lanes 5 and 8 represent mock transformed yeast. Arrows indicate specific GAL4 fusion protein bands. Molecular mass standards were run on a parallel lane (kDa).* ***B.*** *ß-Galactosidase activity as determined in yeast strain Y190, containing the integrated* $UAS_{GAL1}$*-lacZ reporter gene, and transformed with the expression plasmids for GAL4 or GAL4 fusion proteins. Yeast cells were incubated in the absence or presence of DHT* ($10^{-6}$ *M). ß-Galactosidase values represent the mean (± SEM) of three separate determinations.*

## Chapter 6

*Transactivating activity of GAL4-androgen receptor chimeric proteins in S. cerevisiae*

A yeast two-hybrid protein-protein interaction assay, as originally described by Fields & Song (1989), was used to study associations between the different functional AR domains. The two-hybrid system applied, involved the activation of an integrated $UAS_{GAL1}$-lacZ reporter gene. One set of constructs expressed chimeric AR(TAD) (amino acid residues 3-494) proteins, fused to the GAL4(DBD) or the GAL4(TAD). Similar vectors were constructed for the AR(LBD) (amino acid residues 652-910) linked to GAL4(DBD) or GAL4(TAD).

We first assayed whether all components were properly expressed in Y190 yeast cells. To this end, lysates of yeast cells were analyzed by Western blotting, using antibodies directed against the GAL4 part of the fusion proteins, and, in selected cases, against the AR parts. The results are shown in Fig. 6.2A. Immunoblotting with the antibody against the GAL4(TAD) showed a comparable expression level of the GAL4(TAD)AR(TAD) and GAL4(TAD)AR(LBD) chimeric proteins (lanes 1 and 2). Using the antibody against the GAL4(DBD), GAL4(DBD)AR(LBD) could be visualized (lane 4), but not GAL4(DBD)AR(TAD) (lane 3), indicating a lower expression level of the latter. However, GAL4(DBD)AR(TAD) was detectable with an antibody against the AR(TAD) (lane 6). The GAL4(DBD)AR(TAD) expression level was slightly lower than that of the GAL4(TAD)AR(TAD) chimeric protein (lane 7; see also lane 1).

Next, we investigated whether any of these chimeric proteins could activate GAL1 promoter driven LacZ expression, if expressed individually (see Fig. 6.2B). Apparently, no significant ß-galactosidase activity was observed in yeast carrying either one of the AR(LBD) fusion proteins in the absence or presence of DHT. Not surprisingly, expression of GAL4(DBD)AR(TAD) markedly increased reporter gene expression. The transactivating activity of the GAL4(DBD)AR(TAD) chimeric protein was found to be approximately 20% of wild type GAL4 activity, as measured under the same conditions. No significant ß-galactosidase activity was observed in yeast cells carrying GAL4(TAD)AR(TAD). This fusion protein lacks UAS binding potential, and consequently fails to localize to the GAL1-lacZ upstream promoter sequences.

*In vivo ligand dependent androgen receptor LBD-TAD interaction in S. cerevisiae*

We next assessed in the two-hybrid system the putative tethering of the GAL4(TAD)AR(TAD) chimer to the $UAS_{GAL1}$ bound GAL4(DBD)AR(LBD). In the absence of DHT, no significant ß-galactosidase activity was observed in lysates of Y190 cells, which coexpressed both fusion proteins (see Fig. 6.3). However, DHT ($10^{-6}$ M) markedly induced transcription of the LacZ reporter gene. Similar results were obtained, using a construct containing LBD-L(T868A) in the GAL4(DBD)AR(LBD) chimer (data not shown). Cotransfection of either one of the chimeric constructs with the basic GAL4 vectors, lacking AR-sequences, did not result in significant ß-galactosidase activity (data not shown). Taken together, these results indicate an *in vivo* androgen dependent association of the amino-terminal TAD and the LBD of the AR. Co-expression of GAL4(DBD)AR(LBD) and GAL4(TAD)AR(LBD) did not result in the formation of a functionally active complex under the conditions tested.

To evaluate the ligand specificity, and thus obtaining additional evidence for the physiological significance of the AR TAD-LBD interaction, the dose-response curves to a variety of ligands were determined (Fig. 6.4). Experiments were done with both wild type LBD and LBD from AR-L. Both R1881 and DHT were capable of inducing functional wild type and AR-L LBD-

TAD interactions with a similar affinity, although the absolute transcriptional activity induced by the AR-L interaction was slightly higher (data not shown). In the protein-protein interaction assay, identical ligand concentrations were required to obtain similar levels of reporter gene activity as compared to the full-length AR (or AR-L) (see also Fig. 6.1C).

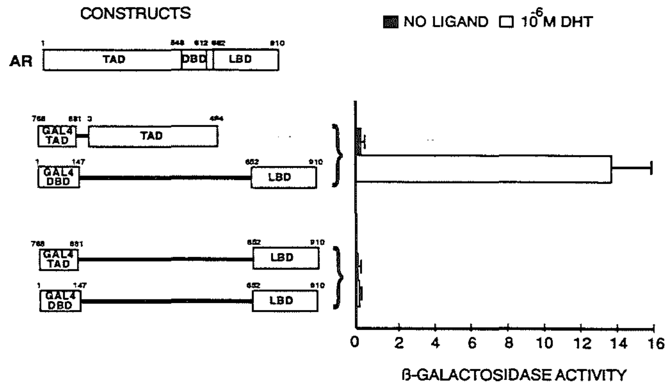



*Fig. 6.3. Transcriptional activity of co-expressed GAL4 fusion proteins in S. cerevisiae Y190. Yeast strain Y190, containing the integrated $UAS_{GAL1}$-lacZ reporter gene, was transformed with the expression plasmids encoding the indicated GAL4 fusion proteins. ß-Galactosidase activity was determined after incubation in the absence or presence of DHT ($10^{-6}$ M). ß-Galactosidase values represent the mean (± SEM) of three separate determinations.*

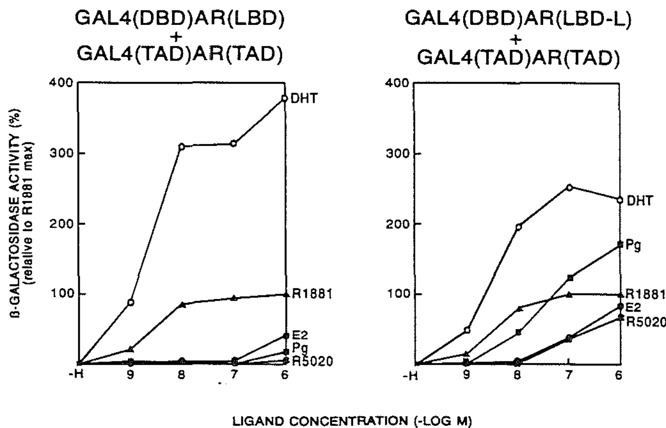



*Fig. 6.4. Effects of various ligands on transcriptional activity of co-expressed GAL4(TAD)AR(TAD) and GAL4(DBD)AR(LBD) or GAL4(DBD)AR(LBD-L) fusion proteins in S. cerevisiae Y190. The yeast strain, containing the integrated $UAS_{GAL1}$-lacZ reporter gene, was transformed with the expression plasmids, encoding the indicated GAL4 fusion proteins. ß-Galactosidase activity was determined upon incubation with increasing concentrations of either R1881, DHT, E2, Pg, or R5020. ß-Galactosidase activity in the presence of $10^{-6}$ M R1881 was set at 100%. Values represent the mean of three separate determinations.*

The progestins Pg and R5020 (almost completely) failed to induce the wild type TAD-LBD interaction, whereas a small induction of ß-galactosidase activity was observed in response to the highest E2 concentration tested ($10^{-6}$ M). However, E2, R5020 and, very efficiently, Pg readily induced the AR-L TAD-LBD interaction. Summarizing, these findings clearly point out the ligand dependency of the functional AR LBD-TAD interaction *in vivo*. Furthermore, the agonistic activity of E2, Pg and R5020 on induction of the AR-L LBD-TAD interaction substantiated the physiological importance of the observations. Interestingly, high concentrations of the ligands Pg, R5020 and E2 were not sufficient to establish wild type AR LBD-TAD interaction, although the full-length, wild type receptor could be activated by these ligands (compare Fig. 6.1C and 6.4). This apparent discrepancy might be contributed to differences in interaction kinetics between separate and linked protein domains and a low affinity ligand.
Because the GAL4(DBD)AR(TAD) fusion protein showed intrinsic transactivating properties (Fig. 6.2), we addressed the question whether AR(TAD) by itself was active in the two-hybrid system, if coexpressed with GAL4(DBD)AR(LBD). As illustrated in Fig. 6.5, AR(TAD) and GAL4(DBD)AR(LBD) were inactive when expressed separately. In contrast, in the coexpression system, a clear DHT induced ß-galactosidase activity could be observed. The activity was less than the GAL4(TAD)AR(TAD) fusion protein activity (approximately 50%) (Fig. 6.3). This indicates that both AR(TAD) and GAL4(TAD) might contribute to the activity in GAL4(TAD)AR(TAD).

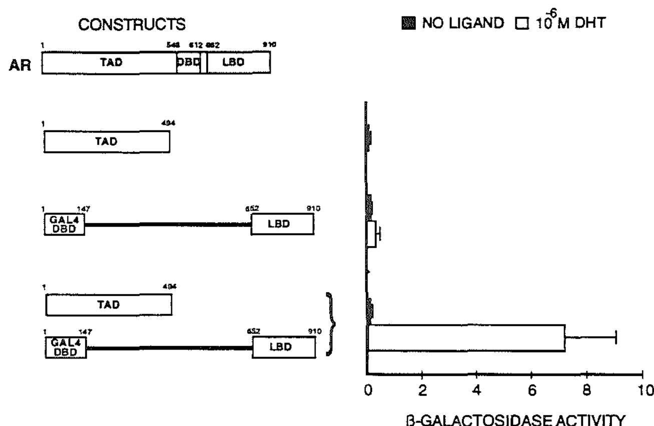



*Fig. 6.5. Transcriptional activity of co-expressed AR(TAD) and GAL4(DBD)AR(LBD) proteins in S. cerevisiae Y190. ß-Galactosidase activity was determined in yeast strain Y190, containing the integrated $UAS_{GAL1}$-lacZ reporter gene, and transformed with the expression plasmid(s) encoding the indicated proteins. ß-Galactosidase values were measured upon incubation in the absence or presence of DHT ($10^{-6}$ M) and represent the mean (± SEM) of three separate determinations.*

*In vivo ligand dependent androgen receptor LBD-LBD interaction in S. cerevisiae*
The high intrinsic transactivating activity of GAL4(DBD)AR(TAD) (see Fig. 6.2) excluded a reliable study of interactions between two AR TADs in the yeast two-hybrid system. This

high background problem could not be overcome by overexpression of GAL4(TAD)AR(TAD), because high concentrations of this protein turned out to be toxic to the yeast cells.

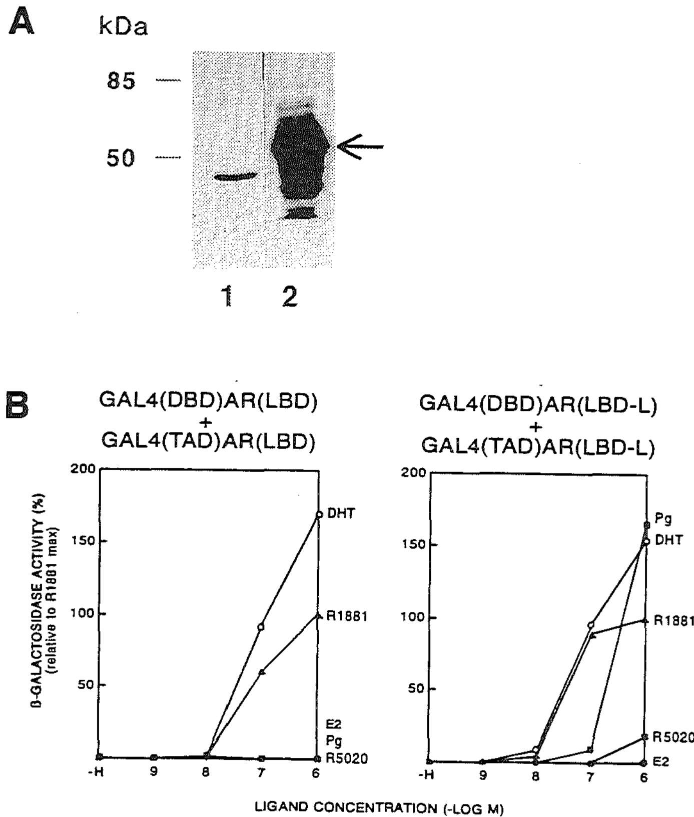



*Figure 6.6. GAL4(TAD)AR(LBD) protein expression levels and transcriptional activity of ligand-dependent LBD-LBD interaction in S. cerevisiae Y190.* ***A.*** *Expression levels of GAL4(TAD)AR(LBD) in cell-free extracts of yeast, either transformed with the low (Lane 1) or high (Lane 2) expression vector. Proteins were visualized by immunoblotting with the monoclonal antibody against GAL4 TAD as described in Materials and Methods. Molecular mass standards were run on a parallel lane (kDa).* ***B.*** *The yeast strain Y190, containing the integrated $UAS_{GAL1}$-lacZ reporter gene, were transformed with the indicated low-expression plasmids, encoding the GAL4(DBD) fusion proteins and the high-expression plasmids, encoding the GAL4(TAD) fusion proteins. β-Galactosidase activity was determined upon incubation with increasing concentrations of either R1881, DHT, E2, Pg, or R5020. β-Galactosidase activity in the presence of $10^{-6}$ M R1881 was set at 100%. Values represent the mean of three separate determinations.*

A similar approach was applied to assess a putative interaction between two AR LBDs. As illustrated in Fig. 6.3, such a functional association could not be observed with low expression

vectors. Evidently, putative LBD-dimers would be constituted of a mixture of transcriptionally inactive GAL4(DBD)AR(LBD) and GAL4(TAD)AR(LBD) homodimers, and transcriptionally active GAL4(DBD)AR(LBD) / GAL4(TAD)AR(LBD) heterodimers. We hypothesized that overexpressing the GAL4(TAD)AR(LBD) chimer would favour its tethering to the $UAS_{GAL1}$-bound GAL4(DBD)AR(LBD), thereby more efficiently competing with the formation of transcriptionally inactive GAL4(DBD)AR(LBD) homodimers at the promoter site. An AR(LBD) cDNA fragment was cloned in the high expression vector pACT2 (see Materials and Methods). Western blot analysis of the chimeric protein showed a 20- to 50-fold higher expression as compared to the originally applied vector (Figure 6A). The chimeric protein migrated slightly slower than the comparable protein expressed from pGAD424, due to the presence of the HA epitope and flanking amino acid residues in this construct (see Materials and Methods).
Y190 cells cotransfected with pGAL4(DBD)AR(LBD) and the high expression vector pACT2-GAL4(TAD)AR(LBD) showed no significant ß-galactosidase activity in the absence of DHT (see Fig. 6.6B). However, increasing concentrations of DHT or R1881 clearly induced LacZ transcription. Similar results were obtained with AR-L LBD constructs. However, compared to the AR TAD-LBD interaction (Fig. 6.3), AR(LBD) dimer formation required not only a much higher GAL4(TAD)AR(LBD) concentration, but also a higher ligand concentration for a maximal response. The absolute level of maximal ß-galactosidase activity measured for LBD-LBD interaction with the high expression vector for GAL4(TAD)AR(LBD) was comparable to the level measured for TAD-LBD interaction using a low expression GAL4(TAD)AR(TAD) vector (Fig. 6.3). For wild type AR(LBD), no interactions were found with the low affinity ligands E2, Pg and R5020. In contrast, Pg, was clearly able to stimulate the LBD-L/LBD-L interaction, reflecting the alteration of ligand specificity in LBD-L.

*In vivo ligand dependent AR TAD-LBD interaction in CHO cells*

The interaction between the AR TAD and LBD was also examined in a mammalian cell protein-protein interaction system. To this end, two expression vectors were constructed, encoding the AR(TAD) and the AR(DBD)(LBD), respectively (see Materials and Methods). Protein expression was assessed by immunoaffinity purification and Western blot analysis of cytosol of CHO cells, transiently transfected with expression plasmids encoding full-length AR, AR(TAD) and AR(DBD)(LBD), using AR specific antibodies. Fig. 6.7A shows the immunodetection of proteins of the appropriate length for the full-length AR, and the receptor fragments AR(TAD) and AR(DBD)(LBD) (110 kDa, 65 kDa and 48 kDa, respectively). Although the expression levels differed markedly, the various proteins could be clearly visualized. Expression levels of the receptor fragments AR(DBD)(LBD) and especially AR(TAD) were high, as compared to full-length AR expression (compare lanes 1, 2 and 4, and 5, 7 and 8, respectively).
To study protein-protein interactions, expression plasmids encoding AR(TAD) and AR(DBD)(LBD) were transiently transfected to CHO cells, together with the AR response reporter plasmid MMTV-LUC. LUC activity was measured in cells incubated in the presence and in the absence of $10^{-9}$ M R1881 (Fig. 6.7B). Separate expression of AR(TAD) or AR(DBD)(LBD) did not result in ligand induced LUC activity. However, coexpression of AR(TAD) and AR(DBD)(LBD) resulted in R1881 induced LUC activity to a similar level as found for the full-length AR (Fig. 6.7B). These results extend the observations made in the yeast system, and indicate that a direct or indirect interaction between the AR(TAD) and

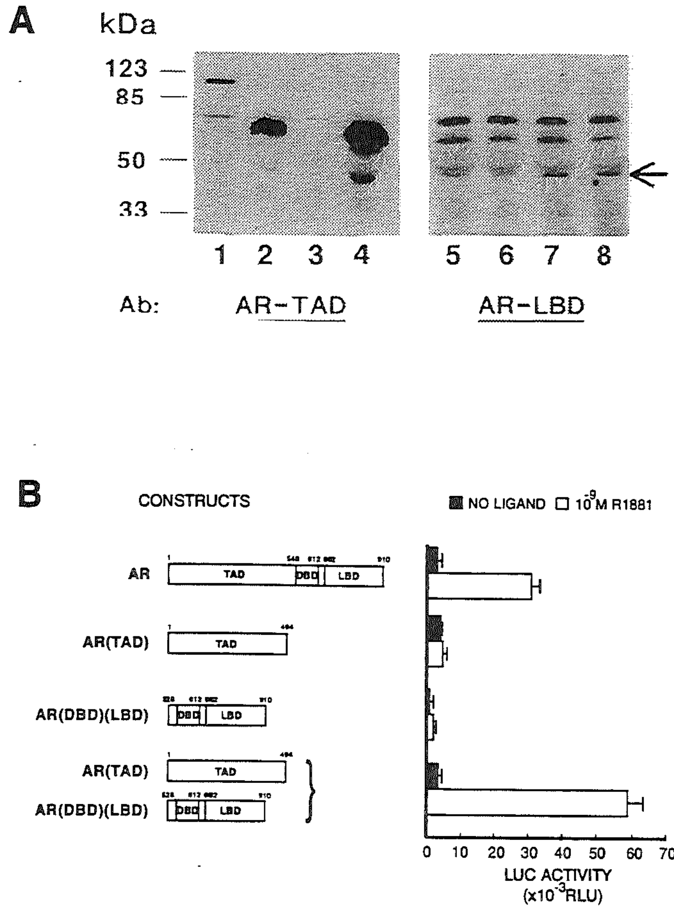
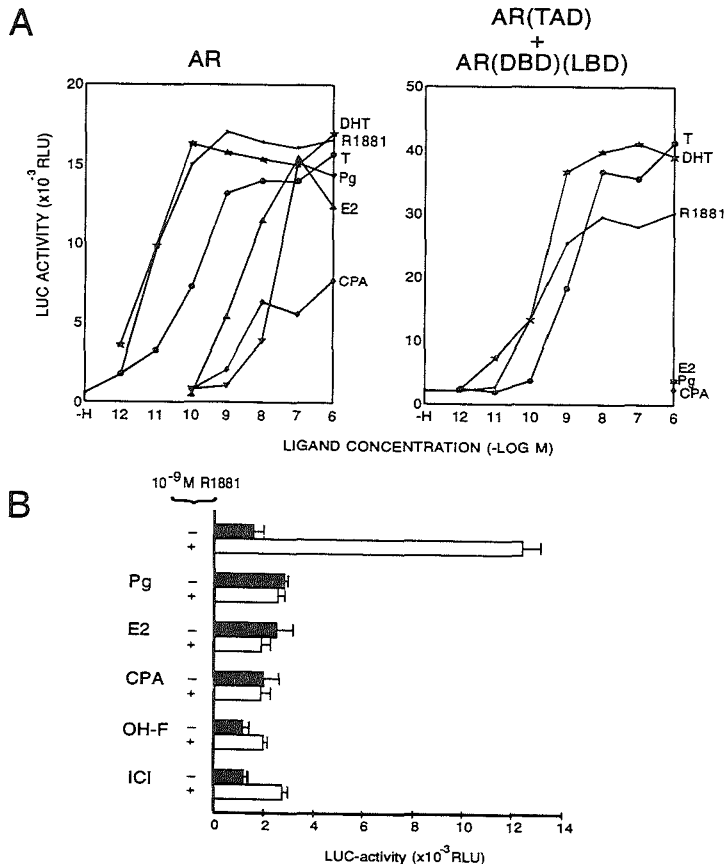



*Figure 6.7. Protein expression and transcriptional activity of the AR fragments AR(TAD) and AR(DBD)(LBD), as compared to full-length AR in transiently transfected CHO cells.* ***A.*** *Cytosols of transfected CHO cells were immunoprecipitated with antibody F39.4.1 and immunoblotted with antiserum SP197 [both against AR(TAD)] (lanes 1-4) or immunoprecipitated with antibody F52.24.4 [against AR(DBD)] and immunoblotted with antiserum SP66 [against AR(LBD)] (lanes 5-8). Lanes 1 and 5: full-length AR; lanes 2 and 6: AR(TAD); lanes 3 and 7: AR(DBD)(LBD); lanes 4 and 8: AR(TAD) + AR(DBD)(LBD). The arrow indicates the specific AR(DBD)(LBD) fragment in lanes 7 and 8. Molecular mass standards were run on a parallel lane (kDa).* ***B.*** *LUC expression was determined in CHO cells transiently cotransfected with the indicated AR expression plasmid(s) and the reporter plasmid MMTV-LUC. After transfection, cells were incubated without hormone or with R1881 ($10^{-9}$ M) for 24 h. Values of a representative experiment are shown, and represent the mean (± SEM) of triplicate determinations.*

AR(DBD)(LBD), which results in the formation of a transcriptionally active complex, can also occur in mammalian cells. In the experiment shown, a high expression level of AR(TAD) was used, but functional complex formation was also observed at lower AR(TAD) levels, albeit less efficiently (data not shown). In the AR(TAD) expression construct, a nuclear localization signal is lacking. As a consequence, AR(TAD) has to be transported to the nucleus by complex formation with AR(DBD)(LBD), or by diffusion, which might be less efficient (Jenster *et al.*, 1993).

In a next series of experiments, we determined the dose-response curves to several ligands on the formation of a functional complex between AR(TAD) and AR(DBD)(LBD) (Fig. 6.8A). R1881 induced LUC activity in a dose-dependent manner. Similar activities were observed with DHT or T. The maximal response was reached at a 10-fold higher ligand concentration, as compared to the dose-response curve for the full-length AR (Fig. 6.8A).

The full-length AR could be (partially) activated by high concentrations of E2, Pg or the antiandrogen cyproterone acetate (CPA). However, these low affinity ligands were unable to stimulate the formation of a transcriptionally active complex in cells cotransfected with the AR(TAD) and AR(DBD)(LBD) expression plasmids. The absence of induction of LUC activity was not due to a loss of hormone binding by AR(DBD)(LBD), because all compounds, including hydroxyflutamide and bicalutamide, could inhibit the LUC activity induced by R1881 (Fig. 6.8B).



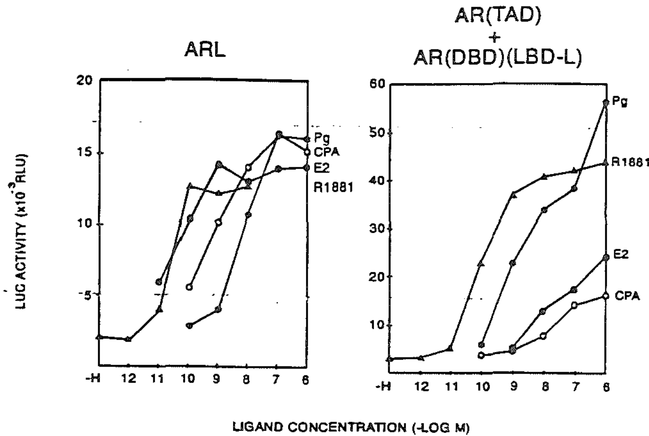



*Figure 6.9. Dose-response curves of transcriptional activity of the mutant AR-L and separate AR(TAD) and AR(DBD)(LBD-L) in the presence of various ligands. LUC expression was determined in CHO cells transiently co-transfected with the indicated AR expression plasmid(s) and the reporter plasmid MMTV-LUC. After transfection, cells were incubated for 24 h without hormone (-H) or in the presence of R1881, Pg, E2 and CPA, respectively. Values of representative experiments are shown, and represent the mean of triplicate determinations.*

In order to investigate the effect of the altered steroid specificity of the mutant AR-L on the interaction of AR(TAD) and AR(DBD)(LBD), the T868A mutation was introduced into the AR(DBD)(LBD) expression plasmid. As expected, stimulation of the MMTV-LUC reporter was induced by R1881, DHT and T in CHO cells (Fig. 6.9, and data not shown). However, as found for wild type AR(LBD) constructs, maximal reporter gene activity was detected at somewhat higher ligand concentrations, as compared to the full-length AR. CPA, E2 and Pg were able to induce LUC activity, however, bicalutamide still acted as a full antagonist (Fig. 6.9, and data not shown). These results paralelled the activating and inhibitory properties of these ligands with the full-length AR-L in CHO cells, albeit at higher ligand concentrations.

***Figure 6.8.** (previous page) Transcriptional activity of the full-length AR and seperate AR(TAD) and AR(DBD)(LBD) fragments in the presence of various ligands. LUC expression was determined in CHO cells transiently co-transfected with the indicated AR expression plasmid(s) and the reporter plasmid MMTV-LUC. After transfection, cells were incubated for 24 h without hormone (-H) or in the presence various ligands. **A.** Dose-response curves of R1881, DHT, T, Pg, E2 and cyproterone acetate (CPA) with wild type AR and separate AR(TAD) and AR(DBD)(LBD) proteins. **B.** For determination of steroid specificity of AR(TAD) and AR(DBD)(LBD) interaction, the cells were incubated in the presence of Pg ($10^{-6}$ M), E2 ($10^{-6}$ M), CPA ($10^{-6}$ M), hydroxyflutamide (OH-F, $10^{-6}$ M), and bicalutamide (ICI, $10^{-6}$ M), respectively, either in the absence (-) or in the presence (+) of $10^{-9}$ M R1881. Values of a representative experiment are shown, and represent the mean (± SEM) of triplicate determinations.*

## Chapter 6

### Discussion

In this study we assessed the *in vivo* association between the AR TAD and the AR LBD, leading to the formation of a transcriptionally active complex. Experiments were performed both in yeast and mammalian protein-protein interaction systems. The functional interaction was strictly hormone dependent, and could be blocked by antiandrogens. The TAD-LBD association was also observed in the AR mutant T868A (AR-L). This mutant AR was originally found in the prostate cancer cell line LNCaP, and can also be activated by estrogens, progestins and several antiandrogens (Veldscholte *et al.*, 1990). The data collected indicate that the TAD-LBD interaction is of physiological relevance, because it can occur at low ligand concentrations, and at low expression levels of the various components. AR LBD-LBD interactions could be measured in the yeast two-hybrid system at high ligand concentrations, and at high expression level of the GAL4(TAD)AR(LBD) chimeric protein. Our findings are summarized in the schematical representation depicted in Figure 10. We propose an intramolecular, direct or indirect interaction between AR TAD and LBD. In addition to a DBD-DBD interaction, a weaker intermolecular LBD-LBD interaction is presumed in AR homodimers binding to the cognate DNA binding site. This concept is comparable to the ER dimerization model (Kraus *et al.*, 1995), but differs in several aspects from a recently proposed model for the AR, which postulates an intermolecular interaction between TAD and LBD, resulting in the binding of AR homodimers in an anti-parallel orientation to the DNA binding site, without LBD-LBD association (Wong *et al.*, 1993; Langley *et al.*, 1995).

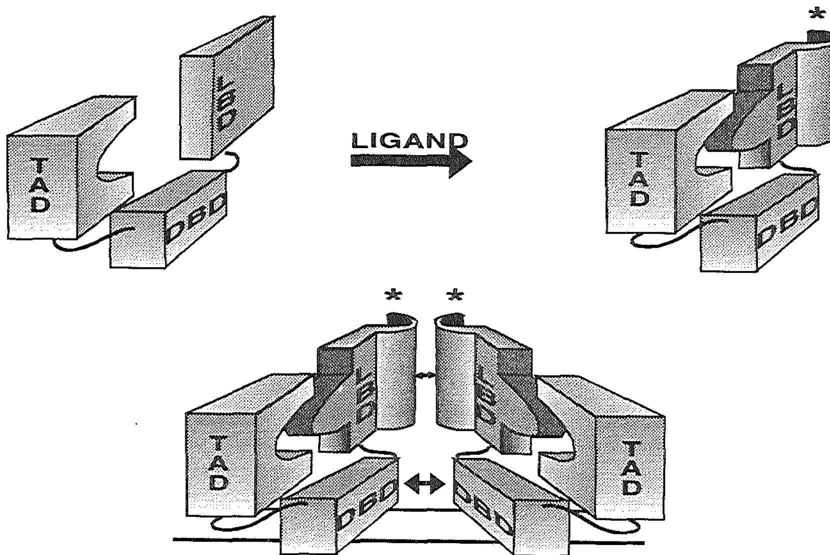



*Figure 6.10. Model of functional interactions between AR domains. Binding of androgen induces conformational changes in the ligand binding domain (LBD) of the AR, leading to a transcriptionally functional interaction between the transcription activation domain (TAD) and ligand binding domain. Upon DNA binding, the DNA binding domains (DBD) and the ligand binding domains of the receptor dimer interact with their counterparts.*

Interactions between two LBDs have been described for several members of the steroid receptor family, especially the ER and the PR, using different experimental approaches (Kumar & Chambon, 1988; Guiochon-Mantel *et al.*, 1989; Fawell *et al.*, 1990). The *in vivo* experiments presented here extend these observations to the AR. However, a drawback of the two-hybrid system is the occurrence of a competition between the formation of transcriptionally inactive and active LBD-LBD complexes. Therefore, the strength of the LBD-LBD interaction might be underestimated.

The formation of LBD-LBD complexes might also affect the efficacy of TAD-LBD interactions. On the one hand, this could result in the underestimation of the strength of the TAD-LBD interaction, because of a competition between the formation of functionally inactive LBD-LBD complexes and active TAD-LBD complexes. On the other hand, it might be that ligand dependent LBD-LBD formation is a prerequisite for TAD-LBD association. However, the observation that functional TAD-LBD association can take place at low concentrations of AR(TAD) or GAL4(TAD)AR(TAD) and low ligand concentrations argues against interference of LBD-LBD with TAD-LBD complex formation. This would implicate that different regions in the AR LBD are involved in LBD-LBD and TAD-LBD interactions, and/or that TAD-LBD association is much stronger than LBD-LBD association.

Obviously, it would be of interest to identify the regions of AR TAD and AR LBD directly involved in the interaction. Unfortunately, mutations in the LBD, in general, lead to loss of ligand binding; mutations in the AR amino-terminal domain might affect the transactivating capacity. Effects of TAD mutations in TAD-LBD associations can be studied in a two-hybrid system, if AR(TAD) is hooked to a much stronger TAD, which largely excludes the contribution of AR(TAD) to the total transactivating activity of chimeric AR(TAD) constructs. In GAL4(TAD)AR(TAD), both TADs seem to contribute equally to the total transactivating capacity of the chimeric protein. Previously, we showed that deletion of the regions 1-188 or 370-528 in the full-length AR hardly affected the transactivating activity, and that almost full activity was retained within the 101-370 fragment (Jenster *et al.*, 1995). We interpreted this as the presence of a ligand dependent TAD (TAU-1) in this region, which interacts with the basal transcription machinery. It seems reasonably to presume that ligand dependent TAU-1 activity depends on TAD-LBD interaction. In this concept, the 101-370 region contains not only TAU-1, but also the TAD-LBD interaction domain. Recently, it has been described that deletion of the 14-150 region or 339-499 region would affect the formation of a transcriptionally active complex of GAL4(DBD)AR(LBD) and VP16(TAD)AR(TAD) (Langley *et al.*, 1995). However, both deletions will hardly affect the transactivating capacity of the full-length AR (Jenster *et al.*, 1995). Elucidation of this apparent discrepancy with the above postulated hypothesis requires further investigation.

In a recent study, interaction between the amino-terminal and the carboxyl-terminal domains of the ER was described (Kraus *et al.*, 1995). It was supposed that this interaction was required for synergistic cooperation between a TAD in the amino-terminal domain (AF-1) and one in the LBD (AF-2). In contrast to the ER, evidence for an AF-2 in the AR LBD is lacking (Jenster *et al.*, 1991; Simental *et al.*, 1991). Our current knowledge favours the concept, that the TAD-LBD interaction in the AR is required for a conformational change in the TAD, which enables TAU-1 to interact with the basal transcription initiation complex. In this regard, it would be of interest to find out whether or not functional TAD-LBD interactions occur for the GR and PR, which have been shown to contain a ligand dependent transactivating domain (AF-2) in the LBD (Hollenberg & Evans, 1988; Danielian *et al.*,

1992).
From our experiments it cannot be deduced, whether the functional TAD-LBD interaction is direct or indirect. So far, *in vitro* experiments with purified AR fragments aimed at investigation of direct protein-protein contacts were unsuccessful (data not shown). Although this suggests that the TAD-LBD interaction is indirect, it cannot be excluded that we were unable to mimic *in vitro* the *in vivo* conditions for direct interaction. Indirect interaction might involve specific proteins, or more general bridging factors or coactivators, associating with AR segments (Halachmi *et al.*, 1994; Cavailles *et al.*, 1994; Onate *et al.*, 1995; Yeh & Chang, 1996). If bridging factors are involved in TAD-LBD interaction, their function must be conserved in yeast proteins.
Essentially, TAD-LBD interaction can be intramolecular and intermolecular. However, kinetics of intramolecular interaction seem favourable to those of intermolecular association. In this model, it has to be assumed that the DBD, which links the TAD to the LBD, does not interfere with such an interaction. Because of the readily exchangeable domain structure of steroid receptors, the absence of this interference by steric hindrance is a real possibility.
Functional TAD-LBD association cannot be detected with the low affinity ligands Pg, E2 and antiandrogens, even at ligand concentrations up to $10^{-6}$ M. However, competition with high affinity ligands does take place, reflected in the inhibition of the formation of a functionally active complex, as studied in CHO cells. Antiandrogen-bound AR exhibits ligand-induced conformational changes that are distinct from those induced by androgens (Kuil *et al.*, 1995). Whether the aberrant conformation of the antiandrogen-bound LBD results in the inhibition of a TAD-LBD interaction or in an association between both domains which is transcriptionally non-productive, remains to be determined. For the ER, antiestrogen-induced changes in the LBD of the ER differ from those induced by estrogens (Beekman *et al.*, 1993), and result in a transcriptionally non-productive association of the TAD and LBD (Kraus *et al.*, 1995). Evidently, our results suggest a novel mechanism of antiandrogen action: blockade of a functional interaction between the TAD and LBD in the full-length AR.

**Acknowledgement**
We thank Drs Picard and Dijkema for plasmid constructs, and Mr. F. van der Panne for skilful photography.

# GENERAL DISCUSSION

## Chapter 7

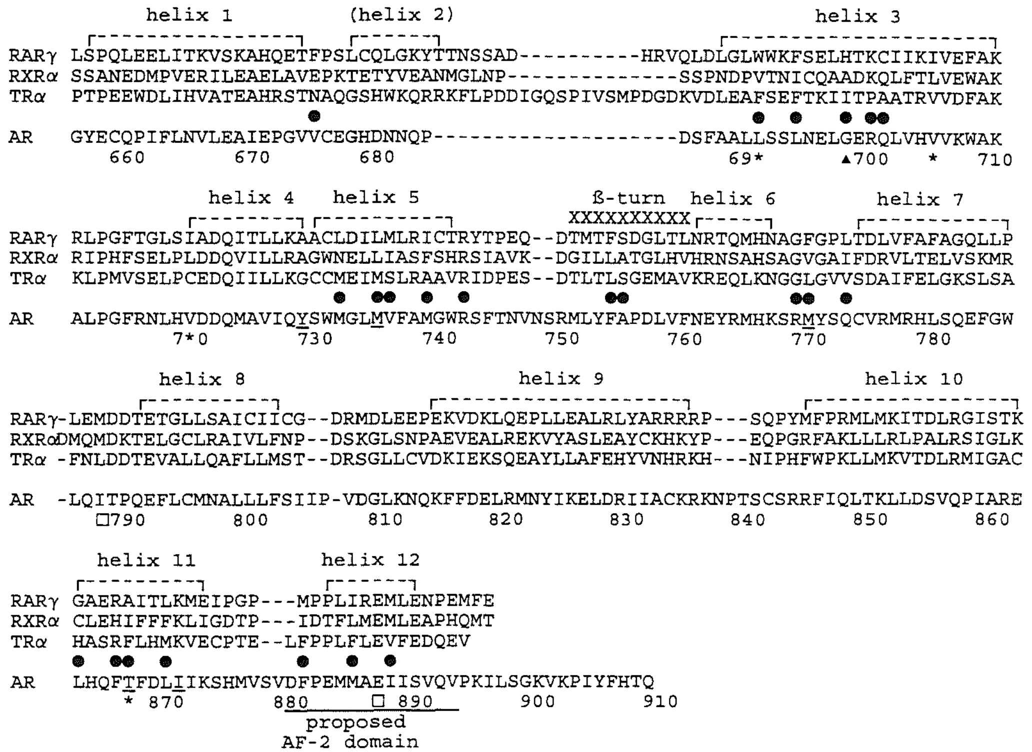
### 7.1 Androgen receptor structure-activity relationships

#### 7.1.1 Three-dimensional structure of the ligand binding domain

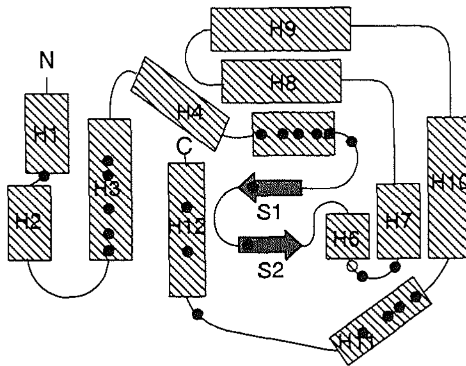
In order to better understand the bioactivities of androgens and antiandrogens and their different interactions with the AR, some of our studies focused on identifying the regions of the AR that are involved in androgen and antiandrogen binding and in discriminating between androgen and antiandrogen action (Chapters 3 and 4). Since the hormone binding domain of the AR is large (approximately 250 amino acid residues), analysis of its structural and functional complexity is challenging. Recently, the three-dimensional structures of the unliganded RXRα (Bourguet *et al.*, 1995) and liganded RARγ (Renaud *et al.*, 1995) and TRα (Wagner *et al.*, 1995) have been determined. Although these receptors belong to two evolutionarily distinct groups (Laudet *et al.*, 1992), the three-dimensional structures are very similar: they all form anti-parallel α-helical sandwiches composed of 11-12 α-helices and 2 ß-sheets (Chapter 2: Fig 2.4). Apart from these common folds, there are two important differences between the unliganded and liganded structures. First, the RARγ and TRα holo-ligand binding domains are more compact than the RXRα apo-ligand binding domain. Second, the α-helix 12, and to some extent also α-helix 11, are folded back towards the main body of the ligand binding domain in its liganded state. Especially this last change led to propose a 'mouse trap-like' mechanism by which the ligand is trapped by electrostatic forces in the ligand binding pocket, whereafter helix 12 seals the pocket like a lid (Wurtz *et al.*, 1996). Based on these recently clarified structures of the RXRα apo- and RARγ and TRα holo-ligand binding domains, an alignment can be constructed of the sequences of the ligand binding domain of these receptors and the AR (Wurtz *et al.*, 1996) to evaluate whether the results of our studies are in agreement with predicted three-dimensional structures of the ligand binding domain (Fig. 7.1A). Although the identity on amino acid residue level between the readily matched sequence alignments is not very high, several experimental findings indicate homology between the three-dimensional structures of the ligand binding domain of the AR and other steroid hormone receptors with those of the retinoid acid/thyroid hormone receptor subfamily.

***Figure 7.1.*** *(next page) A. Sequence alignment of RXRα, RARγ, TRα and AR (adapted from Wurtz et al., 1996). Sequences corresponding to the secondary structure elements found in the RARγ ligand binding domain are indicated by X for the β-turns (β-sheet) and by brackets for α-helices (helices 1 and 3-12): helix 2 is only found in RXRα. Black dots (●) in between the alignments of the AR and other receptors denote amino acid residues in the immediate vicinity of the ligand in RARγ. The underlined residues in the AR correspond to amino acid residues in ER, PR and GR, determined to be in the vicinity of the ligand by affinity labeling studies. The asterisks indicate amino acid residues which were found mutated in prostate cancer cells. The ▲ indicates a residue presumably involved in RU 38486 binding, whereas □ indicates residues which do not impact ligand binding (see text for details and references). B. Model of the structure of the ligand binding pocket. The black dots point to the same amino acid residues as in part A, and located in the immediate vicinity of the ligand in RARγ. In the three-dimensional structure, helix 3 is behind helix 12.*

A




B



### 7.1.2 Similarities in the ligand binding pocket of nuclear hormone receptors

Experimental findings on homology in the three-dimensional structure of ligand binding domains of the nuclear receptor superfamily can be provided by: i) limited proteolytic digestion of the receptor, ii) affinity labeling with steroids, and iii) mutational analysis.
Agonist binding to the AR, as described by us (Chapters 3 and 4) and by others (Kallio *et al.*, 1994; Zeng *et al.*, 1994), as well as for the PR (Allan *et al.*, 1992a,b) and ER (Beekman *et al.*, 1993), resulted in an increased resistance of the whole ligand binding domain against proteolytic degradation, indicative for a more compact structure of this domain in its liganded state. The conformational changes in the ligand binding domain could also be demonstrated in proteolytic analysis of the agonist-bound retinoic acid receptor RXRα (Keidel *et al.*, 1994). As mentioned in the previous paragraph, the protein structures of the liganded RARγ and TRα ligand binding domains are more compact than the unliganded RXRα ligand binding domain. This could explain the experimental findings of increased resistance of the liganded receptors against proteolytic degradation. Furthermore, these results provide evidence for homology in ligand-induced conformational changes between different members of the nuclear hormone receptor superfamily.
Detailed information on contact-sites between the ligand binding domain and its respective ligand could be obtained by affinity labeling studies. Ligand binding of the glucocorticoid and progesterone receptors has been probed by photo-affinity labelling with synthetic steroids having their reactive functions in the A-ring. Triamcinolone acetonide labeled Met622 and Cys754 in rat GR, while dexamethasone mesylate was cross-linked to Cys 656 (Carlstedt-Duke *et al.*, 1988; Byravan *et al.*, 1991). The progestagen R5020 labeled Met759 and Met909 in human PR (Strömstedt *et al.*, 1990). Sequence alignment of the amino acid residues of the PR and GR indicates that Met622 and Met759, respectively on GR and PR, correspond to the same position in the ligand binding domain. In the human ER Cys530 can be covalently labeled with aziridine analogs of estrogen (Harlow *et al.*, 1989). When this amino acid residue Cys530 was mutated to alanine, the Cys381 was labeled (Reese *et al.*, 1992). On basis of sequence homology, these contact-sites between the ligand binding domain and its respective ligand correspond to Tyr730, Met736, Met771, Thr868, Ile873 and Met886 of the human AR. All of the corresponding amino acid residues of the RARγ are in the immediate vicinity of the ligand [Fig 7.1 (Wurtz *et al.*, 1996)], indicative for a possible role of these residues in ligand binding of the AR.
In agreement with a role for position Thr868 in the ligand binding of the AR, mutation of this amino acid residue to Ala868, as present in the LNCaP prostate cancer cell line (Veldscholte *et al.*, 1990) and in a number of advanced prostate tumors (Suzuki *et al.*, 1993; Gaddipati *et al.*, 1994), results in an altered ligand binding specificity. R5020, estradiol, and several antihormones are able to activate the mutant receptor, presumably through altered interaction of these ligands with the ligand binding domain. These altered interactions were demonstrated in limited proteolytic digestion of this mutant receptor (Chapters 3 and 4). The antihormones cyproterone acetate and hydroxyflutamide, which are full agonists for the receptor with Ala868, induced compact structures of the ligand binding domain, indistinguishable from the androgen-induced structure. The importance of Thr868 in ligand binding and androgen receptor action is further emphasized by mutational analyses. Exchange of this residue into a cysteine, as it is in the GR and PR, did not alter the relative binding affinities of the AR for R1881, whereas exchanges to other amino acid residues produced either a receptor with a broad steroid specificity or an inactive receptor (Ris-Stalpers *et al.*, 1993). Other mutations

found in the AR, obtained from prostate cancer specimens, were Leu692 to His692 (Suzuki *et al.*, 1993), Val706 to Met706 (Culig *et al.*, 1993), and Val721 to Met721 (Newmark *et al.*, 1992; Schoenberg *et al.*, 1994). These mutations in the ligand binding domain result in altered steroid binding specificities, indicative for a role of these amino acid residues in determination of ligand binding. The homologous amino acid residues in the RARγ of these AR mutations, with the exception of the Val721 mutation, are near or in the ligand binding pocket of the RARγ [Fig. 7.1 (Wurtz *et al.*, 1996)]. Together these findings support the proposed structural homology in the ligand binding domains of the members of the nuclear receptor superfamily. The amino acid residues Thr868, Ile873, and Met886, identified by ligand affinity labeling and mutational analyses as belonging to the ligand binding pockets of GR, AR, PR and ER, are all located in the proposed lid region of the ligand binding pocket at the C-terminal end of the receptor. The importance of this region in ligand binding is further emphasized by deletion analysis of these receptor proteins. Truncation of the ligand binding domain of the AR with 12 amino acid residues resulted in the inability of androgens to protect this domain against proteolytic degradation (Chapter 4), most likely due to the absence of ligand binding (Jenster *et al.*, 1991). Also truncation of the C-terminal end of the ligand binding domain with either 42 amino acid residues in the PR (Vegeto *et al.*, 1992) or 14 amino acid residues in the GR (Zhang *et al.*, 1996) resulted in a complete loss of ligand binding. These deletion mutations also indicate the limits of the three-dimensional model of the ligand binding domain of steroid hormone receptors, based on sequence homology between members of the nuclear receptor superfamily as shown in Figure 7.1. Although the extreme C-terminal domains of the AR, GR and PR are important in ligand binding and, as such, presumably contain residues which contact the ligand, homologous amino acid residues are neither present in the RARγ, RXRα nor TRα (exemplified for the AR in Fig. 7.1). In addition to its important role in ligand binding, the C-terminal end of the ligand binding domain is implicated in the hormone-dependent activation function (AF-2) of the ER (Danielian *et al.*, 1992), GR (Danielsen *et al.*, 1987; Hollenberg and Evans, 1988), PR (Bocquel *et al.*, 1989), and TR (Barettino *et al.*, 1994). The AF-2 domain is proposed to form an amphipatic helix, within which the highly conserved motif ΦΦXEΦΦ (where Φ represents a hydrophobic amino acid residue) is proposed to mediate interactions between the receptors and transcriptional coactivators (Danielian *et al.*, 1992). Indeed, the AF-2 domain is present in α-helix 12 of the three-dimensional structures of the RXRα (Bourguet *et al.*, 1995), RARγ (Renaud *et al.*, 1995) and TRα (Wagner *et al.*, 1995), which fold back to the ligand binding domain in its liganded state. Mutations of key amino acid residues within the AF-2 domain decrease or abolish ligand-dependent activation (Danielian *et al.*, 1992; Lanz *et al.*, 1994). So far, there is no evidence for the AF-2 in the ligand binding domain of the AR (Jenster *et al.*, 1995). In agreement with the absence of an AF-2 domain in the AR, mutation of the highly conserved glutamate residue Glu888 (represented as E in the conserved motif of the AF-2) towards Gln888 resulted in similar ligand binding characteristics, for both agonists and antagonists, and transactivation capacities as for the wild type receptor [Fig. 7.1 (C. Berrevoets, unpublished results)].

Many natural occurring mutations within the AR have been identified in individuals with the androgen insensitivity syndrome (reviewed in Quigley *et al.*, 1995; Gottlieb *et al.*, 1996). The complete form of this syndrome is characterized by 46,XY karyotype, external female phenotype, intra-abdominal testes, absence of uterus and ovaries, blind ending vagina, and gynaecomastia (Schweikert, 1993). Although the amino acid substitutions in the ligand binding

domain are scattered throughout this domain, the frequency of mutations is highest in the second half of exon 5 of the AR (McPhaul *et al.*, 1992; Gottlieb *et al.*, 1996). This part of the exon encodes for the amino acid residues 730 to 772. The homologues amino acid residues within the RARα are involved in direct ligand binding, and forms the predicted ß-sheets within the ligand binding domain. So, mutations in this structural important region of the AR could easily lead to ligand binding abnormalities.
In general, results of most studies of the ligand binding domains of the steroid hormone receptors fit well with the three-dimensional structural models of the ligand binding domains of the retinoid acid and thyroid hormone receptors, indicating a structural homology between these domains. In addition, the results on the involvement of the extreme C-terminal region of the ligand binding domain in both ligand binding and transactivation show that structural properties of the ligand binding domains could be predicted by these homologies, but on details the receptor proteins differ.

### 7.1.3 Antagonists and the structure of the ligand binding pocket

To characterize regions in the hormone binding domain that are critical in antagonist binding, we analyzed receptor fragments formed upon proteolytic digestion of antiandrogen-bound AR (Chapters 3 and 4). The data obtained provided evidence for two different molecular interactions between antihormones and the ligand binding domain, which impact the cellular mechanism of antihormone action (see also 7.2: Cellular mechanisms of antiandrogen action). The antihormone RU 38486 protected a smaller fragment against trypsinization than agonists did, whereas all other tested antagonists (cyproterone actetate, hydroxyflutamide, ICI 176.334 and RU 23908) protected larger fragments of the AR (Chapters 3 and 4). Furthermore, C-terminal truncation of the AR with 12 amino acid residues abrogated both androgen binding (Jenster *et al.*, 1993) and the induction of proteolysis resisting fragments by androgens and several antiandrogens (Chapter 4). This truncation of the ligand binding domain with 12 amino acid residues hardly effected binding of RU 38486, in analogy with binding of RU 38486 to truncated PR and GR (Vegeto *et al.*, 1992; Zhang *et al.*, 1996). For RU 38486, the unaltered respons of the mutant receptor ARL in a transcription activation assay presumably can be understood by the results of proteolytic digestion of the AR. Proteolytic digestion of RU 38486-bound AR (Chapter 4), but also of the PR (Allan *et al.*, 1992a), resulted in a C-terminal truncation of approximately 40 amino acid residues, i.e. the 4 kDa difference in molecular mass between an androgen- and a RU 38486-bound proteolysis resisting fragment of the AR (Chapter 4). Furthermore, RU 38486 still binds to a mutant PR, deleted of 42 residues at the C-terminus (Vegeto *et al.*, 1992). The wild type AR, used in our studies, contains 910 amino acid residues (Faber *et al.*, 1989). So, the LNCaP-mutation, Thr868 to Ala868, is located at the border of amino acid residues necessary for RU 38486 binding. However, mutation of this residue results in the unaltered respons in transcription activation studies of the mutant receptor ARL upon binding of RU 38486 (Chapter 4). From these results it can be concluded that the C-terminal region of the ligand binding domain is not involved in binding of RU 38486, whereas it is in binding of androgens and the antiandrogens cyproterone acetate, hydroxyflutamide, RU 23908 and ICI 176.334.
So far, little is known about specific amino acid residues involved in direct binding of antihormones to the AR. Only for the position Thr868 and Gly699 within the AR some data are available.
-Thr868: the amino acid residue Thr868 seems to be involved in binding of the antagonists

cyproterone acetate and hydroxyflutamide. Alteration of this amino acid residue towards alanine results, upon binding of these antagonists, in complete transformation of the heat-shock protein-receptor complex in a receptor protein able to induce full agonistic activity (Veldscholte *et al.*, 1990). In contrast, no change in response of the mutant receptor was found in the presence of the antagonists RU 38486 and ICI 176.334.

-Gly699: mutational analysis of the human PR revealed that the single amino acid residue Gly722 determined sensitivity to RU 38486 (Benhamou *et al.*, 1992); mutation of this residue to either cysteine, methionine or leucine resulted in loss of RU 38486 binding, whereas progesterone still binds its respective receptor (Vegeto *et al.*, 1992). Sequence alignment of the human PR, the human GR and the human AR, which all bind RU 38486, revealed the presence of a glycine at the position correponding to human PR Gly722. In contrast, the chicken PR and the human MR have, respectively, a cysteine and an alanine at this position and do not bind RU 38486 (Benhamou *et al.*, 1992). These results again demonstrate the important role of this glycine residue in RU 38486 binding. In addition, mutation of the glycine in the GR resulted in a loss of binding of the agonist dexamethasone, but also of the antagonist RU 38486 (Benhamou *et al.*, 1992; Zhang *et al.*, 1996). These observations suggest that Gly699 in the AR (corresponding to the Gly722 in the PR) is also important in RU 38486 binding. However, mutational analysis of this position in the AR has not been performed to date.

### 7.1.4 Models of the ligand binding pocket(s)

Based upon conformational alterations induced by agonists and antagonists in the ligand binding domain of the AR (Chapters 3 and 4; Kallio *et al.*, 1993; Zeng *et al.*, 1994), but also on those obtained for the PR (Allan *et al.*, 1992a,b) and ER (Beekman *et al.*, 1993), one can speculate about the nature of the ligand binding pocket(s) of steroid hormone receptors. To our opinion, the experimental results obtained by us and others cannot be rationalized any longer by a simple *"lock and key"* model (see also 2.2.2 Antagonist-receptor interactions), in which all ligands interact with the receptor at a single binding pocket in the same (or similar) manner.

In order to explain the various proteolysis resisting fragments of the AR, hydrophobic interactions between the diverse ligands and the ligand binding site have to be distinct. Initial binding to the AR of agonists and most antagonists resulted in a 35 kDa proteolysis resisting fragment, whereas upon the initial binding of RU 38486 to the receptor a 30 kDa fragment was protected against proteolysis (Chapters 3 and 4). As a result, one can propose a *"two-site"* model in which two different ligand binding pockets are present: ligand binding pocket I, which interacts with androgens and most antiandrogens, and a ligand binding pocket II, in which the 11ß-substituted antihormone RU 38486 binds. Although the evidence for two ligand binding pockets is not indisputable, several reports have proposed two binding sites to explain unusual binding behaviour of steroid hormone receptor ligands (Jonas and Bell, 1980; Svec *et al.*, 1989; Shrivastava and Thompson, 1990; Bergmann *et al.*, 1994; Berthois *et al.*, 1994; Teutsch *et al.*, 1995). Additional evidence for the existence of two ligand binding sites can be obtained from the three-dimensional structures of the ligand binding domains of the RXRα (Bourguet *et al.*, 1995), RARγ (Renaud *et al.*, 1995) and TRα (Wagner *et al.*, 1995). A search for a potential binding pocket within the unliganded RXRα revealed two large hydrophobic cavities (Bourguet *et al.*, 1995). These cavities were separated by helix 5 which was part of both sites, and the ligand 9-*cis* retinoic acid could be docked in each of the cavities with only

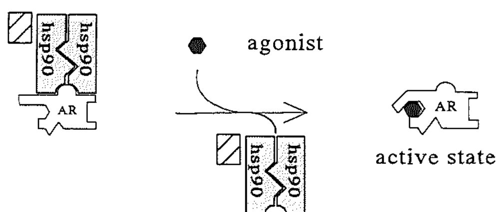
minor conformational adaptations of the side chains. Although one of these sites was favoured, and proved to be a ligand binding site in the RXRγ and TRα, binding of ligand within the other pocket could not be excluded. Therefore, binding of RU 38486 to the AR, and in analogy also to the GR and PR, in a different binding pocket than favoured by agonists could be a possibility, and could also explain the differences in binding for RU 38486 and agonists. Due to the proposed second binding site for RU 38486, and other 11ß-substituted steroids (Vegeto *et al.*, 1992; McDonnell, 1995), hydrophobic interactions of the ligands with helices 11 and 12 in the C-terminal region of the ligand binding domain are unnecessary and may be impossible.
As mentioned in the introduction of this thesis (2.2.2 Antagonist-receptor interactions), experimental data on structure-activity and structure-affinity relationships of various compounds suggested a more dynamic model of ligand-receptor interactions, the *"induced fit"* model. Alternatively to the *"two-site"* model, this *"induced fit"* model could also accommodate the results on the conformational alterations in the ligand binding domain of the AR as detected upon proteolytic degradation. In this model, binding of the ligand to an initially undifferentiated ligand binding site results in extensive conformational changes of the ligand binding domain leading to a mutual adaptation of ligand and receptor. As a consequence, each ligand-receptor complex will have its own conformation, which could give rise to a continuum of transcriptional responses from agonist to full antagonist.

### 7.2 Cellular mechanisms of antiandrogen action

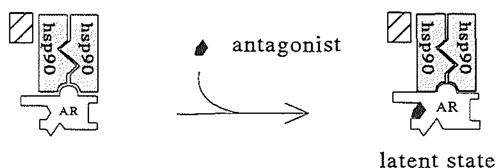
In order to explain the results of our *in vitro* studies on androgen and antiandrogen action in Chapter 4, we proposed a model of the sequential steps in androgen receptor activation (Chapter 4: Fig. 4.7). In this model, binding of the androgen to the inactive AR, complexed with heat-shock proteins, induces an initial conformation change. Upon dissociation of the heat-shock protein-AR complex and a second conformation change, the receptors dimerize and bind to DNA. The DNA-bound receptor interacts with the transcription initiation complex to regulate transcription, i.e. the active state of the receptor (Fig. 7.2A). Potentially, all these steps may be blocked by binding of an antagonist. However, the key-step in this model, especially for antagonist action, is the step from a ligand-occupied complex of the receptor and associated proteins, and a receptor that has a changed conformation and has released these proteins. Based on this key-point of the model, at least two classes of antiandrogens can be postulated. The first class of antagonists blocks the access of agonists to the AR, while the antagonist only induces an initial conformational change of the inactive receptor, complexed with associated proteins. As a consequence, the AR remains in an inactivated, latent state within the cell (Fig. 7.2B). On a theoretical basis, any agonistic activity of this latent antagonist-receptor complex is not feasible. As such, these compounds are full antagonists. Alternatively, the second class of antagonists has a more active role in inhibition of AR activation (Fig 7.2C). Besides the direct competition with the agonist for binding to the receptor, these antagonists function as "pseudoagonists" by mimicking some of the actions of the androgen, but ultimately pushing the AR down a transcriptionally nonproductive pathway. As a result, the antagonist-receptor complex may have additional antagonistic activities such as competing with the androgen-bound receptor for DNA-binding sites and/or associating less productively with the general transcription machinery. The antagonist-bound receptor complexes which bind to DNA can show some agonist activity in a promoter- and cell-specific manner, and are therefore referred to as partial antagonists. The pharmacological

implications of these two classes of antagonist are likely to be different.

A. Agonist action




B. Latent interaction of antagonist and AR




C. Active inhibition of AR activation

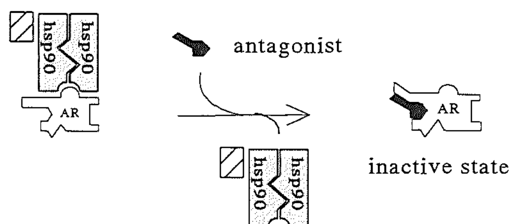



***Figure 7.2.*** *Potential mechanisms of antiandrogen action.* ***A.*** *The AR is present in a latent state within the cell. Binding of the agonist initiates a cascade of events finally leading to a transcriptionally active receptor.* ***B.*** *The androgen receptor antagonist associates with the receptor, and this ligand-receptor complex is kept in an inactivated, latent state.* ***C.*** *Upon binding of the antagonist to the receptor, the ligand acts as a "pseudoagonist" pushing the AR to a transcriptionally non-productive pathway.*

The antiandrogens, tested in the experiments described in this thesis, can be clearly arranged in these categories, based upon the various experimental findings. The antagonists hydroxyflutamide, RU 23908 and ICI 176.334 seem to belong to the first class of antagonist: those that maintain the receptor-complex in an inactivated, latent state. These compounds do neither induce heat-shock protein dissociation from the receptor complex (Chapter 4), nor induce DNA-binding *in vitro* (Wong *et al.*, 1993), which is in accordance to the inability of these antagonists to induce a second conformational change within the receptor protein after the initial change upon binding to the AR (Chapter 4). In addition, no tight nuclear- or DNA-bound receptor molecules can be detected in transient tranfected cell systems (Berrevoets *et al.*, 1993; Chapter 5). Despite these arguments, dissociation of the existing heat-shock protein-

AR complex by hydroxyflutamide, RU 23908 and ICI 176.334 in the intact cell is still not completely excluded. Circumstantial evidence to support inhibition of complex dissociation by antagonists in whole cells can be obtained from studies with the mutant receptor ARL present in LNCaP cells. The antagonist ICI 176.334 interacted with this mutant receptor in all studies, in a similar way as the full antagonists hydroxyflutamide, ICI 176.334 and RU 23908 did interact with the wild type receptor. Binding of ICI 176.334 to the mutant ARL did not result in dissociation of the heat-shock protein-ARL complex *in vitro* (Chapter 4). Furthermore, no tight nuclear- or DNA-bound receptors were detectable in whole cells upon addition of ICI 176.334 (Berrevoets *et al.*, 1993; Chapter 5). In addition to these homologous findings between the wild type AR and mutant ARL, no dissociation of the existing heat-shock protein-receptor complex occurred upon binding of ICI 176.334 to the mutant receptor ARL in whole cells (Veldscholte *et al.*, 1992a). So, the antagonists hydroxyflutamide, RU 23908 and ICI 176.334 most likely interfere with dissociation of the heat-shock protein receptor complex, which results in latent antagonist-receptor complexes.
The antagonists cyproterone acetate and RU 38486 seem to act as "pseudoagonists". Both antagonists could exert partial agonistic activity in transcription activation studies, performed by us and others (Veldscholte *et al.*, 1992a; Kemppainen, 1992; Culig *et al.*, 1993; Chapter 4). In agreement to the partial agonistic activities, both compounds induce DNA binding of the AR, both *in vitro* (Wong *et al.*, 1993) and in whole cells (Chapter 5). Furthermore, cyproterone acetate-bound AR is at least partially transformed into a tight nuclear-bound form in whole cells (Berrevoets *et al.*, 1993). In contrast to these similarities, the RU 38486- and cyproterone acetate-bound AR respond distinct in the induction of proteolysis resisting fragments of the wild type AR (Zeng *et al.*, 1994; Chapter 4), and dissociation of the heat-shock protein-AR complex *in vitro* (Chapter 4). Furthermore, binding of RU 38486 to a mutant AR, truncated at the extreme C-terminal end of the ligand binding domain, results in the appearance of a similar proteolysis resisting fragment upon trypsin digestion as for the RU 38486-bound wild type receptor. Cyproterone acetate, as androgens and other antiandrogens, was unable to protect a fragment of this truncated mutant AR against proteolytic degradation (Chapter 4). Based on these differences between the molecular interactions of cyproterone acetate and RU 38486 with the AR, we classified these antagonists into two different subtypes: the conformation of the extreme C-terminal region of the AR is either aberrant (with RU 38486) or normal (with cyproterone acetate), compared to the conformation of this region in an agonist-bound AR (Chapter 4).
In summary, we have been able to establish, using a series of novel methods, molecular and cellular criteria that clearly distinguish AR agonists from antagonists, and additionally classify the known AR antagonists into three functionally distinct categories. The behavior of known agonists and antagonists in our analysis suggest that these classifications are related to distinct ligand-induced structural alterations within the AR.

## 7.3 Interactions of the N- and C-terminal domains of the androgen receptor

In Chapter 6, the results were described concerning interactions between different domains of the AR, as determined with a two-hydrid system in both yeast and mammalian cells. Agonist-inducible interaction between separately expressed N-terminal and ligand binding domains resulted in a transcriptionally active receptor. In contrast, androgen receptor antagonists were unable to induce such a transcriptionally productive interaction. These findings suggest a role for interaction, either directly or mediated by additional proteins,

between the N-terminal and ligand binding domains in the transactivating function of the AR, which potentially may be inhibited by antagonists.

The AR is believed to exert its transactivation capacity solely through the activation function AF-1 which is located in the large N-terminal domain of the receptor (Jenster *et al.*, 1995). In contrast to other steroid hormone receptors (Kumar *et al.*, 1987; Webster *et al.*, 1988; Tora *et al.*, 1989), no activation function (AF-2) could be subscribed to the ligand binding domain of the AR (Jenster *et al.*, 1991). The absence of a ligand-dependent AF-2 implicates that the ligand binding domain is not directly involved in interaction with the transcription machinery. This would suggest that the sole function of the ligand is to bring the receptor in a form able to bind DNA and that a DNA-bound receptor protein exhibits full transcriptional activity. However, the antagonists cyproterone acetate and RU 38486 induce DNA binding of the receptor in intact cells (Chapter 5), but are unable to activate transcription to the same level as agonists do (Kemppainen *et al.*, 1992; Culig *et al.*, 1993; Chapter 4). So, the ligand binding domain of the AR appears to have a modulatory function in regulation of the transcriptional activity of a DNA-bound receptor by mediating interaction of AF-1 with other transcription factors. Additional support for the interaction between the N-terminal and ligand binding domain comes from kinetic studies of the AR in which the absence of the N-terminal domain accelerated androgen dissociation and decreased the stability of an androgen-bound receptor (Zhou *et al.*, 1995). This ligand-induced stabilization of the AR was highly androgen specific, as a variety of antihormones failed to stabilize the receptor against degradation (Kemppainen *et al.*, 1992; Zhou *et al.*, 1995). Furthermore, the proposed influence of the ligand binding domain on the transactivation function of the receptor could also be demonstrated in transcription activation studies with mutant receptors. If the ligand binding domain is eliminated from the AR, this constitutively active mutant receptor exerts transcriptional activity to a comparable level as the ligand-bound wild type AR. However, the regions in the N-terminal domain involved in interaction with the transcription machinery are completely different in the wild type AR and the mutant receptor without the ligand binding domain (Jenster *et al.*, 1995). Presumably, conformational changes within the N-terminal domain are induced by the interaction with the ligand binding domain. An effect of the N-terminal domain on the structure of the ligand binding domain is less likely: digestion of either the full-length AR or a separate ligand binding domain results in similar proteolysis resisting fragments (Chapter 3).

The absence of a functional interaction between the N-terminal and ligand binding domains in an antagonist-bound receptor supports the concept that antihormones induce conformational changes in the AR that are distinct from those induced by androgens (Chapter 6). In favour of this hypothesis, a functional interaction can be induced by hydroxyflutamide and cyproterone acetate between the N-terminal domain and the LNCaP-mutant ligand binding domain (Thr868 to Ala868). This interaction reflects the agonist activaties of these ligands in the full-length LNCaP mutant ARL (Veldscholte *et al.*, 1992a; Langley *et al.*, 1995; Chapter 6).

Ligand-dependent, productive association of the N-terminal and ligand binding domains is not only an important feature in androgen receptor activation. For the ER, estrogen-induced conformational alterations result in an association of the N-terminal and ligand binding domains and a subsequent synergism between AF-1 and AF-2, necessary to exhibit full transcriptional activity (Beekman *et al.*, 1993; Montano *et al.*, 1995; Kraus *et al.*, 1995). Both estrogens and antiestrogens do induce the association between these two domains, but upon

antiestrogen binding the association was not intrinsically transcriptionally productive (Kraus *et al.*, 1995). These findings contrast to the inability of antiandrogens to induce an association between the N-terminal and ligand binding domains (Langley *et al.*, 1995; Chapter 6), and indicate differences between members of the nuclear hormone receptor superfamily which may be related to the absence of an AF-2 in the ligand binding domain of the AR.

### 7.4 Antiandrogen action *in vitro* versus *in vivo*

Androgen receptor antagonists are characterised by their ability to repress agonist-activated gene transcription. The nonsteroidal antagonists hydroxyflutamide, ICI 176.334 and RU 23908 are full antagonists that show solely antagonistic activity without any agonistic effect in both animal studies (Neri and Monahan, 1972; Furr *et al.*, 1987; Labrie *et al.*, 1993) and in cell-lines, either stably or transient transfected with both AR expression and AR-responsive reporter constructs (Fuhrmann *et al.*, 1992; Kemppainen *et al.*, 1992; Veldscholte *et al.*, 1992a; Chapter 5). In contrast to the full antagonistic action of nonsteroidal antiandrogens, the antagonists cyproterone acetate and RU 38486 show partial agonistic activity in cell lines, transiently transfected with an AR expression plasmid and MMTV reporter construct (Kemppainen *et al.*, 1992; Warriar *et al.*, 1993; Berrevoets *et al.*, 1993; Chapter 5). However, cyproterone acetate shows hardly any agonistic effect in animal studies (Neumann and Töpert, 1986), and in CV-1 cells with stably transfected AR expression and MMTV reporter constructs (Fuhrmann *et al.*, 1992).
These contradictory findings for the effect of the steroidal antihormones on transactivation properties of the AR, with on the one hand the transient transfected cells and on the other hand stable transfected cell lines and animal studies, may be explained in a variety of ways. In general, cell- and promoter-specificities do account for the observed discrepancies in transactivation capacities of an antihormone (Berry *et al.*, 1990; Meyer *et al.*, 1990; reviewed by Katzenellenbogen *et al.*, 1996). However, this explanation for the different transactivation capacities observed for an AR in the presence of cyproterone acetate could be excluded for several *in vitro* studies: in these studies both CV-1 cells and the androgen-responsive MMTV-promoter were used (Fuhrman *et al.*, 1992; Simental *et al.*, 1992; Warriar *et al.*, 1993). Another possibility would be that in contrast to the stable introduced MMTV template, transient introduced template is not assembled into a phased nucleosomal array, and exhibits constitutive binding of several transcription factors, such as nuclear factor I (Piña *et al.*, 1990; Archer *et al.*, 1992; Archer *et al.*, 1994). Therefore, when an antihormone-bound receptor binds to either the stable or the transient introduced MMTV-template, this binding could be functional inequivalent (Archer *et al.*, 1994; Mymryk and Archer, 1995a, b). In the stable transfected cell line, the complex might not interact productively with the cellular apparutus to open up a repressive chromatin structure, which would result in the absence of agonistic activity of the antagonist-bound receptor.
The findings described above, that cyproterone acetate may have agonistic effects in addition to its antagonistic effects, challenge the usefulness of cyproterone acetate in total androgen blockade regimens *in vivo*. However, cyproterone acetate seems not to exert any androgenic effect in available standard biological models at dosages that suppress androgen dependent functions; cyproterone acetate has no virilization effect in female rat foetuses, and causes a reduction in weight of male rat accessory organs (i.e. the ventral prostate and seminal vesicles) not different from the result of castration (reviewed by Schröder, 1993). In contrast to the full antiandrogenic activity of cyproterone acetate at the organ level, promoter specific

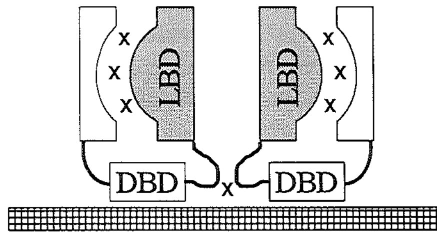
effects of this compound are demonstrated at the cellular level. Whereas cyproterone acetate did not increase the serum levels of androgen-regulated prostate specific antigen (PSA) and prostatic alkaline phosphatase (PAP), partial agonistic effects of this compound have been reported for expression of the androgen-regulated genes prostatic binding protein (PBP) and ornithine decarboxylase (ODC) in rats (Labrie *et al.*, 1993). So, one study in an intact animal showed partial agonistic effects of cyproterone acetate on androgen-regulated gene-expression (Labrie *et al.*, 1993), but in most animal studies no agonistic effects of cyproterone acetate were found (Neumann *et al.*, 1986; Schröder *et al.*, 1993).
Together these results indicate that, from a clinical point of view, animal studies remain indispensable in the research towards potential new receptor antagonists. However, the molecular aspects of antihormone action can be studied more easily in transfected cell systems, and screening for new antagonists is faster in these systems. Furthermore, the *in vitro* studies could pinpoint research goals towards potential agonistic effects of antagonists in intact animals. As such, transfected cell systems are the methods of first choice in the development of new antihormones.

### 7.5 Future investigations

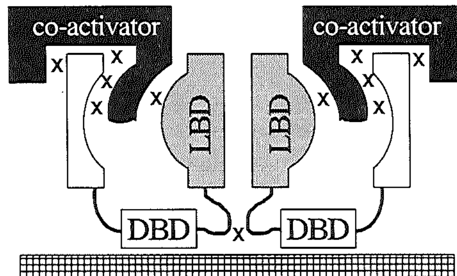
The determination of the three-dimensional structures of the ligand binding domains of the RXRα, RARγ and TRα by crystallography and the prediction of structural homologies between the diverse members of the nuclear receptor superfamily are major achievements in the study of receptor activation (Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995; Wurtz *et al.*, 1996). Despite these achievements, determination of the precise three-dimensional structure of the ligand binding domain of the AR, and other steroid hormone receptors, by crystallography is still a major goal for the future. In addition, a computer modeled structure of the ligand binding domain of the AR can be validated by designing mutations in key amino acid residues and testing of their functional consequences. Recognition of the key amino acid residues which are important in ligand binding will increase our knowledge of the interactions between the ligand and the ligand binding domain. The results of these studies should provide an increased knowledge, applicable in the design of novel agonists but certainly also of antagonists with potential clinical use.
The results described by us (Chapter 6) and others (Langley *et al.*, 1995) suggest that the N-terminal and ligand binding domains may interact with each other in the agonist-bound full-length AR. In Figure 7.3, we have modeled possible interactions between both domains in the context of a full-length receptor. These models should provide the framework for further research towards the association between the N-terminal and ligand binding domain. So far, we were not able to distinguish between a direct interaction of the N-terminal and ligand binding domains within the AR (Fig. 7.3A) and one that involves additional intermediary proteins (Fig. 7.3B). Since we were unable to observe an interaction between separately produced N-terminal and ligand binding domains in immunoprecipitation experiments (C. Berrevoets, data not shown), it is likely that interaction between the domains would involve additional factors such as the recently discovered co-activators (Halachmi *et al.*, 1994; Oñate *et al.*, 1995; Yeh and Chang, 1996). Demonstration that the already discovered or newly isolated additional factors migth be involved in interaction between the N-terminal and ligand binding domains would be a major goal for the future. Furthermore, the present data do not discriminate between an intramolecular (within one receptor molecule) and an intermolecular (between two receptor molecules) interaction. A direct intermolecular interaction, as proposed

by Langley *et al.* (1995), would result in an anti-parallel orientation of a ligand-activated androgen receptor dimer (Fig. 7.3C). Experimental evidence for either a parallel or an anti-parallel orientation of the receptor-dimer is hard to obtain; presumably the answer can only be obtained by analysis of the three-dimensional structure of full-length receptor dimers. In addition, intermolecular interaction may also involve additional intermediary proteins (not shown in Fig. 7.3). Experiments with deletion mutants, as also performed to determine the activation domain(s) of the receptor (Jenster *et al.*, 1995), should reveal the precise regions involved in interaction of the N-terminal and ligand binding domains of the AR.

A. Direct, intramolecular interaction (hormone-induced)




B. Indirect, intramolecular interaction (hormone-induced)




C. Direct, intermolecular interaction (hormone-induced)

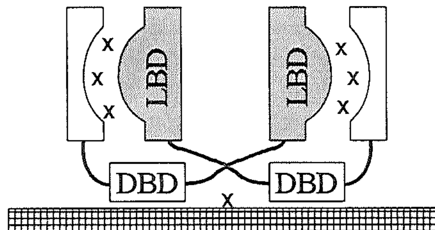



***Figure 7.3.*** *Models for the ligand-dependent, transcriptionally productive interaction of the N-terminal and ligand binding domains of the AR. A description of the model is provided in Section 7.5 Future investigations. X represents protein-protein interactions.*

As a result of the studies described in this thesis, we now have some structural information on the molecular interactions of both agonists and antagonists with the ligand binding domain of the AR, and the differential effects of ligand binding on the transactivation capacity of the AR. However, the determination of the transactivation capacities of the AR is usually performed in transient transfected cell systems on artificial promoters (Veldscholte *et al.*, 1990; Kemppainen *et al.*, 1992; Chapter 4). The AR, in particular when occupied by an antagonist, may exert regulatory effects on gene expression *in vivo* which are different from what can be observed in transient transfected cell sytems. Therefore, the study of specific androgen-regulated promoters in intact androgen target cells should further enhance our knowledge on the molecular mechanisms of androgen and antiandrogen action.

## REFERENCES

Adler, A. J., M. Danielsen, and D. M. Robins. 1992. Androgen-specific gene activation via a consensus glucocorticoid response element is determined by interaction with nonreceptor factors. *Proc Natl Acad Sci U S A* 89: 11660-3.

Agarwal, M. K. 1994. Analysis of steroid receptor domains with the aid of antihormones. [Review]. *Int J Biochem* 26: 341-50.

Ali, S., D. Metzger, J. M. Bornert, and P. Chambon. 1993. Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. *Embo J* 12: 1153-60.

Allan, G. F., X. Leng, S. Y. Tsai, N. L. Weigel, D. P. Edwards, M. J. Tsai, and B. W. O'Malley. 1992a. Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. *J Biol Chem* 267: 19513-20.

Allan, G. F., S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 1992b. Ligand-dependent conformational changes in the progesterone receptor are necessary for events that follow DNA binding. *Proc Natl Acad Sci U S A* 89: 11750-4.

Alnemri, E. S., A. B. Maksymowych, N. M. Robertson, and G. Litwack. 1991. Overexpression and characterization of the human mineralocorticoid receptor. *J Biol Chem* 266: 18072-81.

Amero, S. A., R. H. Kretsinger, N. D. Moncrief, K. R. Yamamoto, and P. R. Pearson. 1992. The origin of nuclear recptor proteins: a single precursor different from other transcription factors. *Mol Endocrinol* 6: 3-7.

Andersen, B., G. C. Kennedy, and J. H. Nilson. 1990. A cis-acting element located between the cAMP response elements and CCAAT box augments cell-specific expression of the glycoprotein hormone α subunit gene. *J Biol Chem* 263: 21874-80.

Archer, T. K., P. Lefebvre, R. G. Wolfer, and G. L. Hager. 1992. Transcription factor loading on the MMTV-promoter: a bimodal mechanism for promoter activation. *Science* 255: 1573-6.

Archer, T. K., E. Zaniewski, M. L. Moyer, and S. K. Nordeen. 1994. The differential capacity of glucocorticoids and progestins to alter chromatin structure and induce gene expression in human breast cancer cells. *Mol Endocrinol* 8: 1154-62.

Arnold, S. F., D. P. Vorojeikina, and A. C. Notides. 1995. Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element. *J Biol Chem* 270: 30205-30212.

Auphan, N., J. A. DiDonato, C. Rosette, A. Helmberg, and M. Karin. 1995. Immunosuppression by glucocorticoids: Inhibition of NF-κB activity through induction of IκB synthesis. *Science* 270: 286-90.

Auricchio, F. 1989. Phosphorylation of steroid receptors. [Review]. *J Steroid Biochem* 32: 613-22.

Bagchi, M. K., S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 1990. Identification of a functional intermediate in receptor activation in progesterone-dependent cell-free transcription. *Nature* 345: 547-50.

Bagchi, M. K., S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 1991. Progesterone enhances target gene transcription by receptor free of heat-shock proteins hsp90, hsp56, and hsp70. *Mol Cell Biol* 11: 4998-5004.

Bai, W., S. Tullos, and N. L. Weigel. 1994. Phosphorylation of Ser530 facilitates hormone-dependent transcriptional activation of the chicken progesterone receptor. *Mol Endocrinol* 8: 1465-73.

Baniahmad, A., and M. J. Tsai. 1993. Mechanisms of transcriptional activation by steroid hormone receptors. [Review]. *J Cell Biochem* 51: 151-6.

Baniahmad, C., Z. Nawaz, A. Baniahmad, M. A. Gleeson, M. J. Tsai, and B. W. O'Malley. 1995. Enhancement of human estrogen receptor activity by SPT6: a potential coactivator. *Mol Endocrinol* 9: 34-43.

Barettino, D., M. M. Vivanco-Ruiz, and H. G. Stunnenberg. 1994. Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. *Embo J* 13: 3039-49.

Barnes, P. J., and I. Adcock. 1993. Anti-inflammatory actions of steroids: molecular mechanisms. [Review]. *Trends Pharmacol Sci* 14: 436-41.

Barrack, E. R. 1987. Steroid hormone receptor localization in the nuclear matrix: interaction with acceptor sites. *J Steroid Biochem* 27: 115-21.

Battmann, T., A. Bonfils, C. Branche, J. Humbert, F. Goubet, G. Teutsch, and D. Philibert. 1994. RU 58841, a new specific topical antiandrogen: a candidate of choice for the treatment of acne, androgenetic alopecia and hirsutism. *J Steroid Biochem Mol Biol* 48: 55-60.

Bauerle, P. A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system [review]. *Ann Rev Immunol* 12: 141-79.

Baulieu, E. E. 1989. Contragestion and other clinical applications of RU 486, an antiprogesterone at the receptor. *Science* 245: 1351-7.

Baumann, H., K. Paulsen, H. Kovacs, H. Berglund, A. P. Wright, J. A. Gustafsson, and T. Hard. 1993. Refined solution structure of the glucocorticoid receptor DNA-binding domain. *Biochemistry* 32: 13463-71.

Baur, E. V., C. Zechel, D. Heery, M. J. S. Heine, J. M. Garnier, V. Vivat, B. Ledouarin, H. Gronemeyer, P. Chambon, and R. Losson. 1996. Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *Embo J* 15: 110-124.

Beato, M. 1989. Gene regulation by steroid hormones. [Review]. *Cell* 56: 335-44.

Beato, M., P. Herrlich, and G. Schutz. 1995. Steroid hormone receptors: Many actors in search of a plot. *Cell* 83: 851-857.

Beck, C. A., P. A. Estes, B. J. Bona, Cacho Ca Muro, S. K. Nordeen, and D. P. Edwards. 1993. The steroid antagonist RU486 exerts different effects on the glucocorticoid and progesterone receptors. *Endocrinology* 133: 728-40.

Beck, C. A., N. L. Weigel, and D. P. Edwards. 1992. Effects of hormone and cellular modulators of protein phosphorylation on transcriptional activity, DNA binding, and phosphorylation of human progesterone receptors. *Mol Endocrinol* 6: 607-20.

Beekman, J. M., G. F. Allan, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 1993. Transcriptional activation by the estrogen receptor requires a conformational change in the ligand binding domain. *Mol Endocrinol* 7: 1266-74.

Benhamou, B., T. Garcia, T. Lerouge, A. Vergezac, D. Gofflo, C. Bigogne, P. Chambon, and H. Gronemeyer. 1992. A single amino acid that determines the sensitivity of progesterone receptors to RU486. *Science* 255: 206-9.

Bergmann, K. E., C. H. Wooge, K. E. Carlson, B. S. Katzenellenbogen, and J. A. Katzenellenbogen. 1994. Bivalent ligands as probes of estrogen receptor action. *J Steroid Biochem Mol Biol* 49: 139-52.

Berrevoets, C. A., J. Veldscholte, and E. Mulder. 1993. Effects of antiandrogens on transformation and transcription activation of wild-type and mutated (LNCaP) androgen receptors. *J Steroid Biochem Mol Biol* 46: 731-6.

Berry, M., D. Metzger, and P. Chambon. 1990. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *Embo J* 9: 2811-8.

Berthois, Y., M. Pons, C. Dussert, Crastes, A de Paulet, and P. M. Martin. 1994. Agonist-antagonist activity of anti-estrogens in the human breast cancer cell line MCF-7: an hypothesis for the interaction with a site distinct from the estrogen binding site. *Mol Cell Endocrinol* 99: 259-68.

Bevan, C. L., B. B. Brown, H. R. Davies, B. A. J. Evans, I. A. Hughes, and M. N. Patterson. 1996. Functional analysis of six androgen receptor mutations identified in patients with partial androgen insensitivity syndrome. *Hum Mol Genet* 5: 265-273.

Bocquel, M. T., J. Ji, T. Ylikomi, B. Benhamou, A. Vergezac, P. Chambon, and H. Gronemeyer. 1993. Type II antagonists impair the DNA binding of steroid hormone receptors without affecting dimerization. *J Steroid Biochem Mol Biol* 45: 205-15.

Bocquel, M. T., V. Kumar, C. Stricker, P. Chambon, and H. Gronemeyer. 1989. The contribution of the N- and C-terminal regions of steroid receptors to activation of transcription is both receptor and cell-specific. *Nucleic Acids Res* 17: 2581-95.

Bodwell, J. E., E. Orti, J. M. Coull, D. J. Pappin, L. I. Smith, and F. Swift. 1991. Identification of phosphorylated sites in the mouse glucocorticoid receptor. *J Biol Chem* 266: 7549-55.

Bohen, S. P. 1995. Hsp90 mutants disrupt glucocorticoid receptor ligand binding and destabilize aporeceptor complexes. *J Biol Chem* 270: 29433-29438.

Bohen, S. P., A. Kralli, and K. R. Yamamoto. 1995. Hold 'em and fold 'em: chaperones and signal transduction. [Review]. *Science* 268: 1303-4.

Bourguet, W., M. Ruff, P. Chambon, H. Gronemeyer, and D. Moras. 1995. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. *Nature* 375: 377-82.

Bresnick, E. H., F. C. Dalman, E. R. Sanchez, and W. B. Pratt. 1989. Evidence that the 90-kDa heat-shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. *J Biol Chem* 264: 4992-7.

Brinkmann, A. O., P. W. Faber, H. C. van Rooij, G. G. J. M. Kuiper, C. Ris, P. Klaassen, J. A. G. M. van der

## References


Korput, M. M. Voorhorst, van Laar J. H., E. Mulder, and J. Trapman. 1989. The human androgen receptor: domain structure, genomic organization and regulation of expression. *J Steroid Biochem* 34: 307-10.

Buratowski, S. 1994. The basics of basal transcription by RNA polymerase II. *Cell* 77: 1-3.

Byravan, S., J. Milhon, S. K. Rabindran, B. Olinger, M. J. Garabedian, M. Danielsen, and M. R. Stallcup. 1991. Two point mutations in the hormone-binding domain of the mouse glucocorticoid receptor that dramatically reduce its function. *Mol Endocrinol* 5: 752-8.

Cadepond, F., Groyer G. Schweizer, Maurel I. Segard, N. Jibard, S. M. Hollenberg, V. Giguere, R. M. Evans, and E. E. Baulieu. 1991. Heat-shock protein 90 as a critical factor in maintaining glucocorticosteroid receptor in a nonfunctional state. *J Biol Chem* 266: 5834-41.

Cairns, C., W. Cairns, and S. Okret. 1993. Inhibition of gene expression by steroid hormone receptors via a negative glucocorticoid response element: evidence for the involvement of DNA-binding and agonistic effects of the antiglucocorticoid/antiprogestin RU486. *DNA Cell Biol* 12: 695-702.

Caldenhoven, E., J. Liden, S. Wissink, A. Van de Stolpe, J. Raaijmakers, L. Koenderman, S. Okret, J.-A. Gustafsson, and P. T. Van der Saag. 1995. Negative cross-talk between relA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol Endocrinol* 9: 401-12.

Carlstedt-Duke, J., P. E. Stromstedt, B. Persson, E. Cederlund, J. A. Gustafsson, and H. Jornvall. 1988. Identification of hormone-interacting amino acid residues within the steroid-binding domain of the glucocorticoid receptor in relation to other steroid hormone receptors. *J Biol Chem* 263: 6842-6.

Carson-Jurica, M., A. T. Lee, A. W. Dobson, O. M. Conneely, W. T. Schrader, and B. W. O'Malley. 1989. Interaction of the chicken progesterone receptor with heat-shock protein (HSP) 90. *J Steroid Biochem* 34: 1-9.

Carson-Jurica, M., W. T. Schrader, and B. W. O'Malley. 1990. Steroid receptor family: structure and functions. [Review]. *Endocr Rev* 11: 201-20.

Castoria, G., A. Migliaccio, S. Green, M. Di-Domenico, P. Chambon, and F. Auricchio. 1993. Properties of purified estradiol-dependent calf uterus tyrosine kinase. *Biochemistry* 32: 1740-50.

Cavailles, V., S. Dauvois, P. S. Danielian, and M. G. Parker. 1994. Interaction of proteins with transcriptionally active estrogen receptors. *Proc Natl Acad Sci U S A* 91: 10009-13.

Cavailles, V., S. Dauvois, F. L'Horset, G. Lopez, S. Hoare, P. J. Kushner, and M. G. Parker. 1995. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *Embo J* 14: 3741-51.

Chambraud, B., M. Berry, G. Redeuilh, P. Chambon, and E.-E. Baulieu. 1990. Several regions of human estrogen receptor are involved in the formation of receptor-heat-shock protein 90 complexes. *J Biol Chem* 265: 20686-91.

Chang, C. S., J. Kokontis, and S. T. Liao. 1988. Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc Natl Acad Sci U S A* 85: 7211-5.

Chen, J. Y., S. Penco, J. Ostrowski, P. Balaguer, M. Pons, J. E. Starrett, P. Reczek, P. Chambon, and H. Gronemeyer. 1995. RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *Embo J* 14: 1187-97.

Clay, C. M., R. A. Keri, A. B. Finicle, L. L. Heckert, D. L. Hamernik, K. M. Marschke, E. M. Wilson, F. S. French, and J. H. Nilson. 1993. Transcriptional repression of the glycoprotein hormone α subunit gene by androgen may involve direct binding of androgen receptor to the proximal promoter. *J Biol Chem* 268: 13556-64.

Cook, E. C., M. C. Wani, Y. W. Lee, P. A. Fail, and V. Petrow. 1992. Reversal of activity profile in analogs of the antiprogestin RU 486: effect of a 16α-substituent on progestational (agonist) activity. *Life Sci* 52: 155-62.

Courey, A. J., D. A. Holtzman, and S. P. Jackson. 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. *Cell* 55: 887-98.

Culig, Z., A. Hobisch, M. V. Cronauer, A. C. Cato, A. Hittmair, C. Radmayr, J. Eberle, G. Bartsch, and H. Klocker. 1993. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. *Mol Endocrinol* 7: 1541-50.

Dahlman-Wright, K., T. Almlof, I. J. McEwan, J. A. Gustafsson, and A. P. Wright. 1994. Delineation of a small region within the major transactivation domain of the human glucocorticoid receptor that mediates transactivation of gene expression. *Proc Natl Acad Sci U S A* 91: 1619-23.

Dalman, F. C., E. H. Bresnick, P. D. Patel, G. H. Perdew, Sj Jr Watson, and W. B. Pratt. 1989. Direct evidence that the glucocorticoid receptor binds to hsp90 at or near the termination of receptor translation in vitro. *J Biol*

*Chem* 264: 19815-21.

Danielian, P. S., R. White, J. A. Lees, and M. G. Parker. 1992. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *Embo J* 11: 1025-33.

Danielsen, M., L. Hinck, and G. M. Ringold. 1989. Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* 57: 1131-8.

Danielsen, M., J. P. Northrop, J. Jonklaas, and G. M. Ringold. 1987. Domains of the glucocorticoid receptor involved in specific and nonspecific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement. *Mol Endocrinol* 1: 816-22.

de Ruiter, P. E., R. Teuwen, J. Trapman, R. Dijkema, and A. O. Brinkmann. 1995. Synergism between androgens and protein kinase-C on androgen-regulated gene expression. *Mol Cell Endocrinol* 110: R1-R6.

de Vos, P. , F. Cleassens, L. Celis, B. Peeters, W. Rombauts, W. Heyns, and G Verhoeven. 1994. Nuclear extracts enhance the interaction fusion proteins containing the DNA-binding domain of androgen and glucocorticoid receptor with androgen and glucocorticoid response elements. *J Steroid Biochem Molec Biol* 48: 317-23.

Delabre, K., A. Guiochon-Mantel, and E. Milgrom. 1993. In vivo evidence against the existence of antiprogestins disrupting receptor binding to DNA. *Proc Natl Acad Sci U S A* 90: 4421-5.

DeMarzo, A. M., S. A. Onate, S. K. Nordeen, and D. P. Edwards. 1992. Effects of the steroid antagonist RU486 on dimerization of the human progesterone receptor. *Biochemistry* 31: 10491-501.

Denis, M., L. Poellinger, A. C. Wikstom, and J. A. Gustafsson. 1988. Requirement of hormone for thermal conversion of the glucocorticoid receptor to a DNA-binding state. *Nature* 333: 686-8.

Denner, L. A., W. T. Schrader, B. W. O'Malley, and N. L. Weigel. 1990. Hormonal regulation and identification of chicken progesterone receptor phosphorylation sites. *J Biol Chem* 265: 16548-55.

Dobson, A. D. W., O. M. Coonely, W. Beattie, B. L. Maxwell, P. Mak, M.-J. Tsai, W. T. Schrader, and B. W. O'Malley. 1989. Mutational analysis of the chicken progesterone receptor. *J Biol Chem* 264: 4207-11.

Dorit, R. L., L. Schoenbach, and W. Gilbert. 1990. How big is the universe of exons? *Science* 250: 1377-82.

Drouin, J., Y. L. Sun, M. Chamberland, Y. Gauthier, Lean A. De, M. Nemer, and T. J. Schmidt. 1993. Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. *Embo J* 12: 145-56.

El-Ashry, D., S. A. Onate, S. K. Nordeen, and D. P. Edwards. 1989. Human progesterone receptor complexed with the antagonist RU 486 binds to hormone response elements in a structurally altered form. *Mol Endocrinol* 3: 1545-58.

Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. [Review]. *Science* 240: 889-95.

Faber, P. W., G. G. J. M. Kuiper, H. C. J. van Rooij, J. A. G. M. van der Korput, A. O. Brinkmann, and J. Trapman. 1989. The N-terminal domain of the human androgen receptor is encoded by one large exen. *Mol Cell Endocrinol* 61: 257-62.

Fanjull, A., M. I. Dawson, P. D. Hobbs, L. Jong, J. F. Cameron, E. Harlev, G. Graupner, X. P. Lu, and M. Pfahl. 1992. A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature* 372: 107-11.

Fawell, S. E., R. White, S. Hoare, M. Sydenham, M. Page, and M. G. Parker. 1990. Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. *Proc Natl Acad Sci U S A* 87: 6883-7.

Fields, S., and O. Song. 1989. A novel system to detect protein-protien interactions. *Nature* 340: 245-6.

Forman, B. M., and H. H. Samuels. 1990. Interaction among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol Endocrinol* 3: 1610-26.

Freedman, L. P. 1992. Anatomy of the steroid receptor zinc finger region. [Review]. *Endocr Rev* 13: 129-45.

Freedman, L. P., B. F. Luisi, Z. R. Korszun, R. Basavappa, P. B. Sigler, and K. R. Yamamoto. 1988. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* 334: 543-6.

Fuhrmann, U., C. Bengtson, G. Repenthin, and E. Schillinger. 1992. Stable transfection of androgen receptor and MMTV-CAT into mammalian cells: inhibition of CAT expression by anti-androgens. *J Steroid Biochem Molec Biol* 42: 787-93.

Furr, B. J. A., B. Valcaccia, B. Curry, J. R. Woodburn, G. Chesterton, and H. Tucker. 1987. ICI 176.334: a novel nonsteroidal peripherally selective antiandrogen. *J Endocrinol* 113: R7-9.

Fuxe, K., A. Harfstrand, L. F. Agnati, Z. Y. Yu, A. Cintra, A. C. Wikstrom, S. Okret, E. Cantoni, and J. A.

## References


Gustafsson. 1985. Immunocytochemical studies on the localization of glucocorticoid receptor immunoreactive nerve cells in the lower brain stem and spinal cord of the male rat using a monoclonal antibody against rat liver glucocorticoid receptor. *Neurosci Lett* 60: 1-6.

Gaddipati, J. P., D. G. McLeod, H. B. Heidenberg, I. A. Sesterhenn, M. J. Finger, J. W. Moul, and S. Srivastava. 1994. Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. *Cancer Res* 54: 2861-4.

Garcia, T., B. Benhamou, D. Gofflo, A. Vergezac, D. Philibert, P. Chambon, and H. Gronemeyer. 1992. Switching agonistic, antagonistic, and mixed transcriptional responses to 11 ß-substituted progestins by mutation of the progesterone receptor. *Mol Endocrinol* 6: 2071-8.

Georgopoulos, C., and W. J. Welch. 1993. Role of the major heat-shock proteins as molecular chaperones. *Ann Rev Cell Biol* 9: 601-34.

Gibson, M. K., L. A. Nemmers, W. C. Beckman Jr, V. L. Davis, S. W. Curtis, and K. S. Korach. 1991. The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. *Endocrinology* 129: 2000-10.

Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucl Acids Res* 20: 1245.

Giguere, V., S. M. Hollenberg, M. G. Rosenfeld, and R. M. Evans. 1986. Functional domains of the human glucocorticoid receptor. *Cell* 46: 645-52.

Goldstein, R. A., J. A. Katzenellenbogen, Z. A. Luthey-Schulten, D. A. Seielstad, and P. G. Wolynes. 1993. Three-dimensional model of the hormone binding domains of steroid receptors. *Proc. Natl. Acad. Sci. USA* 90: 9949-53.

Gorski, J., D. Toft, G. Shyamala, D. Smith, and A. Notides. 1968. Hormone receptors: studies on the interaction of estrogen with the uterus. *Rec Prog Horm Res* 24: 45-81.

Gottlieb, B., M. Trifiro, R. Lumbroso, D. M. Vasiliou, and L. Pinsky. 1996. The androgen receptor gene mutations database. *Nucleic Acids Res* 24: 151-154.

Green, S. 1990. Modulation of oestrogen receptor activity by oestrogens and anti-oestrogens. [Review]. *J Steroid Biochem Mol Biol* 37: 747-51.

Gronemeyer, H. 1991. Transcription activation by estrogen and progesterone receptors. [Review]. *Annu Rev Genet* 25: 89-123.

Gronemeyer, H., B. Benhamou, M. Berry, M. T. Bocquel, D. Gofflo, T. Garcia, T. Lerouge, D. Metzger, M. E. Meyer, and L. Tora. 1992. Mechanisms of antihormone action. [Review]. *J Steroid Biochem Mol Biol* 41: 217-21.

Guiochon-Mantel, A., H. Loosfelt, P. Lescop, S. Sar, M. Atger, M. Perrot-Applanat, and E. Milgrom. 1989. Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. *Cell* 57: 1147-54.

Halachmi, S., E. Marden, G. Martin, H. MacKay, C. Abbondanza, and M. Brown. 1994. Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* 264: 1455-8.

Hard, T., E. Kellenbach, R. Boelens, B. A. Maler, K. Dahlman, L. P. Freedman, Duke J. Carlstedt, K. R. Yamamoto, J. A. Gustafsson, and R. Kaptein. 1990. Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* 249: 157-60.

Harlow, K. W., D. N. Smith, J. A. Katzenellenbogen, G. L. Greene, and B. S. Katzenellenbogen. 1989. Identification of cysteine 530 as the covalent attachment site of an affinity-labeling estrogen (ketononestrol aziridine) and antiestrogen (tamoxifen aziridine) in the human estrogen receptor. *J Biol Chem* 264: 17476-85.

Heck, S., M. Kullmann, A. Gast, H. Ponta, H. J. Rahmsdorf, P. Herrlich, and A. C. Cato. 1994. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *Embo J* 13: 4087-95.

Helmberg, A., N. Auphan, C. Caelles, and M. Karin. 1995. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. *Embo J* 14: 452-60.

Herrlich, P., and H. Ponta. 1994. Mutual cross-modulation of steroid/retinoic acid receptor and AP-1 transcription factor activities. A novel property with practical implications. *Trends Endocrinol Metab* 5: 341-6.

Higuchi, R., B. Krummel, and R. K. Saiki. 1988. A general method for in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucl Acids Res* 16: 7351-67.

Hoeck, W., and B. Groner. 1990. Hormone-dependent phosphorylation of the glucocorticoid receptor occurs

mainly in the amino-terminal transactivation domain. *J Biol Chem* 265: 5403-8.

Hollenberg, S. M., and R. M. Evans. 1988. Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. *Cell* 55: 899-906.

Hu, L. M., J. Bodwell, J. M. Hu, E. Orti, and A. Munck. 1994. Glucocorticoid receptors in ATP-depleted cells. Dephosphorylation, loss of hormone binding, HSP90 dissociation, and ATP-dependent cycling. *J Biol Chem* 269: 6571-7.

Hu, M. C. T., and N. Davidson. 1987. The inducible *lac* operator-repressor system is functional in mammalian cells. *Cell* 48: 455-66.

Hunter, T., and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell* 70: 375-87.

Imakado, S., S. Koike, S. Kondo, M. Sakai, and M. Muramatsu. 1991. The N-terminal transactivation domain of rat estrogen receptor is localized in a hydrophobic domain of eighty amino acids. *J Biochem* 109: 684-9.

Jacq, X., C. Brou, Y. Lutz, I. Davidson, P. Chambon, and L. Tora. 1994. Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. *Cell* 79: 107-17.

Jensen, T., T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut, and E. R. Desombre. 1968. A two-step mechanism for the interaction of estradiol with the rat uterus. *Proc Natl Acad Sci USA* 59: 632-38.

Jenster, G., P. E. de Ruiter, J. A. G. M. van der Korput, G. G. J. M. Kuiper, J. Trapman, and A. O. Brinkmann. 1994. Changes in the abundance of androgen receptor isotypes: effects of ligand treatment, glutamine-stretch variation, and mutation of putative phosphorylation sites. *Biochemistry* 33: 14064-72.

Jenster, G., J. Trapman, and A. O. Brinkmann. 1993. Nuclear import of the human androgen receptor. *Biochem J.* 293: 761-8.

Jenster, G., J. A. G. M. van der Korput, J. Trapman, and A. O. Brinkmann. 1995. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *J Biol Chem* 270: 7341-6.

Jenster, G., J. A. G. M. van der Korput, C. van Vroonhoven, Th. van der Kwast, J. Trapman, and A. O. Brinkmann. 1991. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Mol Endocrinol* 5: 1396-404.

Jonas, T. R., and P. A. Bell. 1980. Glucocorticoid receptor interactions. Studies of the negative co-operativity induced by steroid interactions with a secondary, hydrophobic, binding-site. *Biochem J* 188: 237-45.

Jones, N. 1990. Transcriptional regulation by dimerization: two sides to an incestuous relationship. *Cell* 61: 9-11.

Jorgensen, W. L. 1991. Rusting of the lock and key model for protein-ligand interaction. *Science* 254: 954-5.

Kalkhoven, E., S. Wissink, P. T. Van der Saag, and B. Van der Burg. 1996. Negative interaction between the RelA(p65) subunit of NF-κB and the progesterone receptor. *J Biol Chem.* 271: 6217-23

Kallio, P. J., O. A. Janne, and J. J. Palvimo. 1994b. Agonists, but not antagonists, alter the conformation of the hormone-binding domain of androgen receptor. *Endocrinology* 134: 998-1001.

Kallio, P. J., J. J. Palvimo, M. Mehto, and O. A. Janne. 1994a. Analysis of androgen receptor-DNA interactions with receptors produced in insect cells. *J Biol Chem* 269: 11514-22.

Kallio, P. J., H. Poukka, A. Moilanen, O. A. Janne, and J. J. Palvimo. 1995. Androgen receptor-mediated transcriptional regulation in the absence of direct interaction with a specific DNA element. *Mol Endocrinol* 9: 1017-28.

Kaspar, F., H. Klocker, A. Denninger, and A. C. Cato. 1993. A mutant androgen receptor from patients with Reifenstein syndrome: identification of the function of a conserved alanine residue in the D box of steroid receptors. *Mol Cell Biol* 13: 7850-8.

Kato, S., H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima, D. Metzger, and P. Chambon. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270: 1491-1494.

Katzenellenbogen, J. A., B. W. Omalley, and B. S. Katzenellenbogen. 1996. Tripartite steroid hormone receptor pharmacology: Interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Mol Endocrinol* 10: 119-131.

Keidel, S., P. LeMotte, and C. Apfel. 1994. Different agonist- and antagonist-induced conformational changes in retinoic acid receptors analyzed by protease mapping. *Mol Cell Biol* 14: 287-98.

Kemppainen, J. A., M. V. Lane, M. Sar, and E. M. Wilson. 1992. Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. *J Biol Chem* 267: 968-74.

## References

King, W. J., and G. L. Greene. 1984. Monoclonal antibodies localize the estrogen receptor in the nuclei of target cells. *Nature* 307: 745-47.

Klein-Hitpass, L., A. C. B. Cato, D. Henderson, and G. U. Ryffel. 1991. Two types of antiprogestins identified by their differential action in transcriptionally active extracts fromT47D cells. *Nucleic Acids Res* 19: 1227-34.

Klein-Hitpass, L., S. Y. Tsai, N. L. Weigel, G. F. Allan, D. Riley, R. Rodriguez, W. T. Schrader, M. J. Tsai, and B. W. O'Malley. 1990. The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex. *Cell* 60: 247-57.

Knegtel, R. M., M. Katahira, J. G. Schilthius, A. M. Bonvin, R. Boelens, D. Eib, P. T. van der Saag, and R. Kaptein. 1993. The solution structure of the human retinoic acid receptor-ß DNA-binding domain. *J Biomol NMR* 3: 1-17.

Koleske, A. J., and R. A. Young. 1995. The RNA polymerase II holoenzyme and its implications for gene regulation [review]. *Trends Biochem Sci* 20: 113-6.

Kralli, A., S. P. Bohen, and K. R. Yamamoto. 1995. LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. *Proc Natl Acad Sci U S A* 92: 4701-5.

Kraus, W. L., E. M. McInerney, and B. S. Katzenellenbogen. 1995. Ligand-dependent, transcriptionally productive association of the amino- and carboxyl-terminal regions of a steroid hormone nuclear receptor. *Proc Natl Acad Sci U S A* 92: 12314-18.

Krongrad, A., C. M. Wilson, J. D. Wilson, D. R. Allman, and M. J. McPhaul. 1991. Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells. *Mol Cell Endocrinol* 76: 79-88.

Kuil, C. W., C. A. Berrevoets, and E. Mulder. 1995. Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization - Studies on the mechanism of antiandrogen action. *J Biol Chem* 270: 27569-27576.

Kuil, C. W., and E. Mulder. 1994. Mechanism of antiandrogen action: conformational changes of the receptor. *Mol Cell Endocrinol* 102: R1-R5

Kuil, C. W., and E. Mulder. 1996. Deoxyribonucleic acid-binding ability of androgen receptors in whole cells: Implications for the action of androgens and antiandrogens. *Endocrinology* 137: 1870-1877.

Kuiper, G. G. J. M., and A. O. Brinkmann. 1994. Steroid hormone receptor phosphorylation: is there a physiological role?. [Review]. *Mol Cell Endocrinol* 100: 103-7.

Kuiper, G. G. J. M., P. E. de Ruiter, J. A. Grootegoed, and A. O. Brinkmann. 1991. Synthesis and post-translational modification of the androgen receptor in LNCaP cells. *Mol Cell Endocrinol* 80: 65-73.

Kuiper, G. G. J. M., P. E. de Ruiter, J. Trapman, W. J. Boersma, J. A. Grootegoed, and A. O. Brinkmann. 1993a. Localization and hormonal stimulation of phosphorylation sites in the LNCaP-cell androgen receptor. *Biochem J* 291:95-101.

Kuiper, G. G. J. M., P. E. de Ruiter, J. Trapman, G. Jenster, and A. O. Brinkmann. 1993b. In vitro translation of androgen receptor cRNA results in an activated androgen receptor protein. *Biochem J* 296: 161-7.

Kumar, V., and P. Chambon. 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* 55: 145-56.

Kumar, V., S. Green, G. Stack, M. Berry, J. R. Jin, and P. Chambon. 1987. Functional domains of the human estrogen receptor. *Cell* 51: 941-51.

Kupfer, S. R., K. B. Marschke, E. M. Wilson, and F. S. French. 1993. Receptor accessory factor enhances specific DNA binding of androgen and glucocorticoid receptors. *J Biol Chem* 268: 17519-27.

Kutoh, E., P. E. Stromstedt, and L. Poellinger. 1992. Functional interference between the ubiquitous and constitutive octamer transcription factor 1 (OTF-1) and the glucocorticoid receptor by direct protein-protein interaction involving the homeo subdomain of OTF-1. *Mol Cell Biol* 12: 4960-9.

Labrie, F. 1993. Mechanism of action and pure antiandrogenic properties of flutamide. *Cancer* 73: 3816-27.

Laemmli, U. K. 1970. Changes of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-5.

Langley, E., Z. X. Zhou, and E. M. Wilson. 1995. Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. *J Biol Chem* 270: 29983-29990.

Lanz, R. B., and S. Rusconi. 1994. A conserved carboxy-terminal subdomain is important for ligand interpretation and transactivation by nuclear receptors. *Endocrinology* 135: 2183-95.

LaSpada, A. R., E. M. Wilson, D. B. Lubahn, A. E. Harding, and K. H. Fischbeck. 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 352: 77-9.

Laudet, V., C. Hänni, J. Coll, F. Catzeflis, and D. Stéhelin. 1992. Evolution of the nuclear receptor gene superfamily. *EMBO J* 11: 1003-13.

Le Douarin, B., C. Zechel, J. M. Garnier, Y. Lutz, L. Tora, B Pierrat, D. Heery, H. Gronemeyer, P. Chambon, and R. Losson. 1995. The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J* 14: 2020-33.

Lee, J. W., F. Ryan, J. C. Swaffield, S. A. Johnston, and D. D. Moore. 1995. Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. *Nature* 374: 91-4.

Lee, M. S., S. A. Kliewer, J. Provencal, P. E. Wright, and Evans R. M. 1993. Structure of the retinoic X receptor α DNA-binding domain: a helix required for homodimeric DNA binding. *Science* 260: 1117-21.

Lees, J. A., S. E. Fawell, and M. G. Parker. 1989. Identification of two transactivation domains in the mouse oestrogen receptor. *Nucleic Acids Res* 17: 5477-88.

Lees, J. A., S. E. Fawell, R. White, and M. G. Parker. 1990. A 22-amino-acid peptide restores DNA-binding activity to dimerization-defective mutants of the estrogen receptor. *Mol Cell Biol* 10: 5529-31.

LeGoff, P., M. M. Montano, D. J. Schodin, and B. S. Katzenellenbogen. 1994. Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J Biol Chem* 269: 4458-66.

Lemesle-Varloot, L., T. Ojasoo, J. P. Mornon, and J. P. Raynaud. 1992. A model for the determination of the 3D-spatial distribution of the functions of the hormone-binding domain of receptors that bind 3-keto-4-ene steroids. *J Steroid Biochem Mol Biol* 41: 369-88.

Leppa, S., M. Mali, H. M. Miettinen, and M. Jalkanen. 1992. Syndecan expression regulates cell morphology and growth of mouse mammary tumor cells. *Proc Natl Acad Sci USA* 89: 932-36.

Levine, M., and J. L. Manley. 1989. Transcriptional repression of eukaryotic promoters. *Cell* 59: 405-8.

Lindzey, J., M. Grossmann, M. V. Kumar, and D. J. Tindall. 1993. Regulation of the 5'-flanking region of the mouse androgen receptor gene by cAMP and androgen. *Mol Endocrinol* 7: 1530-40.

Lubahn, D. B., D. R. Joseph, M. Sar, J. Tan, H. N. Higgs, R. E. Larson, F. S. French, and E. M. Wilson. 1988. The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. *Mol Endocrinol* 2: 1265-75.

Luisi, B. F., W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler. 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 352: 497-505.

Mader, S., V. Kumar, Verneuil H. de, and P. Chambon. 1989. Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* 338: 271-4.

Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans. 1995. Overview: The nuclear receptor superfamily: The second decade. *Cell* 83: 835-839.

Marivoet, S., P. van Dijck, G. Verhoeven, and W. Heyns. 1992. Interaction of the 90-kDa heat-shock protein with native and in vitro translated androgen receptor and receptor fragments. *Mol Cell Endocrinol* 88: 165-74.

Martins, V. R., W. B. Pratt, L. Terracio, M. A. Hirst, G. M. Ringold, and P. R. Housley. 1991. Demonstration by confocal microscopy that unliganded overexpressed glucocorticoid receptors are distributed in a nonrandom manner throughout all planes of the nucleus. *Mol Endocrinol* 5: 217-25.

Mason, S. A., and P. R. Housley. 1993. Site-directed mutagenesis of the phosphorylation sites in the mouse glucocorticoid receptor. *J Biol Chem* 268: 21501-4.

McDonnell, D. P. 1995. Unraveling the human progesterone rceptor signalling transduction pathway. *Trends Endocrinol Metab* 6: 133-8.

McDonnell, D. P., D. L. Clemm, T. Hermann, M. E. Goldman, and J. W. Pike. 1995. Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. *Mol Endocrinol* 9: 659-69.

McDonnell, D. P., D. J. Mangelsdorf, J. W. Pike, M. R. Haussler, and B. W. O'Malley. 1987. Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science* 235: 1214-7.

McEwan, I. J., T. Almlof, A. C. Wikstrom, K. Dahlman-Wright, A. P. Wright, and J. A. Gustafsson. 1994. The glucocorticoid receptor functions at multiple steps during transcription initiation by RNA polymerase II. *J Biol Chem* 269: 25629-36.

McEwan, I. J., A. P. Wright, K. Dahlman-Wright, Duke J. Carlstedt, and J. A. Gustafsson. 1993. Direct interaction of the tau 1 transactivation domain of the human glucocorticoid receptor with the basal transcriptional machinery. *Mol Cell Biol* 13: 399-407.

## References

McPhaul, M. J., M. Marcelli, S. Zoppi, C. M. Wilson, J. E. Griffin, and J. D. Wilson. 1992. Mutations in the ligand-binding domain of the androgen receptor gene cluster in two regions of the gene. *J Clin Invest* 90: 2097-101.

Metzger, D., M. Berry, S. Ali, and P. Chambon. 1995. Effectes of antagonists on DNA binding properties of the human estrogen receptor *in vitro* and *in vivo*. *Mol Endocrinol* 9: 579-91.

Meyer, M. E., H. Gronemeyer, B. Turcotte, M. T. Bocquel, D. Tasset, and P. Chambon. 1989. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* 57: 433-42.

Meyer, M. E., A. Pornon, J. W. Ji, M. T. Bocquel, P. Chambon, and H. Gronemeyer. 1990. Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. *Embo J* 9: 3923-32.

Meyer, M. E., Stricker C. Quirin, T. Lerouge, M. T. Bocquel, and H. Gronemeyer. 1992. A limiting factor mediates the differential activation of promoters by the human progesterone receptor isoforms. *J Biol Chem* 267: 10882-7.

Montano, M. M., K. Ekena, K. D. Krueger, A. L. Keller, and B. S. Katzenellenbogen. 1996. Human estrogen receptor ligand activity inversion mutants: Receptors that interpret antiestrogens as estrogens and estrogens as antiestrogens and discriminate among different antiestrogens. *Mol Endocrinol* 10: 230-242.

Montano, M. M., V. Muller, A. Trobaugh, and B. S. Katzenellenbogen. 1995. The carboxyl-terminal F-domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol* 9: 814-25.

Mooradian, A. D., J. E. Morley, and S. G. Korenman. 1987. Biological actions of androgens [review]. *Endocrine Rev* 8: 1-28.

Moudgil, V. K., M. J. Anter, and C. Hurd. 1989. Mammalian progesterone receptor shows differential sensitivity to sulfhydryl group modifying agents when bound to agonist and antagonist ligands. *J Biol Chem* 264: 2203-11.

Moudgil, V. K., and C. Hurd. 1987. Transformation of calf uterine progesterone receptor: analysis of the process when receptor is bound to progesterone and RU38486. *Biochemistry* 26: 4993-5001.

Mowszowicz, I., A. Doukani, and M. Giacomini. 1988. Binding of the androgen receptor to the nuclear matrix of human foreskin. *J Steroid Biochem* 29: 715-9.

Moyer, M. L., K. C. Borror, B. J. Bona, D. B. DeFranco, and S. K. Nordeen. 1993. Modulation of cell signaling pathways can enhance or impair glucocorticoid-induced gene expression without altering the state of receptor phosphorylation. *J Biol Chem* 268: 22933-40.

Mymryk, J. S., and T. K. Archer. 1995a. Dissection of progesterone receptor-mediated chromatin remodeling and transcriptional activation in vivo. *Genes Dev* 9: 1366-76.

Mymryk, J. S., and T. K. Archer. 1995b. Influence of hormone antagonists on chromatin remodeling and transcription factor binding to the mouse mammary tumor virus promoter in vivo. *Mol Endocrinol* 9: 1825-1834.

Nemoto, T., Y. Ohara-Nemoto, and M. Ota. 1992. Association of the 90-kDa heat-shock protein does not affect the ligand-binding ability of androgen receptor. *J Steroid Biochem Mol Biol* 42: 803-12.

Neri, R. O., and M. Monahan. 1972. Effects of a novel nonsteroidal antiandrogen on canine prostatic hyperplasia. *Invest Urol* 10: 123-30.

Neumann, F., and M. Topert. 1986. Pharmacology of antiandrogens. *J Steroid Biochem* 25: 885-95.

Newmark, J. R., D. O. Hardy, D. C. Tonb, B. S. Carter, J. I. Epstein, W. B. Isaacs, T. R. Brown, and E. R. Barrack. 1992. Androgen receptor gene mutations in human prostate cancer. *Proc Natl Acad Sci U S A* 89: 6319-23.

O'Malley, B. W. 1989. Did eucaryotic steroid receptors evolve from intracrine gene regulators? *Endocrinology* 125: 1119-20.

O'Malley, B. W., and O. M. Coonely. 1992. Orphan receptors: in search for a unifying hypothesis for activation. *Mol Endocrinol* 6: 1359-61.

Onate, S. A., S. Y. Tsai, M. J. Tsai, and B. W. Omalley. 1995. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270: 1354-1357.

Orti, E., L. M. Hu, and A. Munck. 1993. Kinetics of glucocorticoid receptor phosphorylation in intact cells. Evidence for hormone-induced hyperphosphorylation after activation and recycling of hyperphosphorylated receptors. *J Biol Chem* 268: 7779-84.

Palvimo, J. J., P. J. Kallio, T. Ikonen, M. Mehto, and O. A. Janne. 1993. Dominant negative regulation of trans-activation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation. *Mol*

*Endocrinol* 7: 1399-407.

Pan, T., L. P. Freedman, and J. E. Coleman. 1990. Cadmium-113 NMR studies of the DNA binding domain of the mammalian glucocorticoid receptor. *Biochemistry* 29: 9218-25.

Paris, F., G. F. Weinbauer, V. Blum, and E. Nieschlag. 1994. The effect of androgens and antiandrogens on the immunohistochemicl localization of the androegn receptor in accessory reproductive organs of male rats. *J Steroid Biochem Molec Biol* 48: 129-37.

Pearce, D., and K. R. Yamamoto. 1993. Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. *Science* 259: 1161-5.

Perrot-Applanat, M., F. Logeat, M. T. Groyer-Picard, and E. Milgrom. 1985. Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology* 116: 1473-84.

Persson, H., C. A. L. Lievre, O. Soder, M. J. Villar, M. Metsis, L. Olson, M. Ritzen, and T Hokfelt. 1990. Expression of β-nerve growth factor receptor mRNA in Sertoli cells down-regulated by testosterone. *Science* 247: 704-7.

Pfahl, M., R. Apfel, I. Bendik, A. Fanjul, G. Graupner, M. O. Lee, Vista N. La, X. P. Lu, J. Piedrafita, and M. A. Ortiz. 1994. Nuclear retinoid receptors and their mechanism of action. [Review]. *Vitam Horm* 49: 327-82.

Pham, T. A., J. F. Elliston, Z. Nawaz, D. P. McDonnell, M. J. Tsai, and B. W. O'Malley. 1991. Antiestrogen can establish nonproductive receptor complexes and alter chromatin structure at target enhancers. *Proc Natl Acad Sci U S A* 88: 3125-9.

Philibert, D., G. Costerousse, M. Galliard-Moguilewski, L. Nedelec, F. Nique, C. Tournemine, and G. Teutsch. 1991. From RU 38486 towards dissociated antiglucocorticoid and antiprogesterone. Antagonists in health and disease ed. Edited by M. K. Agarwal, *Front. Horm. Res.* 19: 1-17

Picard, D., B. Khursheed, M. J. Garabedian, M. G. Fortin, S. Lindquist, and K. R. Yamamoto. 1990. Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature* 348: 166-8.

Picard, D., and K. R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *Embo J* 6: 3333-40.

Pina, B., U. Bruggemeier, and M. Beato. 1990. Nucleosome positioning modulates accessibility of regulatory proteins to the mouse mammary tumor virus promoter. *Cell* 60: 719-30.

Poletti, A., and N. L. Weigel. 1993. Identification of a hormone-dependent phosphorylation site adjacent to the DNA-binding domain of the chicken progesterone receptor. *Mol Endocrinol* 7: 241-6.

Pratt, W. B. 1993. The role of heat-shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. [Review]. *J Biol Chem* 268: 21455-8.

Pratt, W. B., D. J. Jolly, D. V. Pratt, S. M. Hollenberg, V. Giguere, F. M. Cadepond, G. Schweizer-Groyer, M. G. Catelli, R. M. Evans, and E. E. Baulieu. 1988. A region in the steroid binding domain determines formation of the non-DNA-binding, 9 S glucocorticoid receptor complex. *J Biol Chem* 263: 267-73.

Pratt, W. B., E. R. Sanchez, E. H. Bresnick, S. Meshinchi, L. C. Scherrer, F. C. Dalman, and M. J. Welsh. 1989. Interaction of the glucocorticoid receptor with the Mr 90,000 heat-shock protein: an evolving model of ligand-mediated receptor transformation and translocation. [Review]. *Cancer Res* 49: 2222-9s.

Pratt, W. B., and M. J. Welsh. 1994. Chaperone functions of the heat-shock proteins associated with steroid receptors. [Review]. *Semin Cell Biol* 5: 83-93.

Predki, P. F., and B. Sarkar. 1992. Effect of replacement of "zinc finger" zinc on estrogen receptor DNA interactions. *J Biol Chem* 267: 5842-6.

Quigley, A. 1995. Androgen receptor defects: historical, clinical, and molecular perspectives [review]. *Endocr Rev* 16: 271-321.

Rastinejad, F., T. Perlmann, R. M. Evans, and P. B. Sigler. 1995. Structural determinants of nuclear receptor assembly on DNA direct repeats. *Nature* 375: 203-11.

Ray, A., and K. E. Prefontaine. 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NF-κB and the glucocorticoid receptor. *Proc Natl Acad Sci U S A* 91: 752-6.

Ray, A., K. E. Prefontaine, and P. Ray. 1994. Down-modulation of interleukin-6 gene expression by 17 ß-estradiol in the absence of high affinity DNA binding by the estrogen receptor. *J Biol Chem* 269: 12940-6.

Raynaud, J. -P., M. M. Bouton, M. Moquilewsky, T. Ojasoo, D. Philibert, G. Beck, F. Labrie, and J. P. Mormon. 1980. Steroid hormone receptors and pharmacology. *J Steroid Biochem* 12: 143-57.

Raynaud, J. -P., and T. Ojasoo. 1986. The design and use of sex-steroid antagonists [review]. *J Steroid Biochem* 25: 811-33.

## References


Reese, J. C., and B. S. Katzenellenbogen. 1991. Differential DNA-binding abilities of estrogen receptor occupied with two classes of antiestrogens: studies using human estrogen receptor overexpressed in mammalian cells. *Nucleic Acids Res* 19: 6595-602.

Reese, J. C., and B. S. Katzenellenbogen. 1992. Examination of the DNA-binding ability of estrogen receptor in whole cells: implications for hormone-independent transactivation and the actions of antiestrogens. *Mol Cell Biol* 12: 4531-8.

Reese, J. C., C. H. Wooge, and B. S. Katzenellenbogen. 1992. Identification of two cysteines closely positioned in the ligand-binding pocket of the human estrogen receptor: roles in ligand binding and transcriptional activation. *Mol Endocrinol* 6: 2160-6.

Renaud, J. P., N. Rochel, M. Ruff, V. Vivat, P. Chambon, H. Gronemeyer, and D. Moras. 1995. Crystal structure of the RAR-γ ligand-binding domain bound to all-trans retinoic acid. *Nature* 378: 681-689.

Renoir, J. M., C. Radanyi, Testas I. Jung, L. E. Faber, and E. E. Baulieu. 1990. The nonactivated progesterone receptor is a nuclear heterooligomer. *J Biol Chem* 265: 14402-6.

Riehl, R. M., W. P. Sullivan, B. T. Vroman, V. J. Bauer, G. R. Pearson, and D. O. Toft. 1985. Immunological evidence that the nonhormone binding component of avian steroid receptors exists in a wide range of tissues and species. *Biochemistry* 24: 6586-91.

Ris-Stalpers, C., M.C. Verleun-Mooijman, J. Trapman, and A. O. Brinkmann. 1993. Threonine on amino acid position 868 in the human androgen receptor is essential for androgen binding specificity and functional activity. *Biochem Biophys Res Commun* 196: 173-80.

Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targetting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* 64: 615-23.

Roche, P. J., S. A. Hoare, and M. G. Parker. 1992. A consensus DNA-binding site for the androgen receptor. *Mol Endocrinol* 6: 2229-35.

Rusconi, S., and K. R. Yamamoto. 1987. Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. *EMBO J* 6: 1309-15.

Sabbah, M., F. Gouilleux, B. Sola, G. Redeuilh, and E. E. Baulieu. 1991. Structural differences between the hormone and antihormone estrogen receptor complexes bound to the hormone response element. *Proc Natl Acad Sci U S A* 88: 390-4.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanchez, E. R. 1990. Hsp56: a novel heat-shock protein associated with untransformed steroid receptor complexes. *J Biol Chem* 265: 22067-70.

Sar, M., D. B. Lubahn, F. S. French, and E. M. Wilson. 1990. Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology* 127: 3180-6.

Sartorius, C. A., M. Y. Melville, A. R. Hovland, L. Tung, G. S. Takimoto, and K. B. Horwitz. 1994. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. *Mol Endocrinol* 8: 1347-60.

Sartorius, C. A., L. Tung, G. S. Takimoto, and K. B. Horwitz. 1993. Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. *J Biol Chem* 268: 9262-6.

Scheinman, R. I., P. C. Cogswell, A. K. Lofquist, and A. S. Baldwin. 1995b. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. *Science* 270: 283-286.

Scheinman, R. I., A. Gualberto, C. M. Jewell, J. A. Cidlowski, and A. S. Baldwin Jr. 1995a. Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. *Mol Cell Biol* 15: 943-53.

Schena, M., D. Picard, and K. R. Yamamoto. 1991. Vectors for constitutive gene expression in yeast. *Methods Enzym* 194: 389-98.

Schoenberg, M. P., J. M. Hakimi, S. Wang, G. S. Bova, J. I. Epstein, K. H. Fischbeck, W. B. Isaacs, P. C. Walsh, and E. R. Barrack. 1994a. Microsatellite mutation ($CAG_{24\rightarrow18}$) in the androgen receptor gene in human prostate cancer. *Biochem Biophys Res Commun* 198: 74-80.

Schröder, F. H. 1993. Cyproterone acetate - Mechanism of action and clinical effectiveness in prostate cancer treatment. *Cancer* 72: 3810-15.

Schuurmans, A. L. G., J. Bolt, J Veldscholte, and E Mulder. 1990. Stimulatory effects of antiandrogens on LNCaP human prostate tumor cell growth, EGF-receptor level and acid phosphatase secretion. *J Steroid Biochem Molec Biol* 37: 849-53.

Schwabe, J. W., L. Chapman, J. T. Finch, and D. Rhodes. 1993a. The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell* 75: 567-78.

Schwabe, J. W., L. Fairall, L. Chapman, J. T. Finch, R. N. Dutnall, and D. Rhodes. 1993b. The cocrystal structures of two zinc-stabilized DNA-binding domains illustrate different ways of achieving sequence-specific DNA recognition. *Cold Spring Harb Symp Quant Biol* 58: 141-7.

Schwabe, J. W., D. Neuhaus, and D. Rhodes. 1990. Solution structure of the DNA-binding domain of the oestrogen receptor. *Nature* 348: 458-61.

Schweikert, H. U. 1993. The androgen resistance syndrom: clinical and biochemical aspects. *Eur J Pediatr* 152: s50-7.

Segnitz, B., and U. Gehring. 1990. Mechanism of action of a steroidal antiglucocorticoid in lymphoid cells. *J Biol Chem* 265: 2789-96.

Simental, J. A., M. Sar, M. V. Lane, F. S. French, and E. M. Wilson. 1991. Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J Biol Chem* 266: 510-8.

Simental, J. A., M. Sar, and E. M. Wilson. 1992. Domain functions of the androgen receptor. [Review]. *J Steroid Biochem Mol Biol* 43: 37-41.

Sleddens, H. F., B. A. Oostra, A. O. Brinkmann, and J. Trapman. 1992. Trinucleotide repeat polymorphism in the androgen receptor gene (AR). *Nucleic Acids Res* 20: 1427.

Sleddens, H. F., B. A. Oostra, A. O. Brinkmann, and J. Trapman. 1993. Trinucleotide (GGN) repeat polymorphism in the human androgen receptor (AR) gene. *Hum Mol Genet* 2: 493.

Smith, D. F. 1993. Dynamics of heat-shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. *Mol Endocrinol* 7: 1418-29.

Smith, D. F., D. B. Schowalter, S. L. Kost, and D. O. Toft. 1990. Reconstitution of progesterone receptor with heat-shock proteins. *Mol Endocrinol* 4: 1704-11.

Smith, D. F., and D. O. Toft. 1993. Steroid receptors and their associated proteins. [Review]. *Mol Endocrinol* 7: 4-11.

Smith, D. F., L. Whitesell, S. C. Nair, S. Y. Chen, V. Prapapanich, and R. A. Rimerman. 1995. Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Mol Cell Biol* 15: 6804-6812.

Srinivasan, G., N. T. Patel, and E. B. Thompson. 1994. Heat-shock protein is tightly associated with the recombinant human glucocorticoid receptor:glucocorticoid response element complex. *Mol Endocrinol* 8: 189-96.

Srivastava, D., and E. B. Thompson. 1990. Two glucocorticoid binding sites on the human glucocorticoid receptor. *Endocrinology* 127: 3103-8.

Stromstedt, P. E., A. Berkenstam, H. Jornvall, J. -A. Gustafsson, and J. Carlstedt-Duke. 1990. Radiosequence analysis of the human progestin receptor charged with [3H]promegestone. A comparison with the glucocorticoid receptor. *J Biol Chem* 265: 12973-7.

Struhl, K. 1996. Chromatin structure and RNA polymerase II connection: Implications for transcription. *Cell* 84: 179-82.

Suzuki, H., N. Sato, Y. Watabe, M. Masai, S. Seino, and J. Shimazaki. 1993. Androgen receptor gene mutations in human prostate cancer. *J Steroid Biochem Mol Biol* 46: 759-65.

Svec, F., V. Teubner, and D. Tate. 1989. Location of the second steroid-binding site on the glucocorticoid receptor. *Endocrinology* 125: 3103-8.

Tai, P. K., Y. Maeda, K. Nakao, N. G. Wakim, J. L. Duhring, and L. E. Faber. 1986. A 59-kilodalton protein associated with progestin, estrogen, androgen, and glucocorticoid receptors. *Biochemistry* 25: 5269-75.

Takimoto, G. S., D. M. Tasset, A. C. Eppert, and K. B. Horwitz. 1992. Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: analysis with zinc finger mutants and the progesterone antagonist ZK98299. *Proc Natl Acad Sci U S A* 89: 3050-4.

Teutsch, G., F. Goubet, T. Battmann, A. Bonfils, F. Bouchoux, E. Cerede, D. Gofflo, Kelly M. Gaillard, and D. Philibert. 1994. Non-steroidal antiandrogens: synthesis and biological profile of high-affinity ligands for the androgen receptor. *J Steroid Biochem Mol Biol* 48: 111-9.

## References


Teutsch, G., F. Nique, G. Lemoine, F. Bouchoux, E. Cerede, D. Gofflo, and D. Philibert. 1995. General structure-activity correlations of antihormones. *Ann N Y Ac Sci* 761: 5-28.

Teutsch, G., T. Ojasoo, and J. P. Raynaud. 1988. 11 ß-substituted steroids, an original pathway to anithormones. *J Steroid Biochem* 31: 549-65.

Tjian, R., and T. Maniatis. 1994. Transcriptional activation: a complex puzzle with few easy pieces. *Cell* 77: 5-8.

Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon. 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 59: 477-87.

Truss, M., J. Bartsch, and M. Beato. 1994. Antiprogestins prevent progesterone receptor binding to hormone responsive elements in vivo. *Proc Natl Acad Sci U S A* 91: 11333-7.

Truss, M., J. Bartsch, A. Schelbert, R. J. Hache, and M. Beato. 1995. Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promoter *in vivo*. *EMBO J* 14: 1737-51.

Truss, M., and M. Beato. 1993. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. [Review]. *Endocr Rev* 14: 459-79.

Tsai, M. J., and B. W. O'Malley. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. [Review]. *Annu Rev Biochem* 63: 451-86.

Tung, L., M. K. Mohamed, J. P. Hoeffler, G. S. Takimoto, and K. B. Horwitz. 1993. Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. *Mol Endocrinol* 7: 1256-65.

Umesono, K., and R. M. Evans. 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* 57: 1139-46.

van der Kwast, Th., J. Schalken, J. A. Ruizeveld-de Winter, C. van Vroonhoven, E. Mulder, W. Boersma, and J. Trapman. 1991. Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int J Cancer* 48: 189-93.

Vegeto, E., G. F. Allan, W. T. Schrader, M. J. Tsai, D. P. McDonnell, and B. W. O'Malley. 1992. The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* 69: 703-13.

Veldscholte, J., C. A. Berrevoets, A. O. Brinkmann, J. A. Grootegoed, and E. Mulder. 1992a. Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation. *Biochemistry* 31: 2393-9.

Veldscholte, J., C. A. Berrevoets, and E. Mulder. 1994. Studies on the human prostatic cancer cell line LNCaP. *J Steroid Biochem Molec Biol* 49: 341-46.

Veldscholte, J., C. A. Berrevoets, N. D. Zegers, Th. van der Kwast, J. A. Grootegoed, and E. Mulder. 1992b. Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* 31: 7422-30.

Veldscholte, J., C. Ris-Stalpers, G. G. J. M. Kuiper, G. Jenster, C. A. Berrevoets, E. Claassen, H. C. van Rooij, J. Trapman, A. O. Brinkmann, and E. Mulder. 1990. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun* 173: 534-40.

Wagner, R. L., J. W. Apriletti, M. E. Mcgrath, B. L. West, J. D. Baxter, and R. J. Fletterick. 1995. A structural role for hormone in the thyroid hormone receptor. *Nature* 378: 690-697.

Wakeling, A. E. 1992. Steroid antagonists as nuclear receptor blockers. [Review]. *Cancer Surv* 14: 71-85.

Warriar, N., N. Page, M. Koutsilieris, and M. V. Govindan. 1993. Interaction of antiandrogen-androgen receptor complexes with DNA and transcription activation. *J Steroid Biochem Mol Biol* 46: 699-711.

Webster, N. J., S. Green, J. R. Jin, and P. Chambon. 1988. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell* 54: 199-207.

Weigel, N. L., C. A. Beck, P. A. Estes, P. Prendergast, M. Altmann, K. Christensen, and D. P. Edwards. 1992. Ligands induce conformational changes in the carboxyl-terminus of progesterone receptors which are detected by a site-directed antipeptide monoclonal antibody. *Mol Endocrinol* 6: 1585-97.

Wikstrom, A. C., O. Bakke, S. Okret, M. Bronnegard, and J. A. Gustafsson. 1987. Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology* 120: 1232-42.

Wilding, G., M. Chen, and E. P. Gelmann. 1989. Aberrant respons *in vitro* of hormone-responsive prostatecancer cells to antiandrogens. *Prostate* 14: 103-15.

Wittliff, J. L., L. L. Wenz, J. Dong, Z. Nawaz, and T. R. Butt. 1990. Expression and characterization of an

active human estrogen receptor as a ubiquitin fusion protein from Escherichia coli. *J Biol Chem* 265: 22016-22.

Wong, C. I., Z. X. Zhou, M. Sar, and E. M. Wilson. 1993a. Steroid requirement for androgen receptor dimerization and DNA binding. Modulation by intramolecular interactions between the NH2-terminal and steroid-binding domains. *J Biol Chem* 268: 19004-12.

Wong, P., J. Pineault, J. Lakins, D. Taillefer, J. Leger, C. Wang, and M. Tenniswood. 1993b. Genomic organisation and expression of the rat TRPM-2 (clusterin) gene, a gene implicated in apoptosis. *J Biol Chem* 268: 5021-31.

Wurtz, J. M., W. Bourguet, J. P. Renaud, V. Vivat, P. Chambon, D. Moras, and H. Gronemeyer. 1996. A canonical structure for the ligand-binding domain of nuclear receptors. *Nature Struct Biology* 3: 87-94.

Xie, Y. B., Y. P. Sui, L. X. Shan, J. J. Palvimo, D. M. Phillips, and O. A. Janne. 1992. Expression of androgen receptor in insect cells. Purification of the receptor and renaturation of its steroid- and DNA-binding functions. *J Biol Chem* 267: 4939-48.

Yeh, S. Y., and C. S. Chang. 1996. Cloning and characterization of a specific coactivator, ARA(70), for the androgen receptor in human prostate cells. *Proc Natl Acad Sci USA* 93: 5517-5521.

Ylikomi, T., M. T. Bocquel, M. Berry, H. Gronemeyer, and P. Chambon. 1992. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *Embo J* 11: 3681-94.

Zawel, L., and D. Reinberg. 1993. Initiation of transcription by RNA polymerase II: a multi-step process. *Prog Nucleic Acid Res* 44: 67-108.

Zegers, N. D., E. Claassen, C. Neelen, E. Mulder, J. van Laar, M. M. Voorhorst, C. A. Berrevoets, A. O. Brinkmann, Th. van der Kwast, J.A. Ruizeveld-de Winter, J. Trapman, and W. J. A. Boersma. 1991. Epitope prediction and confirmation for the human androgen receptor: generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. *Biochim Biophys Acta* 1073: 23-32.

Zeng, Z., G. F. Allan, C. Thaller, A. J. Cooney, S. Y. Tsai, B.W. O'Malley, and M.-J. Tsai. 1994. Detection of potential ligands for nuclear receptors in cellular extracts. *Endocrinology* 135: 248-52.

Zhang, S. M., X. Y. Liang, and M. Danielsen. 1996. Role of the C terminus of the glucocorticoid receptor in hormone binding and agonist antagonist discrimination. *Mol Endocrinol* 10: 24-34.

Zhou, Z.-X., J.A. Kemppainen, and E. M. Wilson. 1995. Identification of three proline-directed phosphorylation sites in the human androgen receptor. *Mol Endocrinol* 9: 605-15.

Zhou, Z.-X., M. Sar, J. A. Simental, M. V. Lane, and E. M. Wilson. 1994. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. *J Biol Chem* 269: 13115-23.

Zhou, Z. X., M. V. Lane, J. A. Kemppainen, F. S. French, and E. M. Wilson. 1995. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. *Mol Endocrinol* 9: 208-18.

Zilliacus, J., Wright K. Dahlman, J. Carlstedt-Duke, and J. A. Gustafsson. 1992. Zinc coordination scheme for the C-terminal zinc binding site of nuclear hormone receptors. *J Steroid Biochem Mol Biol* 42: 131-9.

Zilliacus, J., A. P. H. Wright, J. Carlstedt-Duke, and J. A. Gustafsson. 1995. Structural determinants of DNA-binding specificity by steroid receptors. *Mol Endocrinol* 9: 389-400.

# SUMMARY

Androgens control the development, differentiation and function of male reproductive and accessory sex tissues, such as seminal vesicle, epididymis and prostate. Changes in cell behaviour induced by androgens require the presence of a specific cellular receptor, the androgen receptor. In the absence of hormone, the androgen receptor resides in a large macromolecular complex. Binding of its respective ligand triggers a complex set of critical events, such as disruption of the macromolecular complex, dimerization, phosphorylation, and binding to DNA, finally leading to modifications in the pattern of gene expression and cell fate.
In addition to their biological role, androgens stimulate the growth of prostate cancer and benign prostate hyperplasia. Hormonal action of agonists can be prevented by inhibition of their formation or by administration of antiandrogens. Antiandrogens antagonize biological actions of agonists by competing for binding to the receptor. Although the antagonists do bind to the receptor, subsequent steps in the process of receptor activation do not proceed normally (**Chapters 1 and 2**). The basis for this deficiency is not entirely clear, but it is assumed that the conformation of the receptor bound to an antagonist is different from that bound to an agonist. The differences in the mechanisms of action between androgens and antiandrogens are the subject of this thesis.

To probe the different conformations of the receptor after binding of androgens and several antiandrogens, limited proteolysis of *in vitro* produced human androgen receptor was used (**Chapters 3 and 4**). The results provide evidence for 5 different conformations of the receptor, as detected by the formation of proteolysis resisting fragments: 1) an initial conformation of the unoccupied receptor not resisting proteolytic attack; and receptor conformations characterized by: 2) a 35 kD proteolysis resisting fragment spanning the ligand binding domain and part of the hinge region, obtained with the antagonists cyproterone acetate, hydroxyflutamide, ICI 176.334 and RU 23908, and in an initial step after agonist binding; 3) a 29 kD proteolysis resisting fragment spanning the ligand binding domain, obtained in the presence of agonists after an activation process; 4 and 5) 30 and 25 kD fragments, derived from conformations 2 and 3, but missing part of the C-terminus, obtained with RU 38486. Concomitantly with the change from conformation 2 to 3 (and of conformation 4 to 5 for RU 38486) dissociation of the 8S-complex of receptor with associated proteins occurred. With a mutant receptor ARL (LNCaP cell mutation in C-terminal region of the ligand binding domain; codon 868, Thr to Ala), the antagonists cyproterone aceate and hydroxyflutamide activated transcription analogous to agonists, and induced the activated receptor conformation 3. A mutant lacking the C-terminal 12 amino acids bound RU 38486 but not androgens, and formed with RU 38486 also conformation 5.
These data imply that, after the initial rapid binding of ligand, androgens induce a conformational change of the receptor, a process that also involves release of associated proteins. RU 38486 induces an inappropriate conformation of the C-terminal end, similar as found for its effect on the progesterone receptor. In contrast, the other antiandrogens act at a different step in the mechanism of action: they do not induce an abnormal conformation, but act earlier and prevent a conformation change by stabilizing a complex with associated proteins.

To address the hypothesis on the different mechanisms of antiandrogen action in whole cells,

the effect of several androgens and antiandrogens on induction of DNA binding for the human wild type androgen receptor and the mutant receptor ARL was examined, and related to the transcription activation ability of these receptors (**Chapter 5**). For measurement of the DNA binding ability of the various ligand-receptor complexes, an androgen receptor expression vector was cotransfected in CHO cells with a promoter interference plasmid CMV-$(ARE)_3$-LUC, containing androgen response element(s) between the TATA box of the cytomegalovirus promoter and the start site of luciferase gene transcription. Expression levels of the androgen receptors are upregulated by some agonists, but receptor expression levels are comparable for all antiandrogens studied. In the presence of androgens, the wild type androgen receptor is capable to reduce promoter activity of the CMV-$(ARE)_3$-LUC plasmid, indicating androgen-dependent DNA binding of the receptor. The full antagonists hydroxyflutamide, ICI 176.334 and RU 23908 block androgen receptor binding to DNA. The antagonists cyproterone acetate and RU 38486 induce approximately 50% of the DNA binding found for androgens. In a transcription activation assay, the RU 38486-bound receptor was almost inactive whereas the receptor complexed with cyproterone acetate showed partial agonistic activity. Interaction of the antagonists cyproterone acetate, hydroxyflutamide and RU 23908 with the mutant receptor ARL resulted in both a DNA-bound and a transcriptionally active receptor.
In conclusion: transformation of the androgen receptor to a DNA binding state in whole cells is blocked by several antiandrogens. Furthermore, studies with the antiandrogens cyproterone acetate and RU 38486 showed that DNA binding alone is not sufficient to accomplish full transcriptional activity. Full activity requires additional changes, presumably in the protein structure of the receptor.

The transactivation activity of the androgen receptor is subscribed to the N-terminal domain. This activation function in the N-terminal domain of the androgen receptor is considered to be ligand-independent. However, in the studies described in Chapters 3-5 it was found that some androgen receptor antagonists induce binding of the receptor to DNA, but still do not fully activate the androgen receptor. This might indicate that androgens, but not antiandrogens, function by mediating an interaction of the N-terminal domain with other transcription factors. As a consequence, the ligand binding domain has to interact with the N-terminal domain, either directly or indirectly via other proteins. In a cooperative study with the Department of Pathology of the Erasmus University Rotterdam the presumed domain interactions were first investigated in the yeast two-hybrid system, a system developed for the study of protein-protein interactions (**Chapter 6**). Therefore, the ligand binding domain and the N-terminal domain of the androgen receptor were separately linked to both the GAL4 DNA binding domain and GAL4 transactivating domain. In the absence of ligand, no interaction between the various domains was detected. In the presence of androgens, but not in the presence of low affinity ligands such as progesterone and estradiol, a clear interaction was found between the ligand binding domain and N-terminal domain of the androgen receptor. Interaction between two ligand binding domains could be demonstrated, but only when a high expression vector was used and at high ligand concentrations. Putative interaction between N-terminal domains could not be measured due to a high endogenous activity of the fusion protein of GAL4 DNA-binding domain and androgen receptor N-terminal domain. In a mammalian system, co-transfection of an androgen receptor mutant lacking the N-terminal, transactivation domain and a separate N-terminal domain resulted in transcription activation upon androgen binding. No functional interaction was found between the separate domains in the presence

of antiandrogens or in the absence of hormone. Use of the ligand binding domain of mutant ARL in the assay resulted in a change of ligand specificity in the domain interaction: cyproterone acetate and hydroxyflutamide were able to induce functional interactions between the N-terminal domain and the ligand binding domain of ARL. The domain interactions induced by these compounds reflect their transactivating capacity with the full-length mutant ARL. In the mammalian system, maximum activity in the protein-protein interaction experiments was obtained at an approximately 10-fold higher ligand concentration than needed for wild type androgen receptor activation.
In conclusion, these findings implicate interaction between the N-terminal domain and ligand binding domain, which forms an essential step in androgen receptor activation. Furthermore, antiandrogens have the potency to either inhibit this interaction or promote the formation of a non-functional complex.

Finally, the results, as described in the previous chapters, are discussed in the perspective of past, present, and future research (**Chapter 7**).

# SAMENVATTING

De geslachtshormonen testosteron en dihydrotestosteron (androgenen) zijn betrokken bij ontwikkeling, differentiatie en functioneren van de mannelijke geslachtsorganen en kenmerken. Androgenen oefenen hun werking uit door te binden aan een specifieke receptor, de androgeenreceptor die aanwezig is in de kern van hun doelwitcellen. Deze androgeenreceptor bevindt zich in een niet actieve vorm in een complex met verschillende andere eiwitten. Binding van androgenen aan de receptor resulteert in een aantal structurele veranderingen in het receptoreiwit, leidend tot een dissociatie van het niet actieve complex van de receptor en geassocieerde eiwitten. Vervolgens bindt de androgeenreceptor aan hormoon respons elementen, specifieke gebieden van het DNA in de promotor van androgeen-gereguleerde genen. Deze androgeen- en DNA-gebonden receptoren reguleren de produktie van het boodschapper RNA. Deze boodschapper RNAs coderen voor eiwitten die uiteindelijk zorgdragen voor de cellulaire respons, bijvoorbeeld celgroei. De androgeenreceptor, evenals andere steroïdhormoonreceptoren, kan dan ook worden opgevat als een hormoon-gereguleerde transcriptiefactor.
De werking van androgenen via hun receptor is evenwel niet altijd gewenst. Met name benigne prostaat hyperplasie, een aandoening van de prostaat die leidt tot een obstructie van de urinebuis, en de groei van prostaatkanker worden gestimuleerd door androgenen. Eén van de mogelijkheden om stimulerende effecten van androgenen op deze aandoeningen te verhinderen is gebruik te maken van zogenaamde antiandrogenen. Deze antiandrogenen verdringen de androgenen van de androgeenreceptor, hierbij een inactief antiandrogeen-receptor complex vormend. De moleculaire mechanismen achter het proces van androgeenreceptor activatie en inhibitie zijn echter nog grotendeels onbekend. Het onderzoek, beschreven in dit proefschift, betreft dan ook enerzijds de rol die androgenen hebben in de activatie van hun receptor in doelwitcellen, en anderzijds de wijze waarop antiandrogenen met de receptor interacteren en zo de activerende werking van androgenen kunnen blokkeren. Hieruit voortvloeiende kennis over de moleculaire werkingsmechanismen van androgenen en antiandrogenen kan leiden naar betere medicamenten die androgeenwerking tegengaan. Deze achtergronden van dit proefschrift zijn beschreven in **Hoofdstuk 1**.

De huidige kennis omtrent de structuur van steroïdhormoonreceptoren en de verschillende stappen in het activatiemechanisme van deze receptoren in het algemeen, en de androgeenreceptor in het bijzonder, is samengevat in **Hoofdstuk 2**. Tevens worden er verschillende mogelijke mechanismen van werking gepresenteerd, waarop antihormonen hun antagonistische werking op de receptor kunnen uitoefenen.
Een mogelijke verklaring voor de antagonistische werking van antiandrogenen is dat de androgenen en antiandrogenen verschillende conformaties van de androgeenreceptor induceren. Door een afwijkende conformatie zal een antiandrogeen-gebonden receptor geen produktie van boodschapper RNA kunnen initiëren. Conformatieveranderingen, zoals geïnduceerd door binding van het (anti)hormoon, werden onderzocht met behulp van proteolytische afbraak van de receptor en analyse van de daarbij gevormde fragmenten. Tevens werd de eerste stap in het activatiemechanisme van de androgeenreceptor, namelijk de dissociatie van het inactieve complex van de receptor en zijn geassocieerde eiwitten, bestudeerd (**Hoofdstukken 3 en 4**). Wanneer een androgeen aan de receptor gebonden is, wordt het gehele hormoon-bindende gedeelte van de receptor beschermd tegen afbraak door proteolytische enzymen. Verder zijn androgenen in staat het complex van receptor en geassocieerde eiwitten te dissocieren. Binding

van één van de antihormonen cyproteronacetaat, RU 23908, ICI 176.334 en hydroxyflutamide aan de receptor resulteert in een groter beschermd fragment van de receptor dan in aanwezigheid van de androgenen, waarbij tevens het complex van de receptor met zijn geassocieerde eiwitten intact blijft. Echter, niet alle antihormonen interacteren op deze wijze met de androgeenreceptor. Het antihormoon RU 38486 is, net als de androgenen, in staat om het niet-actieve complex van de receptor met zijn geassocieerde eiwitten uiteen te laten vallen. Verder beschermt RU 38486 een kleiner fragment van het hormoon-bindend gedeelte van de receptor tegen proteolytische afbraak dan androgenen. Concluderend duiden deze resultaten erop dat er verschillen zijn in de werkingsmechanismen van antihormonen. Het antihormoon RU 38486 lijkt te werken door inductie van een verkeerde receptorstructuur, waardoor, na binding van het antihormoon-receptorcomplex aan het DNA, gereguleerde produktie van boodschapper RNA niet plaats kan vinden. De antihormonen cyproteronacetaat, RU 23908, ICI 176.334 en hydroxyflutamide daarentegen remmen de activatie van de androgeenreceptor doordat ze de dissociatie van de receptor en zijn geassocieerde eiwitten verhinderen.
De bovenstaande hypothese, betreffende de verschillende werkingsmechanismen van antihormonen, vormde de aanleiding om inductie van DNA-binding van de receptor geïnduceerd door verschillende antihormonen te bestuderen in een intacte cel (**Hoofdstuk 5**). Deze binding van de receptor aan het DNA in de intacte cel is bestudeerd met behulp van een promotor interferentie methode. Deze methode is gebaseerd op het niet gelijktijdig kunnen binden van de androgeenreceptor en basale transcriptiefactoren op een permanent actieve promotor. Verdringing van basale transcriptiefactoren door de DNA-gebonden receptoren resulteert in dit specifieke geval dus juist in een verlaagde produktie van boodschapper RNA. Dit RNA codeert voor het eiwit luciferase dat biochemisch makkelijk detecteerbaar is. Een lage luciferase activiteit wijst dus op binding van de receptor aan het DNA. Het vermogen van de verschillende (anti)hormonen tot inductie van DNA-binding van de receptor werd vergeleken met de mate waarin deze (anti)hormoon-receptor complexen in staat zijn tot transcriptie-activatie, gemeten op een positief-gereguleerde promotor. In overeenstemming met de hypothese dat sommige antihormonen de dissociatie van het inactieve complex van de receptor met zijn geassocieerde eiwitten niet kunnen induceren in de intacte cel, bleken de antihormonen RU 23908, ICI 176.334 en hydroxyflutamide niet in staat te zijn de activiteit van de permanent actieve promotor in het promotor interferentie systeem te onderdrukken. De antagonisten RU 38486 en cyproteronacetaat waren, evenals androgenen, wel in staat om via de receptor de activiteit van de promotor te onderdrukken, duidend op inductie van DNA-binding door deze verbindingen. De laatstgenoemde antagonisten induceren in tegenstelling tot de androgenen echter nauwelijks transcriptie-activatie op de positief-gereguleerde promotor. Uit deze resultaten kan worden geconcludeerd dat in de intacte cel inductie van DNA-binding van de receptor door hormonen niet voldoende is om een volledig actieve receptor te verkrijgen. Volledige activatie van de receptor vereist additionele veranderingen, waarschijnlijk in de eiwitstructuur van de receptor.
Het N-terminaal gelegen deel van de androgeenreceptor is verantwoordelijk voor transcriptie-activatie. Volgens de literatuur functioneert dit domein onafhankelijk van het C-terminaal gelegen hormoon-bindend domein. Echter, uit de studies beschreven in de Hoofdstukken 3 tot en met 5 blijkt dat de antagonisten cyproteronacetaat en RU 38486 binding van de receptor aan het DNA induceren, maar geen of een onvolledige transcriptie-activatie induceren. Dit impliceert dat androgenen direct betrokken zijn bij transcriptie-activatie, mogelijkerwijs door een interactie te bewerkstelligen tussen het N-terminaal gelegen transcriptie-activatie domein

en het C-terminaal gelegen hormoon-bindend domein. In **Hoofdstuk 6** worden resultaten beschreven die erop duiden dat deze gepostuleerde interactie tussen de receptordelen inderdaad optreedt. Hiertoe werden het N-terminale transcriptie-activatie domein van de receptor en het C-terminale deel bestaande uit de DNA- en hormoon-bindend domeinen van de receptor als twee afzonderlijke eiwitten tot expressie gebracht in zowel gistcellen als zoogdiercellen. In beide celtypen zijn androgenen, in tegenstelling tot antiandrogenen, in staat een interactie te induceren tussen de twee losse delen van de receptor. Zowel de volledige androgeenreceptor als de samen tot expressie gebrachte delen van de receptor zijn in staat tot transcriptie-activatie, alhoewel er een tienvoudig hogere concentratie androgeen nodig is om de combinatie van N- en C-terminale delen te activeren.
In **Hoofdstuk 7** tenslotte worden de resultaten zoals beschreven in voorgaande hoofdstukken gerelateerd aan de in de literatuur bekende gegevens over de werkingsmechanismen van androgenen en antiandrogenen.

## LIST OF PUBLICATIONS RELATED TO THIS THESIS

C.W. Kuil, and E. Mulder. 1994. Mechanism of antiandrogen action: conformational changes of the receptor. *Mol Cell Endocrinol* 102: R1-R5.

C.W. Kuil, and E. Mulder. 1995. Effects of antiandrogens on the conformation of the androgen receptor. *Ann N Y Acad Sci* 761: 351-4.

C.W. Kuil, C.A. Berrevoets, and E. Mulder. 1995. Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization. *J Biol Chem* 270: 27569-77.

C.W. Kuil, and A.O. Brinkmann. 1996. Androgens, antiandrogens and androgen receptor abnormalities. *Europ Urol* 29: 78-82.

C.W. Kuil, and E. Mulder. 1996 Deoxyribonucleic acid-binding ability of androgen receptors in whole cells: Implications for the mechanism of action of androgens and antiandrogens. *Endocrinology* 137: 1870-7.

E. Mulder, J. Veldscholte, and C.W. Kuil. 1996. Molecular mechanisms of androgen antagonists. In "Hormone dependent cancer" (eds. B.S. Katzenellenbogen and J.R. Pasqualini), Marcel Dekker Inc., New York, USA: 357-72.

P. Doesburg, C.W. Kuil, C. Berrevoets, K. Steketee, E. Mulder, A.O. Brinkmann, and J. Trapman. 1996. Functional *in vivo* interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. Accepted for publication in *Biochemistry*. P.D. and C.W.K. contributed equally to this study.

## CURRICULUM VITAE

De schrijver van dit proefschrift, Cor Kuil, werd geboren op 18 juni 1966 te Heemskerk. Van 1978 tot 1983 volgde hij het Hoger Algemeen Voortgezet Onderwijs aan het Pius X College te Beverwijk. Een opleiding Atheneum B werd in mei 1985 met goed gevolg afgesloten aan de eerder genoemde scholengemeenschap, waarna de studie werd voortgezet aan de Landbouw-universiteit te Wageningen in de studierichting Biologie. Het propaedeusediploma werd behaald in september 1986. In de doctoraalfase (orientatie cel) participeerde hij in onderzoek aan de Vakgroepen Fysiologie van Mens en Dier (Dr. J.J.A.M. Mattheij), Experimentele Diermorfologie en Celbiologie (Dr. L. Verburg-van Kemenade) en Moleculaire Biologie (Dr. S.C. de Vries / Ir. F.A. van Engelen). Zijn stage-periode werd doorgebracht bij de Endocrinological Research & Developmental Laboratories (Dr. J. Weusten) van N.V. Organon te Oss. In juni 1991 behaalde hij het ingenieursdiploma (cum laude).
Zijn militaire dienstplicht werd vervuld van juli 1991 tot juli 1992 bij het Korps Commando Troepen te Roosendaal, alwaar hem in oktober 1991 de groene baret werd uitgereikt.
Van juli 1992 tot juli 1996 werkte hij aan de Vakgroep Endocrinologie & Voortplanting van de Erasmus Universiteit Rotterdam aan het onderzoek dat in dit proefschrift beschreven staat.
Sinds september 1996 is hij, in tijdelijke dienst van de Koninklijke Nederlandse Academie van Wetenschappen (KNAW), werkzaam bij het Hubrecht Laboratorium [Nederlands Instituut voor OntwikkelingsBiologie (NIOB)] te Utrecht. Het door de Europese Gemeenschap gefinancieerde onderzoek betreft de ontwikkeling van een nieuwe generatie van ontstekingsremmende steroïden gebaseerd op moleculaire interacties tussen de glucocorticoïdreceptor en andere transcriptiefactoren.

## NAWOORD

In tegenstelling tot wat de enkele naam op de voorkant van dit proefschrift doet vermoeden, is de inhoud het resultaat van samenwerking tussen meerdere personen. Daarom wil ik in dit nawoord dan ook een aantal personen bedanken die een bijdrage hebben geleverd aan het tot stand komen van dit boekje.
Allereerst wil ik noemen Eppo Mulder, mijn co-promotor, die mij niet alleen de grondbeginselen van wetenschappelijk onderzoek heeft bijgebracht, maar ook de vrijheid heeft gegeven om niet voor de hand liggende experimenten toch uit te voeren. Tevens is het enorme verschil tussen mijn eerste schrijfselen en de uiteindelijke inhoud van dit boekje voor een groot deel zijn verdienste. Naast mijn co-promotor ben ik ook mijn promotor Anton Grootegoed zeer erkentelijk. Zijn vermogen om het onderzoek, dat zich steeds meer toespitste op details, telkens weer in een breder kader te plaatsen, heeft mijn blikveld verruimd.
Een speciaal woord van dank is er voor Cor Berrevoets, en niet alleen voor de technische hulp en ondersteuning gedurende de afgelopen jaren. Jouw enthousiaste streven naar een verdere optimalisering van experimenten is bewonderenswaardig. Tevens wil ik hier ook alle (ex-)medewerkers van het Androdroom en de rest van de afdeling Endocrinologie & Voortplanting bedanken voor de prettige samenwerking en de gezelligheid, zowel in het lab als daarbuiten. De interactie met de afdeling Pathologie, met name Jan Trapman en Paul Doesburg, die resulteerde in het tot stand komen van hoofdstuk 6 van dit boekje is een leerzame periode geweest.
Uiteraard dienen ook Els, René en verdere familie en vrienden hier genoemd te worden. Bij hen kon ik immers ontspannen, relativeren en bijtanken. Vooral degenen die mij in de afgelopen jaren hebben meegemaakt, hebben altijd belangstelling getoond en hebben mij door moeilijke perioden gesleept. Ook die bijdrage aan mijn proefschrift is onmisbaar geweest.