Differences in DNA Binding Characteristics of the Androgen and Glucocorticoid Receptors Can Determine Hormone-specific Responses*

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Steroid hormones are important endocrine messengers that activate their receptors, which translocate to the cell nucleus and regulate gene expression mainly after interaction with DNA sequences, called response elements (1, 2). The steroid receptors are a subfamily of the nuclear receptor superfamily, a large group of structurally homologous transcription factors. A problem with the explanation of the specificity of these hormone responses arose when several studies pointed out that the class I receptors (androgen receptor (AR), progesterone receptor, and mineralocorticoid receptor) have identical consensus response elements (3, 4) and that their DNA-binding domains were highly conserved (5). This contrasts with the fact that the in vivo expression of several genes is specifically controlled by only one steroid hormone (6).

Several possible mechanisms have been described to explain the steroid specificity of transcriptional control, e.g. steroid metabolism, tissue-specific receptor presence (7), influence of coactivator complexes (8), and chromatin structure (9, 10). In addition, more recent reports indicate that the AR on the one hand and the GR, progesterone receptor, and mineralocorticoid receptor on the other exhibit different DNA binding characteristics (11–15). One AR-specific response element was found in the promoter of the rat probasin gene (PB-ARE-2) (12, 16, 17). Probasin is an androgen-regulated protein exclusively expressed in the dorsolateral epithelium of the prostate (18). Two cis-acting androgen response elements (PB-ARE-1 and PB-ARE-2) were identified in the promoter and were shown to be necessary for its androgen regulation.

The core DNA-binding domain (DBD) of the nuclear receptors is composed of two zinc finger modules (19). The N-terminal zinc finger is involved in specific DNA interaction, whereas the C-terminal zinc finger provides DNA-dependent dimerization (20). Our earlier results indicated that residues in the second zinc finger and a C-terminal extension (CTE) of 12 amino acids determine the difference in the PB-ARE-2 binding between the AR and the GR (21). This was the first clear indication of a direct involvement of the CTE residues in the specificity of DNA binding for steroid receptors. The CTE described here overlaps with the T-box described for other members of the nuclear receptor family (e.g. 9-cis retinoic acid receptor (RXR), thyroid receptor (TR), and nerve growth factor-inducible protein B) (22–24). Structural studies showed that in these receptors the T-box residues form an α-helix, which interacts with the DNA phosphate backbone and which is also involved in the DNA-dependent heterodimerization. Structural studies of the estrogen receptor DBD and GR-DBD, however, did not clarify the involvement of this region in DNA binding (20, 25–28). The RXR and the TR bind in a “head-to-tail” orientation on a response element with a direct repeat structure (half-site, 5′-AGGTCA-3′), whereas the steroid receptors bind in a “head-to-head” orientation on a response element organized as an inverted repeat (half-site estrogen receptor, ARE, androgen response element; CTE, C-terminal extension; DBD, DNA-binding domain; NLS, nuclear localization signal; DR, direct repeat; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; RXR, 9-cis retinoic acid receptor; TR, thyroid receptor.)
5′-AGGTCA-3′; class I receptors, 5′-TGTTCT-3′).

In the present study, the difference in DNA binding between the AR and the GR is analyzed in detail by determining the identity of the amino acids involved and the characteristics of the response element responsible for the exclusion of the GR from binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction and modifying enzymes were obtained from either Life Technologies, Inc., Amersham Pharmacia Biotech, Promega (Madison, WI), Takara Shuzo Co. Ltd. (Shiga, Japan), Eurogentec, (Belgium), or Roche Molecular Biochemicals (Mannheim, Germany). DNA oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (Milligen Corp., Bedford, MA) or purchased from Eurogentec. R1881 (methyltrienolone), dexamethasone, aldosterone, and progesterone were purchased from Sigma, [methyl-3H]estradiol, dexamethasone, aldosterone, and progesterone were purchased from Life Technologies, Amersham Pharmacia Biotech, and X-Omat S x-ray films from Eastman Kodak Co.

**Plasmids**—The cDNA encoding the rat AR was described by Chang et al. (29), and that for the rat GR was described by Hollenberg et al. (30). fAGA is derived from the full-size rat AR by swapping the AR-DBD for the GR-DBD (29), and that for the rat GR was described by Hollenberg et al. (10 s), the extracts were cleared by centrifugation (12,000 × g for 10 min) of pPBARE1luc, pPB2-IR1luc, pDRIluc, and pIDRluc are regulated by the ARE hexamers are underlined). The pES vector was generated by cloning of the SmaI site of pES, generating pPBASEI1uc, pPB2RI1uc, pDR1uc, and pD2R1uc are reporter constructs containing response elements derived from either Life Technologies, Amersham Pharmacia Biotech, or Takara Shuzo Co. Ltd. (Shiga, Japan). Site-directed mutagenesis of the AR and the GR is analyzed in detail by determining the identity of the amino acids involved and the characteristics of the response element responsible for the exclusion of the GR from binding.

**Preparation of COS-7 Nuclear Extracts Containing Full-size Receptor Constructs**—The protocol for the preparation of nuclear extracts has been described by Andrews and Faller (32) and was used with some modifications. Briefly, 106 COS-7 cells were plated in 10 cm Petri dishes and transfected with 2.5 μg of expression plasmid. The cells were incubated (1 h) before harvesting with 10–3× hormone, after which the medium was removed, and the cells were washed twice with 3 ml of ice-cold phosphate-buffered saline. The cells were collected in 1.5 ml of ice-cold phosphate-buffered saline per dish, transferred to an Eppendorf tube, and pelleted by centrifugation (10 s). The phosphate-buffered saline was removed, and the cells were resuspended in 400 μl of ice-cold buffer containing 10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. After 10 min of incubation on ice, the mixture was vortexed (30 s), and the nuclei were collected by a short spin in a microcentrifuge. The supernatant was then frozen in liquid nitrogen and the cells were resuspended in 400 mM KCl, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride. After 20 min of incubation on ice and a short vortexing (10 s), the extracts were cleared by centrifugation (12,000 × g) at 4°C for 2 min. The supernatant was then frozen in liquid nitrogen and stored at −80 °C. Western blotting was performed on these extracts described (33).

**Gal Shift Assays**—The double-stranded oligonucleotides containing the ARE sequences (described in Table II) were labeled with [α-32P]dATP by a fill-in reaction by the Klenow fragment of DNA-polymerase to a specific activity of 5000 cpm/μmol. The response elements with a direct repeat sequence organization are derived from the DR element 5′-AGCTTCTAGTCTCTTGATGTTCTGAATGAGCT-3′ (Table II).

**RESULTS**

**The AR-DBD Is the Main Determinant for the Specific PB-ARE-2 Recognition**—The difference in PB-ARE-2 recognition between the AR and the GR was first observed using only their DBDs (named AR1 and GR1) (12, 21). To analyze the influence of the other receptor domains on the difference in DNA binding, chimerical full-size AR and GR constructs with swapped DBDs (AGA, GR-DBD residues 433–537, the GR1 construct comprises the corresponding rat GR-DBD amino acids 432–533). The different mutated GR1 and AR1 constructs generated are described in Fig. 2. The different receptor fragments were purified as described by Schoenmakers et al. (21).

**Preparation of COS-7 Nuclear Extracts Containing Full-size Receptor Constructs**—The protocol for the preparation of nuclear extracts has been described by Andrews and Faller (32) and was used with some modifications. Briefly, 106 COS-7 cells were plated in 10 cm Petri dishes and transfected with 2.5 μg of expression plasmid. The cells were incubated (1 h) before harvesting with 10–3× hormone, after which the medium was removed, and the cells were washed twice with 3 ml of ice-cold phosphate-buffered saline. The cells were collected in 1.5 ml of ice-cold phosphate-buffered saline per dish, transferred to an Eppendorf tube, and pelleted by centrifugation (10 s). The phosphate-buffered saline was removed, and the cells were resuspended in 400 μl of ice-cold buffer containing 10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. After 10 min of incubation on ice, the mixture was vortexed (30 s), and the nuclei were collected by a short spin in a microcentrifuge. The supernatant and the nuclei were then pelleted. The supernatant was then frozen in liquid nitrogen and stored at −80 °C. Western blotting was performed on these extracts.
In transient cotransfection assays, the pC3AREluc reporter construct is induced 4–5-fold by all the chimerical receptors, depending on the ligand-binding domain present (Table I), we could confirm the involvement of the 12 CTE amino acids (Fig. 2 and Ref. 21) demonstrate that for the AR, residues of the hinge region as well as of the N-terminal part of the second zinc finger are involved. We therefore analyzed the importance of all residues that differ between the AR-DBD and the GR-DBD in these two regions.

In our earlier study, we demonstrated that the first zinc finger of the AR is not involved in the specific recognition of the PB-ARE-2 (21). However, experiments with the chimerical DBD constructs G/A and A/G (Fig. 2 and Ref. 21) demonstrate that for the AR, residues of the hinge region as well as of the N-terminal part of the second zinc finger are involved. We therefore analyzed the importance of all residues that differ between the AR-DBD and the GR-DBD in these two regions.

We have already described the necessity of a C-terminal extension of 12 amino acids for specific and high affinity binding of the AR to the PB-ARE-2 (21). With the constructs AR28.1 and 28.2 (Table I), we could confirm the involvement of the 12 CTE residues in high affinity DNA binding. These constructs are the result of a deletion of residues forming the nuclear localization signal, which partially overlaps with the CTE (36). Note that AR28.1 and 28.2, kindly provided by Dr. A. O. Brinkman, are derived from the human AR, which differs from the rat AR at three residues in the extreme C terminus of the hormone receptor fragment. Deletion constructs showed, however, that deletion of this part of the protein had no influence on the DNA binding by the rat AR-DBD (21). Six of the 12 CTE amino acids differ between the AR and the GR. Systematic mutations of the AR-specific residues to the GR homologues resulted in a num-

Fig. 1. Gel shift and transfection assays with different receptor constructs. A, gel shift assays with full-size receptor constructs that were overexpressed in COS-7 cells. Cell extracts were incubated with labeled C3(1) ARE and PB-ARE-2, followed by an electrophoretic separation of the free DNA from the protein-DNA complexes on a non-denaturating polyacrylamide gel. Competition is performed with 300-fold excess cold C3(1) ARE (lanes 2). The supershift is obtained by adding a specific antibody against the AR or the GR (lanes 3). The free (F), specific (*), supershifted (S), and nonspecific (N) binding complexes are indicated. B, COS-7 cells were transfected with the reporter constructs pC3AREluc or pPBARE2luc together with the indicated receptor constructs. The cells were incubated for 48 h with DCC-treated medium containing ethanol (open bars), 1 nM R1881, or 1 nM dexamethasone (black bars), depending on the ligand-binding domain present in the co-transfected receptor constructs. The luciferase values are given relative to the empty reporter construct, and the mean of at least three independent experiments (±S.E.) is shown. The absolute induction values were obtained by dividing the reporter activity in the presence of hormone by that seen in the absence of hormone and are mentioned in the figure as fold induction. Luciferase activities were corrected for transfection efficiency using the β-galactosidase activities resulting from co-transfection with a CMV-β-galactosidase reported plasmid. C, Western blot analysis of COS-7 cell extracts to control the expression levels of the chimerical receptor constructs (33). The transfected cells were incubated with 1 nM hormone for 24 h. For all constructs, an antibody against the N-terminal domain was used (AR and AGA, anti-AR; GR and GAG, anti-GR).
number of AR1 mutants, schematically illustrated in Table I. The apparent dissociation constants of these constructs for the PB-ARE-2 and C3(1) ARE indicate that mainly the mutation of Gly-610 (and to a lower extent, mutation of Leu-617) abrogates the PB-ARE-2 binding.

In the C-terminal part of the second zinc finger of the GR-DBD, an α-helix (amino acids 492–503) was described (20, 26). In the ARHm construct, all the nonhomologous residues of the α-helical structure in the C-terminal side in the AR-DBD (amino acids 594–605) were swapped for those of the GR (Fig. 2). This construct had DNA binding capacities similar to those of the wild type AR1, indicating that the putative helix in the C-terminal region of the second zinc finger had no influence on the difference in DNA sequence recognition between the AR and the GR. However, for high affinity DNA binding to PB-ARE-2, a correct conformation of the helix is necessary. This is illustrated by the mutation of Leu-599 of the AR to the GR homologue Tyr-497 (AR599Y) (Fig. 2). In the x-ray structure of the GR-DBD, Tyr-497 and Leu-501 are oriented toward each other. When a tyrosine is present at both places, as in AR599Y, steric hindrance might prevent the correct folding of the helix, influencing the overall DBD-conformation. We tested this hypothesis by combining the Leu-599 to Tyr with the Tyr-603 to Leu mutation in AR1. As expected, the AR599Y/603L regained its affinity for the PB-ARE-2 leading to the conclusion that Tyr-497 and Leu-501 in the GR are two complementary residues. This is in contrast with the mutated AR construct, AR599Y/610E, which has lost its affinity for PB-ARE-2. The exchange of another nonconserved residue in the putative α-helix of AR1, namely Glu-604, for that of the GR homologue glutamine had no effect on the DNA specificity.

To identify the other residues in the second zinc finger necessary for the AR-specific PB-ARE-2 binding, we introduced the GR homologues in the AR1. However, none of the mutated AR1 constructs displayed a dramatic decrease in affinity for PB-ARE-2 or C3(1) ARE (data not shown). Therefore, we took an alternative approach based on the observation that the G/A chimera, containing only part of the second zinc finger and the hinge region of the AR, has a low affinity for the PB-ARE-2. In an attempt to restore the binding to the PB-ARE-2, mutations were introduced in G/A (Fig. 2). The apparent dissociation constants of these constructs indicate that Thr-585 (position 483 in GR1) might be involved in high affinity binding of the AR to PB-ARE-2.

Direct Repeat Recognition by the Androgen Receptor—In Table II, synthetic oligonucleotides containing either inverted or direct repeats of the left or right half-site of the PB-ARE-2 are listed (PB2-IR1, PB2-IR2, and PB2-DR2). The apparent dissociation constants of DBD binding to these synthetic elements demonstrate that the left half-site of the PB-ARE-2 sequence determines the AR-specific binding and prevents GR-binding. PB2-IR1, which is recognized with high affinity by both DBDs (Fig. 3), can be considered as an inverted as well as a direct repeat. The PB-IR2 and PB-DR2 are not recognized by the GR-DBD as a dimer, whereas the AR-DBD recognizes these sequences, although with lower affinity (Fig. 3 and Table II). Gel shift assays with COS-7 nuclear extracts containing the full-size AR or GR confirm the results of the DBD constructs (data not shown).

In comparative gel shift assays with AR1 and GR1, we demonstrated that the AR-DBD, but not the GR-DBD, is able to bind a direct repeat of the consensus core recognition sequence 5’-TGTTCT-3’ (DR, Table II and Fig. 3). Note that GR1 binds DR mainly as a monomer and that AR1 forms monomeric and dimeric complexes with the DR sequence with apparently similar affinity. Most mutations in the right half-site of this direct repeat (IDR, DR2, and DR3) decrease the affinity of AR1, whereas two point mutations in the left half-site (DRI) increase the affinity of AR1. In contrast, under similar conditions, none of these imperfect direct repeats is recognized with high affinity by GR1, although monomeric binding is observed (Fig. 3). Note that for DRI and DR14, only a dimeric complex and no monomeric AR1-DNA or GR1-DNA complexes are detected, whereas IDR, DR2, and DR3 are only bound as monomer by both DBDs (Fig. 3 and results not shown).
specific recognition of direct repeats by the AR-DBD was confirmed in gel shift assays with the full-size AR that also binds both DR and DRI but not DRI2 (Fig. 4). The full-size GR recognizes none of the direct repeats with high affinity. Even in the presence of the specific antibody, only a very weak band of the DNA-protein complex is visible.

IDR and DRI are typical examples of DR sequences containing mutations in the right and the left half-sites, respectively. The androgen responsiveness of reporter constructs under control of the PB-IR1, IDR, or DRI sequences in transient transfection assays correlated well with the in vitro determined receptor binding (Table III). Both GR and AR were able to induce pPB2-IR1Luc, whereas only androgens could enhance the transcription from pDR1Luc. pDR1Luc was hardly responsive to either androgens or dexamethasone. The C3(1) ARE, PB-ARE-2, and PB-ARE-2 constructs were taken as control constructs.

**Direct Repeat Binding by Chimerical AR and GR Constructs**—The importance of the AR-DBD for the recognition of response elements with a direct repeat configuration was further illustrated with the chimerical full-size AR and GR, GAG and fAGA. From gel shift assays with COS-7 nuclear extracts containing these chimeras, it became clear that it is mainly the AR-DBD that determines the high affinity binding to direct repeat elements (Fig. 5). Indeed, the fAGA construct binds the C3(1) ARE well but has a low affinity for the direct repeat sequences. The GAG construct, however, is able to bind the PB-ARE-2 and the elements DRI and DR.

The AR-DBD Dimer Involves Only One CTE for High Affinity DNA Binding—The DR sequence recognition by AR1 and the different functions of the CTEs between the AR and the GR in specific and high affinity DNA binding led to the hypothesis of a head-to-tail configuration of the AR on DR sequences, rather than a head-to-head configuration as seen for the GR-DBD (20). This would imply that only one of the two DBDs needs to have its CTE making contacts with the other DBD. We tested this hypothesis by analyzing the DNA binding of mixtures of AR1 with AR(607) (AR-DBD lacking the CTE) or AR(611) (AR-DBD with 4 CTE residues), as well as GR1 with GR(505) (GR-DBD lacking the CTE) or GR(509) (GR-DBD with four CTE residues) (Fig. 6). At low concentrations, at which AR(607) did not bind the C3(1) ARE by itself, it was able to bind as a heterodimer with AR1. In addition, although AR(611) can bind as a dimer to C3(1) ARE, it preferentially binds as a heterodimer with AR1. Under similar conditions, GR1/GR(505) heterodimers are not detectable on the C3(1) ARE, and only a very weak GR1/GR(509) heterodimeric complex appears. For PB-ARE-2, only the AR(611) forms a heterodimeric complex with AR1 in conditions under which neither AR(607) nor AR(611) alone can bind PB-ARE-2.

**DISCUSSION**

The Androgen Receptor DNA-binding Domain Determines the Affinity for PB-ARE-2—The difference in binding of PB-ARE-2 between the AR and the GR has been demonstrated in gel shift assays, as well as in transient transfection assays (21). The involvement of the different androgen receptor domains on the specific binding to the PB-ARE-2 was analyzed by the use of chimerical receptor constructs AGA and GAG. In gel shift assays, we demonstrated that the presence of the AR-DBD determines the high affinity for the PB-ARE-2 (Fig. 1). In transfection assays, the GAG, in contrast with the wild type GR, was also able to transactivate a PB-ARE-2-based reporter construct. An unexpected result was seen for the AGA-DBD...
struct, for which only a weak decrease in transactivation capacities was observed as compared with the wild type AR, whereas no clear binding of AGA to the PB-ARE-2 was observed in gel shift assays (Fig. 1). The chimerical constructs AGA and GAG seemed to have a slightly higher expression level as compared with AR and GR. However, another possible explanation for the weak induction of pPBARE2luc by AGA involves the AR-specific interactions of the ligand-binding and N-terminal domains with each other, as well as with coactivators (37–42). These differences in receptor-specific functions of the ligand-binding domain and the N-terminal domain are without a doubt involved in hormone-specific expression of genes containing response elements recognized by all four class I receptors (31). However, in the case of the androgen-specific probasin gene, the changed DNA binding capacities of GAG demonstrate that the AR-DBD is the main determinant for this specificity.

Amino Acids Determining the AR-specific Interaction with PB-ARE-2—In a detailed mutation analysis of both the AR- and the GR-DBD, we have identified three amino acids, Thr-585, Gly-610, and Leu-617 (AR-relative numbering) that determine the difference in DNA binding specificity between the AR and the GR. None of these residues is located in the first zinc finger of the AR-DBD, a surprising observation because the DNA-recognition helix (20) with the three P-box residues determining the difference in DNA sequence recognition between the estrogen receptor and the GR, is situated in the first zinc finger (43). Another important observation is that the replacement of Thr-585, Gly-610, or Leu-617 in the AR-DBD by their GR homologues affected only the PB-ARE-2 binding and did not result in a dramatic decrease in the binding affinity for the nonspecific C3(1) ARE. This strongly supports the hypothesis that different binding configurations of the AR exist on these two AREs.

One of the residues that determine the high affinity for the PB-ARE-2 in the AR-DBD, Thr-585, is located in the second zinc finger. Its GR homologue (Ile-483) was shown to be involved in the dimerization by interaction with the conserved Ala-476 in the other monomer (20). This is the first indication of a difference in dimerization interaction between the AR and the GR. The other residues involved in the DNA specificity (Gly-610 and Leu-617) are located in a 12-amino acid-long CTE of the AR-DBD. The AR-DBD amino acid sequence resembles most closely the GR-DBD and, based on the three-dimensional structure of the latter, a model of the AR-DBD structure was proposed (44), but the structure of the CTE in the GR-DBD is not well defined (20, 26, 27). For other nuclear receptors (e.g. TR and RXR) this region was shown to be involved in dimerization, as well as in making contacts with the phosphate backbone (22–24). However, these nuclear receptors bind response elements of a direct repeat nature. In the case of the AR the involvement of the 12 CTE amino acids in high affinity DNA binding also depends on the nature of the response element (21). Whereas for the GR-DBD the complete CTE is always necessary for high affinity binding, the AR only requires four residues of the CTE for recognition of a nonspecific response element (C3(1) ARE) and the complete CTE for binding to the PB-ARE-2. This difference is confirmed by the heterodimer complexes formed by AR1 with the AR constructs without and with four residues of the CTE (Fig. 6). We therefore postulate the existence of different binding conformations of the AR-DBD on these two AREs. These het-
erodimeric complexes are not detected for the GR. The involvement of only one complete CTE in stable DNA binding also indicates that the AR-DBD is able to bind DNA in a head-to-tail configuration, a binding configuration in which one CTE can provide necessary dimerization interactions. This would be in analogy with the heterodimer complex described for, for example, the DNA-binding domains of the TR and the RXR (22).

Mutation and deletion of the amino acids, which constitute the NLS, resulted in a decreased or even a loss of DNA binding (Fig. 2, AR28.1 and AR28.2). This result indicates that the NLS is an integral part of the DBD. Earlier reports studying the NLS with identical mutations in full-length human AR mention decreased transcription activation as a result of a loss of nuclear import (36). Our results indicate that loss of transcription could also be the result of a lack of DNA binding. Finally, two mutations in the CTE were identified in patients with prostate cancer: Arg-612 to Gly (45) and Lys-613 to Thr (46), indicating that the CTE region could be involved in the correct functioning of the androgen receptor.

**Sequence Requirements for Specific Recognition by the AR**—The difference in DNA binding characteristics between the AR and the GR is clearly illustrated by the binding experiments with the direct repeat of the consensus half-site 5′-TGTTC-T3′ (Tables II and III and Fig. 3). However, cooperativity on a perfect direct repeat seems to be inefficient, because the monomeric and dimeric binding complexes appear with similar kinetics. The mutation analysis of the DR sequence suggests that a hierarchy regulates the DBD binding to the two core half-sites. The right half-site must be occupied first, upon which conformational changes in the AR-DBD render it possible for the left half-site to be recognized cooperatively by the second DBD. Our results suggest that this can only occur when the left half-site of the response element has equal or lesser affinity for the DBD as compared with the right half-site. This hypothesis is confirmed by the mutations introduced in the left half-site, creating a lower affinity half-site (3, 6, 47) and increasing the affinity of the AR-DBD because only dimeric complexes are observed (e.g. DRI in Table II and Fig. 3). If the left half-site has a markedly higher affinity than the right half-site, the left site will be occupied first, and conformational changes will be unsuitable for recognition of the lower affinity right half-site. This phenomenon would explain the formation of only a monomeric AR-DBD DNA complex on DRI2, DRI3 and DRI5 (Table II and Fig. 3). For the GR, a binding mechanism similar to inverted repeats in a head-to-head orientation has been put forward (48, 49). This is in agreement with structural differences between the monomers in the GR DBD-DNA crystal structure and between DNA-bound GR-DBDs versus DBDs in solution (20, 26).

The mutation analysis of the perfect direct repeat of 5′-TGTTC-T3′ further indicates that the mutation of thymine at position –4 to adenine (DRI4) has a positive effect on both the AR and GR binding, although the AR specificity remains (Table II). This is not surprising because adenine is also present at position –4 in the consensus GRE, and mutation to thymine resulted in a loss of affinity of the GR (3). From the co-crystal structure of the GR-DBD bound to an inverted repeat element, it is known that thymine at position –4 of the consensus GRE directly contacts Val-462 (20), and this valine and Ser-459 were shown to restrict the DNA binding when adenine is present at position –4. The high affinity of the AR-DBD for DRI, DR and PB-ARE-2 is therefore somewhat surprising because both Val-462 and Ser-459 are conserved in the AR. However, the GRE in the co-crystal is bound by two GR-DBD monomers in a head-to-head configuration. One possible explanation for the high affinity AR binding to elements with thymine at position –4 might be that the AR binds in a head-to-tail configuration (Fig. 7). In this concept, the thymine at position –4 becomes the equivalent of the thymine at position +5. Results reported by other groups indicate that nucleotide variations at position +5 have little effect on DNA binding by class I receptors (3, 6, 20, 47). This equivalence would also explain why the mutation to adenine in DRI5 has no effect (Table II).

In the accompanying paper by Verrijdt et al. (50), androgen-specific response elements in the promoters of the slp-gene and of the sc-gene were shown also to contain a thymine at position –4. Mutation of this nucleotide to adenine resulted in promoter constructs inducible by both androgens and glucocorticoids (14, 50). Taken together, these data indicate that thymine at position –4 is an important determinant for the difference in DNA binding between GR and AR.

The involvement of nucleotides and amino acids, other than those described here, in AR-specific DNA binding to the PB-ARE-2 cannot be excluded because combinations of different nucleotides in response elements will lead to subtle changes in DNA backbone structure as well as result in other possibilities for sequence-specific interactions. The variability of the natural AREs certainly emphasizes this point. All of our data suggest that the AR binds the nonspecific AREs, organized as inverted repeats, in a head-to-head orientation, as described for the estrogen receptor and GR. Specific AREs, organized as direct repeats, however, seem to be recognized by the AR in a head-to-tail orientation (Fig. 7). Because other androgen-specific response elements with a direct repeat nature have now been identified (12, 14, 15, 50), we propose that this difference in DNA binding could be a more general mechanism of steroid specificity.
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