Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains

Erik SCHOENMAKERS*, Philippe ALEN*, Guy VERRIJDT*, Ben PEETERS*, Guido VERHOEVEN†, Wilfried ROMBAUTS* and Frank CLAESSENS*[1]

*Division of Biochemistry, Faculty of Medicine, Campus Gasthuisberg, University of Leuven, B-3000 Leuven, Belgium, and †Laboratory for Experimental Medicine and Endocrinology, Faculty of Medicine, Campus Gasthuisberg, University of Leuven, B-3000 Leuven, Belgium

The androgen and glucocorticoid hormones evoke specific *in vivo* responses by activating different sets of responsive genes. Although the consensus sequences of the glucocorticoid and androgen response elements are very similar, this *in vivo* specificity can in some cases be explained by differences in DNA recognition between both receptors. This has clearly been demonstrated for the androgen response element PB-ARE-2 described in the promoter of the rat probasin gene. Swapping of different fragments between the androgen- and glucocorticoid-receptor DNA-binding domains demonstrates that (i) the first Zn-finger module is not involved in this sequence selectivity and (ii) that residues in the second Zn-finger as well as a C-terminal extension of the DNA-binding domain from the androgen receptor are required. For specific and high-affinity binding to response elements, the DNA-binding domains of the androgen and glucocorticoid receptors need a different C-terminal extension. The glucocorticoid receptor requires 12 C-terminal amino acids for high affinity DNA binding, while the androgen receptor only involves four residues. However, for specific recognition of the PB-ARE-2, the androgen receptor also requires 12 C-terminal residues. Our data demonstrate that the mechanism by which the androgen receptor binds selectively to the PB-ARE-2 is different from that used by the glucocorticoid receptor to bind a consensus response element. We would like to suggest that the androgen receptor recognizes response elements as a direct repeat rather than the classical inverted repeat.

Key words: androgen response element, androgen specificity, glucocorticoid response element, probasin, steroid receptors.

INTRODUCTION

The nuclear receptors (NRs) constitute a very large family of transcription factors that are characterized by a well-conserved DNA-binding domain (DBD), defined as a fragment encompassing two Zn-finger-like modules [1]. These DBDs bind as dimers to two hexameric sequences orientated as direct or inverted repeats. The first Zn-finger is responsible for the sequence recognition through specific interactions of several amino acids with the DNA [2]. The most important determinants of the difference in sequence specificity between the glucocorticoid receptor (GR) and the oestrogen receptor (ER) [3] are the P-box residues in the first Zn-finger. The second Zn-finger module contains the D-box, which provides most of the protein–protein interactions necessary for DNA-dependent receptor dimerization [4,5]. Structural studies of DBD–DNA complexes [2,6] have demonstrated several additional interactions, outside the P- and D-boxes, between the DBDs and the bases or the phosphate backbone of the response elements, as well as intra- and intermolecular protein–protein interactions, within the DBD dimer.

For some members of the NR superfamily (thyroid-, vitamin D-, transcription factor Rev-erbβ-, retinoid X receptor), a C-terminal extension (CTE) of the DBD, called the T-box, is involved in the stabilization of the binding to direct repeat elements [7–10]. This T-box contains 12 amino acids and forms an α-helical structure [8,11] that is poorly conserved within the NR family. DNA-binding studies showed no direct involvement of a CTE in the DNA binding by steroid receptors, and the crystal and NMR structures of the GR-DBD do not reveal an α-helix in this region [2,6].

On the basis of the similarity of their consensus binding sites (5′-GGTACAnnnTGTTCT-3′), the GR, androgen (AR), mineralocorticoid (MR) and progesterone (PR) receptors can be considered as a subfamily of the NR superfamily [12]. Not surprisingly, their DBDs are highly conserved (up to 90 %) and the P- and the D-boxes are nearly identical. This left the question open how the different steroids evoke their specific *in vivo* responses, especially in target cells containing more than one type of receptor. Steroid metabolism or differential expression of receptors only offers a partial explanation for the difference, for example, between glucocorticoids and mineralocorticoids (reviewed in [13]). Another factor controlling the steroid specificity might be the local chromatin structure, as shown for the mouse mammary-tumour-virus long-terminal-repeat

Abbreviations used: AR, androgen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; NR, nuclear receptor; PR, progesterone receptor; ER, oestrogen receptor; TR, thyroid receptor; RXR, 9-*cis*-retinoid acid receptor; DBD, DNA-binding domain; GST, glutathione S-transferase; K_S: apparent dissociation constant; ARE, GRE, androgen and glucocorticoid response element respectively; CTE, C-terminal extension; *slp*, sex-limited-protein gene; DMEM, Dulbecco's modified Eagle's medium; DCC, DMEM containing 5 %-dextran-coated charcoal-stripped fetal-bovine serum; h, human; r, rat.

[1] To whom correspondence should be addressed (e-mail frank.claessens@med.kuleuven.ac.be).

promoter [14,15]. Another possible mechanism leading to differential steroid-responsiveness of specific enhancers was discovered in studies of the androgen responsive unit of the sex-limited-protein gene, *slp*. It was observed that the androgen-specificity was due to the exclusion of the GR from binding to the *slp* enhancer by other transcription factors interacting with flanking elements of the *slp*-ARE-3 [16] (ARE is androgen response element).

Although no major differences in DNA-binding specificity have been detected between the GR, AR, MR and PR, some differences have been reported. It was, for example, demonstrated that the AR-DBD, and not the GR-DBD, has the ability to bind to an element with a direct repeat configuration (DR1-type) [17]. This element diverges from ARE consensus, as determined previously [18] and none of the AREs described thus far resembles such a DR1. We reported a functional ARE in the promoter of the androgen-dependent rat probasin gene (PB-ARE-2 [19]) to be recognized by the AR and not by the GR [20]. In the present study we analysed in detail which characteristics of the AR are required for its specific interaction with the PB-ARE-2.

EXPERIMENTAL

Materials

Restriction and modifying enzymes were purchased from GIBCO–BRL Life Technologies, Pharmacia Biotech Inc., Promega, Takara Shuzo Co. Ltd. and Boehringer Mannheim. Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (Milligen Corp., Bedford, MA, U.S.A.). Hormones were purchased from Sigma.

Plasmid constructs

The cDNA encoding the rat AR was described by Chang et al. [21], and that for the rat GR by Hollenberg et al. [22]. The cDNA for the human MR and the human PR were kindly given by Professor R. Evans, The Salk Institute/GEL, San Diego, CA, U.S.A. fAGA is derived from the full-size rat AR (rAR) by swapping the AR-DBD with that of the rGR (AR amino acids 537–619). For the construction of the reporter constructs pPBluc3 and pC3luc3, we have cloned the *Sph*I–*Bgl*II fragment from pBLCAT2 constructs, described in [20], in the pGL3-basic vector (Promega). Each construct contains two copies of the corresponding ARE upstream of the TATA-box of the tk-promoter directing transcription of the luciferase gene. All receptor cDNA fragments were amplified by PCR with specific oligonucleotides, cut with *Eco*RI and *Bam*HI, and subsequently cloned in the corresponding restriction sites of the pGEX-2TK (Pharmacia Biotech Inc.). The fragments were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* BL21. AR1 consists of the rAR amino acids 533–637 [23], GR1 consists of the rGR amino acids 432–533 [24], MR1 is the human MR (hMR)-DBD from 595 to 696 [25] and PR1 is the hPR-DBD from 559 to 660 [26]. In the AGg and GAa constructs, the first Zn-fingers of AR1 (Asp^{533} to Ala^{569}) and GR1 (Ala^{432}–Ala^{467}) were swapped. As a nomenclature for the chimaeric constructs, capital letters represent the origin of a Zn-finger and a small letter represent the hinge region (A for AR and G for GR). AGa, for example, is comparable with AR1, except that the second Zn-finger was swapped for that of the GR (Val^{469}–Met^{505}). In $G^G/_A a$ and $A^A/_G g$, part of the second Zn-finger and the hinge region were swapped between AR1 and GR1. The deletion mutants are schematically represented with numbers indicating the C-terminal amino acid (Table 1). The AAg(517) and GGa(619) constructs were derived from AR(619) and GR(517) in which the twelve C-terminal residues were swapped. The construct AR(619m) was derived from AR(619) by exchanging the three C-terminal amino acids between the AR (Leu^{617}, Gly^{618}, Asn^{619}) with those of the GR (Lys^{515}, Ile^{516}, Lys^{517}).

Production and purification of full-size receptors

The vaccinia-virus expression vectors (pMS56) containing the cDNA of the GR and of the AR were used, and the purification and determination of specific activity were performed as described previously [27].

Purification of receptor fragments

The bacteria were grown in 50 ml of Luria broth up to an A_{600} of 0.5–0.6, the culture was then maintained at 25 °C and the protein production induced by adding 0.2 mM isopropyl β-D-thiogalactoside in the presence of 10 μM $ZnCl_2$. After 2 h the bacteria were collected by centrifugation (5 min, 1500 ***g***), dissolved in 3 ml of PBS (140 mM NaCl/2.7 mM KCl/10.1 mM Na_2HPO_4/1.8 mM KH_2PO_4, pH 7.3) containing 10 μM $ZnCl_2$ and disrupted by ultrasonic treatment in the AT200-Bioruptor sonicator (Cosmo Bio, Seraing, Belgium). The high-speed supernatant of the lysate was applied to a GSH–Sepharose 4B column. The column was washed with 10 bed vol. of PBS containing 10 μM $ZnCl_2$ and the GST portion was cleaved off on the column by thrombin digestion (2 units, 1.5 h). The proteins were stored

Table 1 Comparison of the affinities of the DBDs with the fold induction by the full-size AR, GR, PR and MR

Values are means ± S.E.M. for at least three independent measurements.

	K_S (nM) by gel-shift assays*		Fold induction†			
			COS cells		CV1 cells	
Receptor	PB-ARE-2	C3(1) ARE	PB-ARE-2	C3(1) ARE	PB-ARE-2	C3(1) ARE
AR	23 ± 5	5 ± 1	11 ± 3	14 ± 2	5.5 ± 0.7	5.9 ± 0.7
GR	165 ± 10	21 ± 3	1.6 ± 0.2	7 ± 1	1.1 ± 0.2	7.8 ± 1
MR	88 ± 6	16 ± 3	2.1 ± 0.2	7 ± 1	1.3 ± 0.2	4.7 ± 0.6
PR	290 ± 30	33 ± 8	1.9 ± 0.4	6.9 ± 0.8	1.5 ± 0.2	5.1 ± 0.9
fAGA	–	–	5 ± 1	17 ± 5	–	–

* The apparent dissociation constants of the DBD constructs are determined as described in the Experimental section.
† Transient transfection assays were performed as described in the Experimental section.

in PBS containing 10 μM $ZnCl_2$ and 15% glycerol at −80 °C. To exclude artefacts in the purification process, the purity and size of the proteins obtained were always monitored by SDS/PAGE and the concentration was determined with Coomassie Protein Assay Reagent (Pierce).

The specific activity of AR1 and GR1 was determined in gel-shift assays in which labelled C3(1) ARE was competed for by increasing amounts of excess unlabelled C3(1) ARE in the presence of a constant amount of protein [the C3(1) gene codes for a component of the prostatic binding protein and contains an ARE in the first intron] [28]. The purified preparations of AR1 and GR1 appeared to contain 55% biologically active proteins. Since all purified receptor constructs bind the C3(1) ARE with similar affinity, we assume that they contain similar amounts of active protein.

Gel-shift assays and determination of the apparent dissociation constants (K_s)

The probes (5000 c.p.m./fmol) were labelled with [α-^{32}P]dATP (Amersham) by a fill-in reaction with the Klenow enzyme. X-Omat S X-ray films were purchased from Kodak. In gel-shift assays, constant amounts (20000 c.p.m.) of labelled double-stranded oligonucleotides were incubated (20 min at room temperature) with increasing amounts of protein (ranging from 0.25 ng up to 10 μg) in 20 μl of binding buffer [10 mM Hepes (pH 7.9)/2.5 mM $MgCl_2$/0.05 mM EDTA/10% glycerol/50 mM NaCl/50 ng poly(dI-dC)/0.1% Triton/1 mM dithiothreitol]. Subsequently, free and bound probe were separated by electrophoresis for 90 min at 120 V in a 5% non-denaturing polyacrylamide gel. The percentage of retarded probe for each protein concentration was determined by scanning dried gels with a PhosphorImager (Molecular Dynamics) and used for calculation of the apparent dissociation constant (K_s). The isolated DNA-binding domains bind as dimers to the DNA and involve co-operative protein–protein interactions between the monomers. To take this into account, the formula for Hill kinetics was used for the calculation of the dissociation constants. For an accurate comparison of the DNA affinities of the different receptor constructs, the K_s values were based on data from at least three independent protein preparations.

Cell culture and DNA transfection

CV-1 cells and COS cells, obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5%-dextran-coated charcoal-stripped fetal bovine serum (DCC). Reporter plasmids were co-transfected with expression vectors for rAR, rGR, hMR or hPR with the FuGENE system (Boehringer Mannheim). A β-galactosidase expression plasmid (CMV-β-gal, Stratagene) was used as internal control of the transfection efficiency. For the transfection assay of CV1 cells, 0.5 μg of reporter plasmid together with 50 ng of receptor expression vector, 0.25 μg CMV-β-gal and 0.5 μg carrier DNA were mixed with 50 μl DCC containing 1.5 μl FuGENE transfection reagent. After 15 min incubation at room temperature, the mixture was added dropwise on to the cells (16-mm-diameter culture dish containing 4×10^5 cells in 500 μl DMEM). After overnight incubation at 37 °C, the medium was replaced with DMEM containing 5% fetal-bovine serum and the cells were grown in the absence or presence of 1 nM R1881 (methyltrienolone, a synthetic androgen hormone), dexamethasone, aldosterone or progesterone for 48 h. The same procedure was used for the transfection of COS cells, with the following amounts of DNA: 35 ng of reporter plasmid, 25 ng of receptor expression vector, 50 ng of CMV-β-gal and 0.75 μg of carrier DNA. Luciferase activity was measured with the luciferase assay system from Promega, and β-galactosidase activity was measured with the β-galactosidase system from Clontech.

RESULTS

PB-ARE-2 is a specific ARE

In previous DNA-binding studies, we demonstrated that the PB-ARE-2 (Figure 1A) is specifically recognized by the AR-DBD and not by the GR-DBD [20]. To exclude the possibility that this selective binding in gel-shift assays was an artefact of the Protein

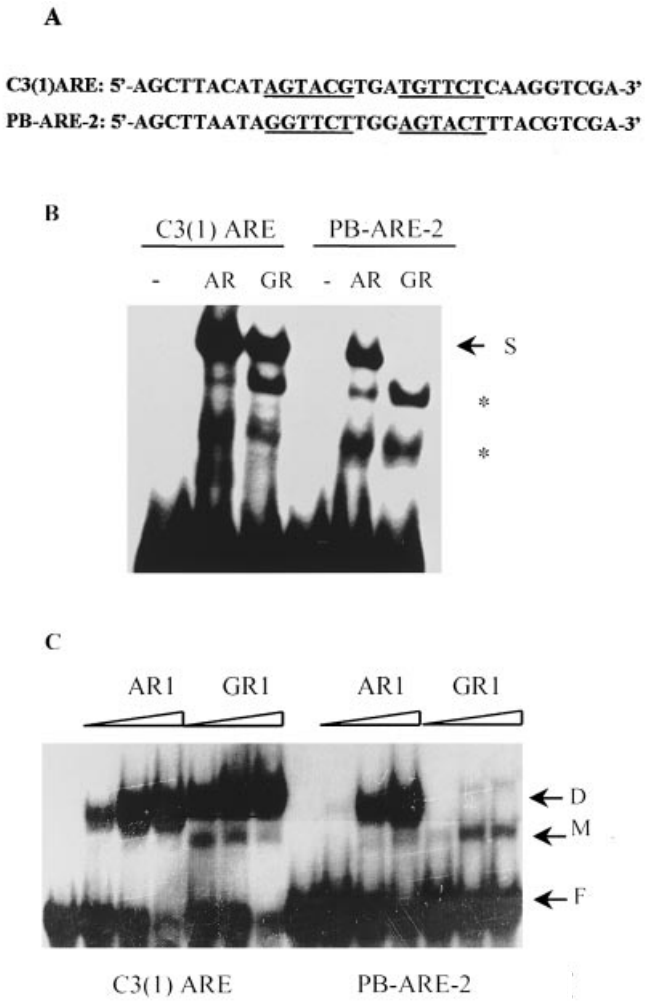
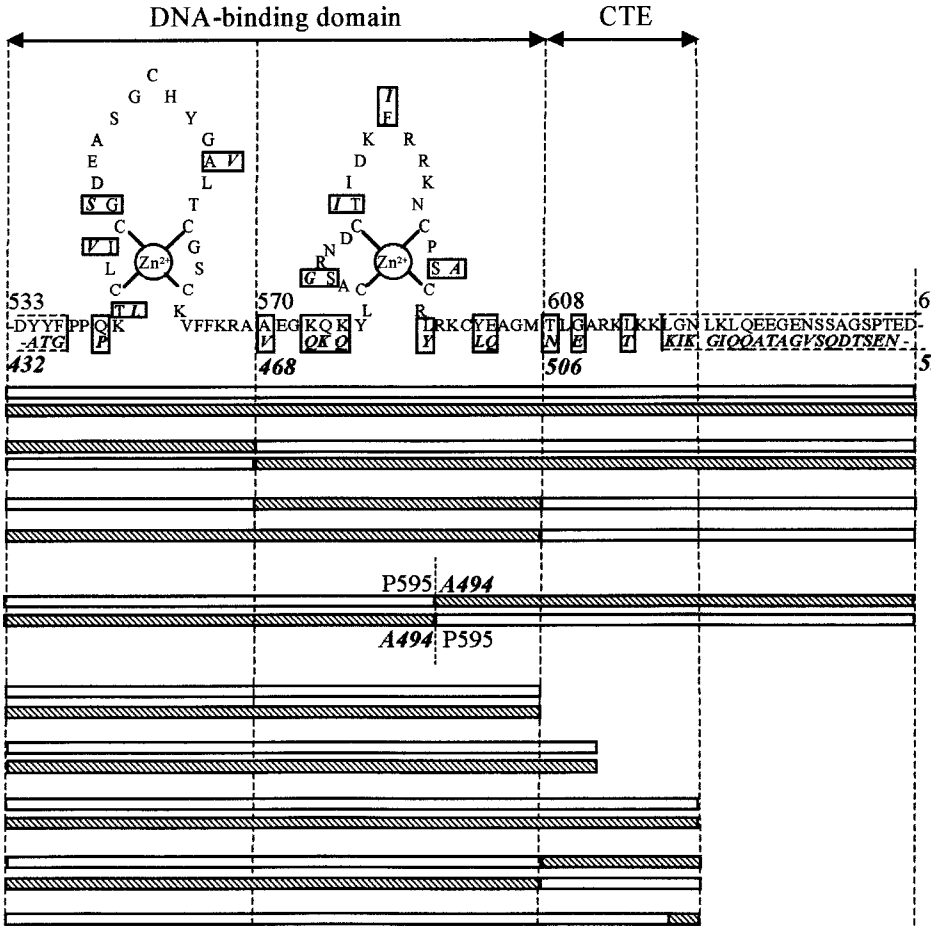


Figure 1 Comparison of the binding of the full-size and DBD fragments of the AR and GR to the C3(1) ARE and the PB-ARE-2

(**A**) Sequence of the oligonucleotides containing the C3(1) ARE and the PB-ARE-2 motifs used in the present study. The core motif-like sequences are underlined. (**B**) Comparative gel-shift assay of the C3(1) ARE and the PB-ARE-2 probe interaction with the full-size hAR and hGR. Radiolabelled probes (2×10^5 c.p.m.) were incubated with the purified receptors as described in the Experimental section. Free and protein-bound DNA were separated on a 5% non-denaturating polyacrylamide gel and revealed by autoradiography. The specifically (S) and the not specifically (*) retarded probes are indicated. (**C**). Comparative gel-shift assay of the C3(1) ARE and the PB-ARE-2 probe interaction with the DBD of the AR (AR1) and the GR (GR1). Radiolabelled probes (2×10^5 c.p.m.) were incubated with 0, 135, 400 or 800 fmol of purified AR1 or GR1 in a total volume of 20 μl for the binding reaction. The protein–DNA complex was separated from the free probe by electrophoresis on a 5% non-denaturing polyacrylamide gel. The gel was dried and exposed to X-ray film to reveal the free probe (F) and the monomeric (M) and dimeric (D) complexes (indicated by arrows).

Table 2 Summary of the apparent dissociation constants of all AR- and GR-DBD constructs

Sequence alignment of the DNA-binding domain and the N-terminal part of the hinge region of the rAR [21] and the rGR (in ***bold and italic***) [24]. The two Zn-finger modules and the CTE are indicated. Differences in amino acid sequence between the AR and the GR are boxed. Deletion and substitution mutants of the AR- and GR-DBD are depicted; white bars indicate parts of the AR, whereas striped bars indicate parts of the GR. The dissociation constants (in nM) of the different protein constructs for the PB-ARE-2 and the C3(1) ARE are given at the right. These values were determined as described in the Experimental section. Values are mean ± S.E.M.



	Dissociation constants (nM)	
	PB-ARE-2	C3(1) ARE
AR1	23 (±5)	5 (±1)
GR1	165 (±10)	21 (±3)
GAa	34 (±8)	19 (±3)
A***Gg***	280 (±40)	28 (±4)
A***G***a	150 (±10)	10 (±1)
GGa	120 (±10)	15 (±1)
A(A/***G***)***g***	90 (±10)	20 (±5)
G(***G***/A)a	99 (±6)	9 (±1)
AR(607)	> 1000	>1000
GR(505)	> 1000	>1000
AR(611)	> 1000	12 (±5)
GR(509)	> 1000	>1000
AR(619)	31 (±7)	6 (±1)
GR(517)	240 (±30)	9 (±1)
AA***g(517)***	200 (±20)	22 (±2)
GGa(619)	270 (±10)	21 (±2)
AR(619)m	98 (±17)	17 (±3)

A fusion constructs used in that study, we performed a more elaborate analysis. The difference in specificity was confirmed in gel-shift assays with purified AR- and GR-DBDs (Figure 1C), as well as with the full-size AR and GR expressed in HeLa cells by means of a vaccinia expression system [27] (Figure 1B). The C3(1) ARE [28] (Figure 1A), used as a positive control in all experiments, was recognized by both AR and GR, while the PB-ARE-2 was only bound with high affinity by the AR. In gel-shift assays using competition, the specific recognition of the responsive elements by the DBD fragments and the partially purified full-size receptor was shown (results not shown) [27].

For functional comparison of the PB-ARE-2 with the non-specific C3(1) ARE, we have cloned two copies of each ARE upstream of the TATA-box of the tk-promoter controlling the transcription of a luciferase reporter gene. This resulted, respectively, in the constructs pPBluc3 and pC3luc3. These constructs were co-transfected with expression vectors for the GR, PR, MR and AR. Stimulation with the specific steroids revealed that only androgens induced both reporter constructs (Table 1). The GR, MR and PR did not transactivate the PB-ARE-2-based reporter construct, although the inductions of the construct containing the C3(1) ARE were comparable with those observed with the AR. The latter observation clearly demonstrates the presence of functional receptors under all conditions.

To verify whether the specificity was due to the DNA-binding domain, we replaced the AR-DBD in the full-size AR with that of the GR (residues 537–619), resulting in the construct fAGA. In transient transfection assays, the pPBluc3 was transactivated by the fAGA construct to a level similar to that mediated by the AR itself. However, the reporter was at least three times less responsive, as compared with pC3luc3, a situation that is intermediate between the GR and AR activation (Table 1). In gel-shift assays, the isolated DBD fragments of the AR, GR, MR and PR (AR1, GR1, MR1 and PR1) bound the C3(1) ARE with high affinity, whereas under similar conditions the PB-ARE-2 was only recognized by AR1 with high affinity (Table 1). This confirmed that the AR-DBD fragment contains sufficient information for specific recognition of the PB-ARE-2.

Contributions of the two Zn-finger modules to the sequence-specific DNA-binding of AR1

Binding assays with chimaeric constructs in which the first Zn-fingers were swapped between AR1 and GR1 (AGg and GAa)

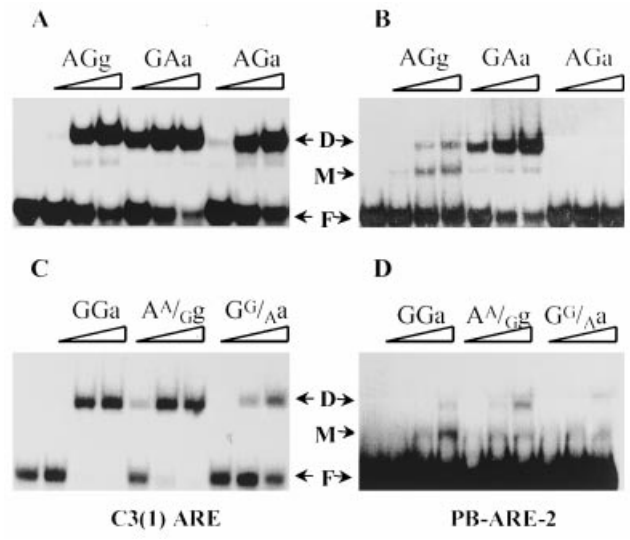


Figure 2 Involvement of the second Zn-finger of the AR in the specific recognition of the PB-ARE-2

The Figure shows the comparative-gel shift assay of C3(1) ARE (**A** and **C**) and PB-ARE-2 (**B** and **D**) by the AGg, GAa, AGa (**A** and **B**); GGa, AA/Gg and GG/Aa (**C** and **D**). The constructs are described in Table 2 and in the text. Radiolabelled probes (2×10^5 c.p.m.) were incubated with 0, 135, 400 or 800 fmol of purified AR1 or GR1 in a total volume of 20 μl for the binding reaction. The protein–DNA complex was separated from the free probe by electrophoresis on a 5% non-denaturating polyacrylamide gel. The gel was dried and exposed to X-ray film to reveal the free probe (F) and the monomeric (M) and dimeric (D) complexes (indicated by arrows). Note that the exposure time of the autoradiograph shown in (**D**) was increased in order to reveal the retarded bands.

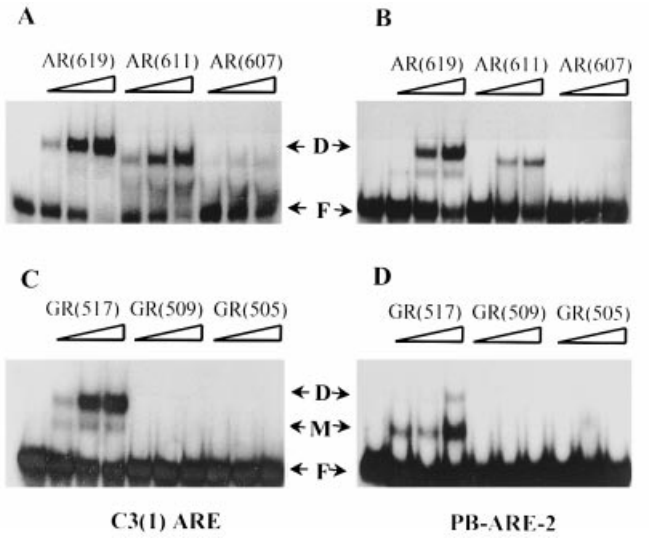


Figure 3 Involvement of AR-DBD (TE and GR-DBD) CTE in DNA binding

The Figure shows a comparative gel-shift assay of the C3(1) ARE (**A** and **C**) and the PB-ARE-2 (**B** and **D**) by AR(619), AR(611) and AR(607) (**A** and **B**); GR(517), GR(509) and GR(505) (**C** and **D**). The constructs are described in Table 2 and in the text. Radiolabelled probes (2×10^5 c.p.m.) were incubated with 0, 135, 400 or 800 fmol of purified AR1 or GR1 in a total volume of 20 μl for the binding reaction. Protein–DNA complex was separated from the free probe by electrophoresis on a 5% non-denaturating polyacrylamide gel. The gel was dried and exposed to X-ray film to reveal the free probe (F) and the monomeric (M) and dimeric (D) complexes (indicated by arrows). Note that the exposure time of the autoradiograph shown in (**D**) was increased in order to reveal the retarded bands.

showed that the affinity of GAa for both AREs was comparable with that of AR1, whereas AGg did not recognize the PB-ARE-2, although it bound the C3(1) ARE with high affinity (Figure 2 and Table 2). This indicates that the first Zn-finger of the AR is not directly involved in the specific recognition of the PB-ARE-2, but that the second Zn-finger and the hinge-region fragment determine the specificity.

The role of the second Zn-finger was analysed in more detail using AR-DBD constructs in which this finger was swapped for that of the GR. This resulted in a construct, AGa, which lost its affinity for the PB-ARE-2 (Figure 2 and Table 2). In addition, the hinge-region fragment of the AR was unable to confer affinity for the PB-ARE-2 to the GR-DBD, since the construct GGa has low affinity for this ARE, although it has high affinity for the C3(1) ARE (Figure 2 and Table 2).

In the constructs $A^A/_Gg$ and $G^G/_Aa$, the C-terminal part of the second Zn-finger, which forms an α-helix in the crystal structures of the GR-DBD, together with the hinge region, were swapped between AR1 and GR1 (Table 2). Both constructs lost their affinity for the PB-ARE-2, but not for the C3(1) ARE (Figure 2). In conclusion, our results indicate that at least two separate segments, one in the hinge region and one in the N-terminal part of the second Zn-finger, are involved in the specific binding of the AR to the PB-ARE-2.

A CTE of the DBD is necessary for high-affinity binding of the AR-DBD and the GR-DBD to their response elements

In AR1 and GR1, a large fragment of the hinge region was included because it was also present in the Protein A fusions we used previously [20]. Most of the sequence divergence between AR1 and GR1 is concentrated within this fragment and, from

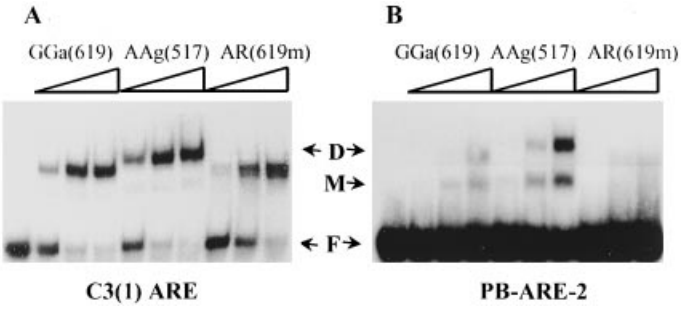


Figure 4 Involvement of the AR-DBD CTE in the specific recognition of PB-ARE-2

The Figure shows a comparative gel-shift assay of the C3 (1) ARE (**A**) and the PB-ARE-2 (**B**) by the GGa(619), AAg(517) and AR(619m) constructs (for nomenclature, see Table 2). Radiolabelled probes (2×10^5 c.p.m.) were incubated with 0, 135, 400 or 800 fmol of purified AR1 or GR1 in a total volume of 20 μl for the binding reaction. The protein–DNA complex was separated from the free probe by electrophoresis on a 5% non-denaturating polyacrylamide gel. The gel was dried and exposed to X-ray film to reveal the free probe (F) and the monomeric (M) and dimeric (D) complexes (indicated by arrows).

the GR-DBD/glucocorticoid response element (GRE) structure studies [2,6], there are no indications that it would be involved in DNA recognition. However, removal of this region, generating the receptor fragments AR(607) and GR(505), resulted in a complete loss of affinity for the C3(1) ARE (Figure 3 and Table 2). Surprisingly, when both constructs were analysed as a GST fusion product, they did recognize the C3(1) ARE with high affinity (results not shown). From a subsequent deletion analysis, it became clear that, for the GR-DBD, a CTE of at least twelve

amino acids had to be included to restore the affinity for the C3(1) ARE [GR(517)]. In contrast, a CTE of only four amino acids [AR(611)] was sufficient to restore the binding of the AR-DBD to the C3(1) ARE, whereas a CTE of 12 amino acids was required in order to restore its high affinity for the PB-ARE-2. The influence of the CTE on the specific binding of the AR to the PB-ARE-2 was further illustrated by the constructs AAg(517) and GGa(619), in which the 12 C-terminal residues were swapped between AR(619) and GR(517) (Figure 4 and Table 2). Both constructs have a low affinity for the PB-ARE-2, whereas the high affinity for the C3(1) ARE remained. In addition, the AR(619m) construct, in which only the last three amino acids of the CTE from AR(619) were exchanged with those of the GR, recognized the C3(1) ARE with high affinity, but not the PB-ARE-2 (Figure 4 and Table 2).

DISCUSSION

Previous studies clearly showed that the consensus sequence for the AR binding site is similar to the GRE/progesterone response element consensus [12,18]. This concept was confirmed in comparative DNA-binding studies between the AR- and the GR-DBDs for a number of AREs [20,29]. An exception to this rule is the PB-ARE-2, which is only recognized with high affinity by the AR and not by the GR [20]. We have confirmed this difference in affinity for the PB-ARE-2 in gel-shift assays with the full-size AR and GR as well as with the GR-DBD and the AR-DBD (Figure 1). The calculated K_S also demonstrates a low affinity of the GR-DBD for the PB-ARE-2 (K_S 165 ± 10 nM, mean $\pm$ S.E.M.) as compared with the C3(1) ARE (K_S 21 ± 3 nM, mean $\pm$ S.E.M.).

In transient transfection assays with PB-ARE-2 and C3(1) ARE containing reporter plasmids and in gel-shift assays with the DBD-receptor fragments, we demonstrate that the PB-ARE-2 is a specific ARE, since it is only recognized by the AR and not by the GR, MR or PR (Table 1). Transfection assays with a mutated AR, called fAGA, further suggests that determinants of selectivity reside at least partially within the amino acids 533–619 of the AR, a fragment existing of the two Zn-finger modules of the DBD and 12 amino acids from the hinge region. We cannot exclude a role for the N-terminal domain and the ligand-binding domain in the selectivity of the full-size receptors. This was, for example, observed for the specific recognition of the HRE3 of the *slp* enhancer [30]. The molecular basis for the differences in the DNA-binding between AR and GR was more thoroughly studied by comparing the binding of mutant DBD fragments to the AREs in gel-shift assays and by calculating their apparent dissociation constants (K_S).

The dissociation constants from the chimaeric constructs demonstrate that the first Zn-finger module is not involved in the difference in specificity between AR1 and GR1 (Table 2). This is surprising, since, for the difference between ER- and GR-DBDs, it was shown that exchanging only three residues of this module, called the P-box, was sufficient to change the sequence specificity [3]. Swapping the C-terminal segment of the second Zn-finger together with the fragment of the hinge region between AR1 and GR1 ($A^A/_Gg$ and $G^G/_Aa$; Figure 2) resulted in a loss of affinity of both constructs for the PB-ARE-2. In conclusion, these observations indicate that both the N-terminal part of the second Zn-finger and part of the hinge region are involved in the AR specificity. All constructs retained high affinity for the C3(1) ARE, indicating that the chimaeric constructs all have the correct conformation.

It has been reported for the ER α-isoform and the GR that, besides the two zinc fingers, at least 30 amino acids of the hinge region were necessary for high-affinity DNA binding [22,31,32]. In a more detailed deletion analysis we demonstrated the requirement of a CTE for the DNA binding that differs in length between the AR and the GR. While the AR-DBD has to be extended by only four amino acids to regain high-affinity binding for the C3(1) ARE, the GR-DBD clearly needs 12 amino acids. However, for the specific recognition of the PB-ARE-2, the AR also needs a CTE of 12 amino acids. This might be the reflection of a different binding mode of the AR-DBD to the C3(1) ARE versus the PB-ARE-2.

The involvement of a CTE in high-affinity DNA binding has also been described for the vitamin D receptor, the peroxisome-proliferator-activated receptor, thyroid receptor (TR), human oestrogen-related receptor, Rev-erbβ and nerve-growth-factor-inducible protein B [7–10,33,34]. However, these receptors are known to bind mainly as heterodimers with the 9-*cis*-retinoid acid receptor (RXR) to direct repeats. Previous studies have shown that the AR might recognize inverted repeats as well as direct repeats as response elements. The PB-ARE-2 can also be considered as an imperfect direct repeat to which GR1 and GR(517) can bind as monomers to one half-site (Figure 1C and Figure 3D), while the different AR constructs recognize the PB-ARE-2 as a dimer rather than a monomer. This suggests that the AR, and not the GR, can bind in a head-to-head, as well as in a head-to-tail, configuration, similar to the RXR/TR heterodimeric DNA complex [17,20]. For the GR, only the first configuration has been reported [2], and most GREs resemble inverted repeats, although a GRE of the DR15 configuration was recently reported [35]. In the head-to-tail binding of the RXR/TR heterodimer on direct repeats, a CTE of 12 amino acids of only the TR, but not of the RXR, is involved in the dimerization. Structural studies of RXR/TR-DBD heterodimer binding to DNA showed the presence of an α-helix in the TR, situated at the position of this CTE, also called T-box. This helix is oriented towards the minor groove of the DNA and is supposed to stabilize the complex by providing extra protein–DNA as well as protein–protein interactions [8]. For the GR-DBD, no α-helical structure in this C-terminal region is seen in the crystallographic and NMR studies [2,6]. The observation that the AR-DBD needs a CTE of different length for high-affinity binding either to the C3(1) ARE or to the PB-ARE-2 is another strong indication of a different dimerization mode for these AREs. Indeed, for the AR-DBD the deletion of eight amino acids of the CTE, the swapping of the entire [AAg(517)], or even only part of the CTE [AR(619) mutation Leu-Gly-Asn → Lys-Ile-Lys], results in a loss of affinity for the PB-ARE-2, whereas all these constructs still recognize the C3(1) ARE with high affinity (Table 2). Moreover, the C3(1) ARE and the PB-ARE-2 are bound with high affinity by heterodimers of either, AR1 with AR(607) or AR1 with AR(611), under conditions where homodimers of AR(607) or AR(611) are hardly detectable (results not shown). This is not the case for GR1, GR(505) and GR(509), indicating that these GR- and AR-DBD constructs recognize the same sequences with a different overall configuration, since the AR-DBD needs only one CTE for high-affinity DNA binding. We postulate that the difference in DNA specificity between the AR and GR for the PB-ARE-2 is due to alternative protein–protein interactions. By analogy with RXR/retinoic acid receptor and RXR/TR heterodimers [36], the CTE and the second Zn-finger probably contribute to a stabilizing interface between the two monomer AR-DBDs when bound to DNA.

The present study demonstrates that the PB-ARE-2 is an AR-specific response element that is not recognized by the GR, the MR or the PR. Surprisingly, this specificity is not determined by the first Zn-finger, but resides in the second Zn-finger and a small

CTE. Deletion analysis shows that this CTE is required for the high-affinity DNA-binding. This region has a different function in the AR versus the GR, a function that depends for the AR on the nature of the ARE. Our results suggest that a further classification of the steroid-receptor family can be made, since we can discriminate the AR from the GR, PR and MR on the basis of the DNA-binding selectivity.

We thank H. Debruyn and R. Bollen for their excellent technical assistance and V. Feytons for the synthesis of numerous oligonucleotides. We also thank Professor R. Evans, Dr. S. Hollenberg and Dr. C. Chang for the expression plasmids of the nuclear receptors. This work was supported by the Geconcerteerde Onderzoeksactie van de Vlaamse Gemeenschap and by grants of the Inter Universitaire Attractie Pool, the Belgium Cancer Funds, Fonds voor Geneeskundig en Wetenschappelijk Onderzoek and Vlaamse Wetenschappelijke Stichting. P. A. was supported by a scholarship of the Vlaams Institute voor Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie. F.C. is a senior assistant of the Fonds voor Wetenschappelijk Onderzoek.

REFERENCES

1 Evans, R. (1988) Science **240**, 889–895
2 Luisi, B., Xu, W., Otwinski, Z, Freedman, L., Yamamoto, K. and Sigler, P. (1991) Nature (London) **352**, 497–505
3 Zilliacus, J., Wright, A., Norinder, U., Gustafsson, J.-A. and Carlstedt-Duke, J. (1992) J. Biol. Chem. **267**, 24941–24947
4 Dahlman-Wright, K., Wright, A., Gustafsson, J.-A. and Carlstedt-Duke, J. (1991) J. Biol. Chem. **266**, 3107–3112
5 Umesono, K. and Evans, R. (1989) Cell **57**, 1139–1146
6 Remerowski, M., Kellenbach, E., Boelens, R., van der Marel, G., VanBoom, J., Maler, B., Yamamoto, K. and Kaptein, R. (1991) Biochem. **30**, 11620–11624
7 Hsieh, J.-C., Jurutka, P., Selznick, S., Reeder, M., Haussler, C., Whitfield, G. and Haussler, M. (1995) Biochem. Biophys. Res. Commun. **215**, 1–7
8 Rastinejad, F., Perlmann, T., Evans, R. and Sigler, P. (1995) Nature (London) **375**, 203–211
9 Terenzi, H., Alzari, P. and Zakin, M. (1998) Biochem. **37**, 11488–11495
10 Wilson, T., Paulsen, R., Padgett, K. and Milbrandt, J. (1992) Science **256**, 107–110
11 Lee, M., Kliewer, S., Provencal, J., Wright, P. and Evans, R. (1993) Science **260**, 1117–1120
12 Beato, M. (1989) Cell **56**, 335–344
13 Funder, J. W. (1993) Science **259**, 1132–1133
14 Archer, T., Zaniewski, E., Moyer, M. and Nordeen, S. (1994) Mol. Endocrinol. **8**, 1154–1162
15 Lambert, J. and Nordeen, S. (1998) J. Biol. Chem. **273**, 32708–32714
16 Adler, A., Scheller, A. and Robins, D. (1993) Mol. Cell. Biol. **13**, 6326–6335
17 Zhou, Z., Cordons, J. and Brown, T. (1997) J. Biol. Chem. **272**, 8227–8235
18 Roche, P., Hoare, S. and Parker, M. (1992) Mol. Endocrinol. **6**, 2229–2235
19 Rennie, P., Bruchovsky, N., Leco, K., Sheppard, P., McQueen, S., Cheng, H., Snoek, R., Hamel, H., Bock, M., Chang, C. et al. (1993) Mol. Endocrinol. **7**, 23–36
20 Claessens, F., Alen, P., Devos, A., Peeters, B., Verhoeven, G. and Rombauts, W. (1996) J. Biol. Chem. **271**, 19013–19016
21 Chang, C., Kokontis, J. and Liao, S. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7211–7215
22 Hollenberg, S., Giguere, V., Segui, P. and Evans, R. (1987) Cell **49**, 39–46
23 Tan, J., Joseph, D., Quarmby, V., Lubahn, D., Sar, M., French, F. and Wilson, E. (1988) Mol. Endocrinol. **2**, 1276–1285
24 Miesfeld, R., Rusconi, S., Godowski, P., Maler, B., Orket, S., Wikstroem, A., Gustafsson, J.-A. and Yamamoto, K. (1986) Cell **46**, 389–399
25 Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E. and Evans, R. (1987) Science **237**, 268–275
26 Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. and Chambon, P. (1990) EMBO J. **9**, 1603–1614
27 De Vos, P., Schmitt, J., Verhoeven, G and Stunnenberg, H. (1994) Nucleic Acids Res. **22**, 1161–1166
28 Claessens, F., Celis, L., Peeters, B., Heyns, W., Verhoeven, G. and Rombauts, W. (1989) Biochem. Biophys. Res. Commun. **164**, 833–840
29 Rundlett, S. and Miesfeld, R. (1995) Mol. Cell. Endocrinol. **109**, 1–10
30 Scheller, A., Hughes, E, Golden, K. and Robins, D. (1998) J. Biol. Chem. **273**, 24216–24222
31 Rusconi, S. and Yamamoto, K. (1987) EMBO J. **6**, 1309–1315
32 Mader, S., Chambon, P. and White, J. (1993) Nucleic Acids Res. **21**, 1125–1132
33 Hsu, M.-H., Palmer, C., Song, W., Griffin, K. and Johnson, E. (1998) J. Biol Chem. **273**, 27988–27997
34 Sem, D., Casimiro, D., Kliewer, S., Provencal, J., Evans, R. and Wright, P. (1997) J. Biol. Chem. **272**, 18038–18043
35 Aumais, J. P., Lee, H. S., DeGannes, C., Horsford, J. and White, J. H. (1996) J. Biol. Chem. **271**, 12568–12577
36 Zechel, C., Shen, X.-Q., Chen, J.-Y., Chen, Z.-P., Chambon, P. and Gronemeyer, H. (1994) EMBO J. **13**, 1425–1433

Received 18 February 1999/12 April 1999; accepted 12 May 1999