Change of Specificity Mutations in Androgen-selective Enhancers

EVIDENCE FOR A ROLE OF DIFFERENTIAL DNA BINDING BY THE ANDROGEN RECEPTOR*

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The androgen and glucocorticoid receptors recognize identical DNA motifs, leaving unanswered the question of how steroid specificity of transcriptional regulation is established in cells containing both receptors. Here, we provide evidence that subtle differences in low affinity DNA recognition might be a crucial element in the generation of steroid-specific responses. Here we identify simple hormone response elements in the mouse sex-limited protein enhancer and the human secretory component androgen response unit to be essential for the androgen specificity of both enhancers. We describe specific in vitro binding to these motifs by the DNA-binding domain of the androgen but not the glucocorticoid receptor. Both elements can be considered partial direct repeats of the 5′-TGTCTT-3′ core binding motif. In addition, we show that specific point mutations in their left half-sites, essentially changing the nature of the repeats, strongly enhance the glucocorticoid sensitivity of the respective enhancers, whereas they have no effect on their androgen responsiveness. Accordingly, these mutations allow specific binding of the glucocorticoid receptor DNA-binding domain to both elements in vitro. With these experiments, we demonstrate that differential recognition by the androgen receptor of nonconventional steroid response elements is, at least in some cases, an important mechanism in androgen-specific transcriptional regulation.

Steroid hormones exert their effects on the expression of target genes by activating their cognate steroid receptors (SRs).† These are ligand-inducible, sequence-specific transcription factors that influence gene expression by binding to their response elements in regulatory DNA regions within steroid responsive genes. Being members of the steroid hormone receptor subfamily of the nuclear receptors, the androgen receptor (AR) and glucocorticoid receptor (GR) have a highly conserved DNA-binding domain (DBD) and hence recognize identical DNA motifs. (1–3). Steroid hormone receptors, with the exception of the estrogen receptor, recognize a partial palindromic repeat, spaced by three nucleotides, of a 5′-TGTCTT-3′ monomer binding motif, with which they interact as a homodimer (4–7). The question of how the specificity of transcriptional responses to the different steroid hormones is established still remains partly unanswered to date. Tissue-specific hormone metabolism and/or receptor expression (8), receptor-specific recruitment of coactivators (9, 10) or receptor-specific interactions with nonreceptor proteins that are structural components of androgen responsive regions (11–13) can account for the differential regulation of gene expression in many situations, but not all. Evidence exists that specific binding of the AR to single nonconventional response elements might be an alternative mechanism involved in androgen-specific transcriptional control (14–17). Indeed, the classical high affinity palindromic steroid receptor binding sites do not discriminate between binding of the DNA-binding domains of the AR and the GR in in vitro binding assays (14, 18). In the rat probasin (PB) promoter and in the human secretory component (SC) androgen response unit (ARU), however, single hormone response elements (HREs), slightly differing from the aforementioned high affinity binding sites, have been described to be bound by the AR-DBD but not by the GR-DBD (15, 16, 19).

The promoter of the rat probasin gene is specifically up-regulated by androgens and not by glucocorticoids in transient transfection assays (19). This up-regulation was attributed to cooperative binding of the AR to two AR binding sites (AR binding sites 1 and 2) within the 286-base pair proximal pb promoter (19–21). Of these sites, AR binding site 2 (from now on referred to as PB-ARE-2) has a high in vitro affinity for the AR-DBD, whereas the affinity of the GR-DBD is low (14).

Our group has recently identified an androgen-specific enhancer located approximately 3.5 kilobase pairs upstream of the human sc promoter (16). The core enhancer consists of four 5′-TGTCTT-3′-like SR monomer interaction sites (in Ref. 16, called cores 1–4) and a high affinity binding site for nuclear factor I. The enhancer was also shown to specifically confer androgen and not glucocorticoid responsiveness to the sc proximal promoter. A motif composed of the cores 1 and 2 (sc-ARE1.2), in which both half-sites are essentially arranged as a partial direct repeat with a three nucleotide spacer, was found to be the main AR-DBD interaction site. The GR-DBD does not interact with this motif in vitro. The introduction of a point mutation in sc-ARE1.2, essentially changing the nature of the repeat from direct to partially palindromic (the T at position −4 relative to the central nucleotide was changed to an A) allows the GR-DBD to bind the motif and abrogates the specificity of the steroid responsiveness of the sc upstream enhancer.

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§ The abbreviations used are: SR, steroid hormone receptor; AR, androgen receptor; GR, glucocorticoid receptor; ARE, androgen response element; HRE, hormone response element; ARU, androgen response unit; DBD, DNA-binding domain; IF, induction factor; Luc, luciferase; PB, probasin; SC, secretory component; SLP, sex-limited protein; TK, thymidine kinase; GRE, glucocorticoid response element.
A third androgen-specific enhancer has been described upstream of the mouse sex-limited protein (mouse SLP) promoter (22). This enhancer was found to respond only to androgen and not glucocorticoid stimulation when inserted in front of the heterologous thymidine kinase (TK) promoter (23). Within the enhancer fragment, three putative steroid receptor interaction sites were identified, two of which (HRE2 and HRE3) were found to be necessary but not sufficient for androgen induction because introduction of a mutation, destroying AR binding, in each of the motifs separately abolishes the androgen response of the enhancer (12). HRE3 was found to be a high affinity binding site for the AR. It does not, however, discriminate between binding of the AR and the GR in vitro (12, 18). Furthermore, as an isolated element, it confers both androgen and glucocorticoid responsiveness to the heterologous TK promoter (23). Androgen specificity of the slp enhancer was therefore attributed to the selective action of other factors interacting with distinct regions within the enhancer. An octamer transcription factor, as well as NFκB and, more recently, AML3/ CBFA1, has been proposed to play an important role in the androgen specificity of the slp enhancer (11, 13, 25). High affinity steroid receptor binding to or transactivation through the slp-HRE2 element could not be demonstrated.

Starting from the similarities between the AR-specific binding motifs sc-ARE1.2 and PB-ARE2, we examined which characteristics of these DNA motifs would determine AR-specific DNA recognition. We also investigated whether a change in the binding characteristics of AR- and GR-DBDs would be reflected by an altered steroid responsiveness of the sc enhancer, as well as of the isolated elements in transient transfection experiments. We have extended these findings to the slp-HRE2 and its role in the functionality of the slp enhancer. We thus provide evidence that AR binding to specific DNA-elements might be more generally involved in androgen-specific transcriptional regulation than has originally been assumed (7).

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and modifying enzymes were purchased from Life Technologies, Inc., Amersham Pharmacia Biotech, Promega Corp. (Madison, WI), and Roche Molecular Biochemicals. Dexamethasone and methyltrienolone (R1881) were purchased from Sigma. Polymerase chain reactions were performed on a Progene thermocycler (Techne, Cambridge, United Kingdom) using Takara Taq DNA polymerase (Takara, Shuzo Co. Ltd., Shiga, Japan). The pGEM-15Zf(−) cloning vector and the pGL3 luciferase reporter vector were purchased from Promega Corp. [α-32P]dNTP was purchased from Amersham Pharmacia Biotech. Luciferase Reporter Plasmids—The pTK-TATA-Luc reporter vector was made by inserting a SalI/BglII fragment from the modified pTK-CAT vector (14) in the BglII/SmaI-digested pGL3 basic reporter vector. Wild-type sc upstream enhancer fragments were cloned in the pGEM15Zf(−) cloning vector as described in Ref. 16 and subsequently cloned as XbaI/SacI fragments in the NheI/SacI-digested pTK-TATA-Luc vector. The mutated sc upstream enhancer fragments were generated by polymerase chain reaction using the appropriate mutant upstream primer (containing a BamHI site) and the reverse PCR2 primer (as described in Ref. 16, and containing an EcoRI site at the 5′-end). After digestion, the polymerase chain reaction product was cloned in the EcoRI/BamHI-digested pGEM15Zf(−) cloning vector. XbaI/SacI fragments from the resulting plasmids were cloned in the pTK-TATA-Luc vector, resulting in the insertion of the enhancer fragment in the correct orientation immediately in front of the TK-TATA-box. The wild-type and mutant slp enhancer fragments (nucleotides −1984 to −1902) were generated by hybridizing two synthetic oligonucleotides, overlapping each other over 20 base pairs at their 3′-ends and containing EcoRI and Klenow fragments at their 5′-ends, followed by a fill-in reaction using the Klenow fragment of DNA polymerase I. The resulting fragments were cloned as EcoRI/BamHI fragments in pGEM15Zf(−) and subsequently as XbaI/SacI fragments in the correct orientation in the pTK-TATA-Luc vector. The pb promoter driven luciferase reporter plasmid was made by insertion of a 454-base pair HindIII/BamHI fragment containing the pb promoter (from position −426 to position +28) into the SmaI/BglII-digested pGL3 basic vector.

Oligonucleotides containing the wild-type and mutant sc-ARE1.2 and slp-HRE2 motifs, as well as the wild-type C3(1) ARE and the PB-ARE2, as they were used in band shift assays and all containing NheI/XhoI 3′-overhanging ends, were cloned in the pBluescript-KS−digested TK-TATA-Luc, creating the TATA box of four copies of the ARE reporter plasmids used in this study were checked by sequence analysis on the ALF express sequencer using the cycle sequencing method (Amersham Pharmacia Biotech).

**RESULTS**

The sc Upstream ABU, the slp Enhancer, and the pb Promoter Are Androgen-specific Regulatory DNA Regions—Luciferase reporter constructs driven by the TK minimal promoter and containing the sc enhancer (16; pSCT-TATA-Luc) or the slp enhancer (pSPL-TATA-Luc) or driven by the pb proximal pro-
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Fig. 1. Androgen specificity of the sc upstream ARU, the slp C3(1) fragment, and the pb promoter. HeLa cells were transiently transfected with the indicated reporter constructs and co-transfected with either an AR or GR expression plasmid, as appropriate. Cells were incubated in the absence (shaded bars) or presence of either 1 nm R1881 (open bars) or 10 nm dexamethasone (filled bars). Luciferase activities were adjusted according to the β-galactosidase activity in the same sample. Activities are depicted relative to the activity of the pSC-TATA-Luc construct in the presence of androgens, which was included in each experiment and which was set to 100. IFs are the ratios of luciferase activities in the stimulated over the average of the nonstimulated duplicates of the same reporter construct, co-transfected with the same SR expression plasmid. Reported values are the averages (± S.E. values) of at least three independent experiments performed in duplicate. The orientations of the SR binding sites identified elsewhere (16, 19, 23, 25, 26) are indicated in the schematic representation given on the left; filled arrows indicate the positions and orientations of the high affinity (right) half-sites, and open arrows indicate those of the low affinity (left) half-sites. The open box indicates that the motif matches the consensus poorly.

The functional specificity of both elements was investigated in transient transfection assays in HeLa cells (Fig. 1). Average IFs upon androgen stimulation were 40, 32, and 29 for the pSC-TATA-Luc, the pSLP-TATA-Luc, and the pPB-Luc vectors, respectively, whereas their glucocorticoid inductions were 6.4, 3.1, and 5.8, respectively. These findings are in agreement with previously reported results (16, 21, 23). In control experiments, a reporter construct containing the 204-base pair PvuII/SstI fragment of the first intron of the C3(1) gene (26), pC8(1)-TATA-Luc, and a reporter construct driven by the mouse mammary tumor virus long terminal repeat was induced by androgens as well as glucocorticoids, in agreement with previous reports (Refs. 2, 3, 26, and 27; Fig. 1 and data not shown).

The sc-ARE1.2 and slp-HRE2 Are AR-Specific Response Elements—The sc-ARE1.2, the PB-ARE-2, and the slp-HRE2 have been shown previously to be essential for the functioning of their respective regulatory regions (12, 16, 19). Specific interaction of the AR-DBD with the rat PB-ARE2 is further explored in the accompanying paper (28). Here, we investigate binding of the rat AR- and GR-DBD to the sc-ARE1.2 and the slp-HRE2 in band shift assays using concentrations of receptor DBD ranging from 3.4 nM to 5.6 μM (Fig. 2). The C3(1) ARE, a high affinity SR binding motif, was used as a positive control for the binding assay (Fig. 2A). Binding of the AR-DBD showed normal allosteric Hill kinetics for all three oligonucleotides. For AR- and GR-DBD binding to the C3(1) ARE, apparent dissociation constants (Kd values) of 20 and 37 nM, respectively, were calculated. For AR-DBD binding to the sc-ARE1.2 and slp-HRE2 motifs, Kd values were 251 and 166 nM, respectively. However, no interaction of the GR-DBD to either of the latter motifs could be demonstrated, even at concentrations as high as 5.6 μM.

The functional specificity of both elements was investigated in transient transfection assays in HeLa cells using TK-TATA-driven reporter constructs containing four copies of the respective AREs (Fig. 2B). A luciferase reporter construct containing the C3(1) ARE was induced 15.3- and 50-fold by androgens and glucocorticoids, respectively. A reporter construct containing the sc-ARE1.2 was induced 12.9-fold upon androgen stimulation and only 3.6-fold by glucocorticoids. The reporter construct containing the slp-HRE2 was induced 15.9-fold by androgens and was not responsive to glucocorticoid stimulation (IF: 1.6).

The sc-ARE1.2 Motif Is Essential for the Androgen Specificity of the sc ARU—To investigate whether the sc-ARE1.2 by itself is the predominant factor in the androgen specificity or whether additional mechanisms are required to impose androgen specificity on the enhancer, the element was replaced with either the slp-HRE2, the PB-ARE-2, or the C3(1) ARE motif. None of these mutations had a dramatic effect on the androgen-stimulated transcriptional activity in co-transfected HeLa cells (Fig. 3A). Glucocorticoid-stimulated transcriptional activity remained low when the sc-ARE1.2 motif was replaced by either the PB-ARE-2 or the slp-HRE2. Replacement of the sc-ARE1.2 motif with the C3(1) ARE, however, dramatically increased glucocorticoid-stimulated transcriptional activity: GR-stimulated luciferase activity of the motif was increased by more than 10-fold higher than that of the wild-type construct (Fig. 3A). The glucocorticoid induction factor was elevated more than 13-fold in the C3(1) ARE mutated reporter construct compared with the wild-type vector (IFs were 6.4 for the wild-type and 95 for the C3(1) ARE substituted construct). Glucocorticoid induction factors remained unaltered when the sc-ARE1.2 motif was
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Trivial names, sequences, and $K_s$ values for AR- and GR-DBD binding to the wild-type and mutated sc-ARE1.2 motifs are indicated. Numbering is relative to the central nucleotide of the three-nucleotide spacer. The hexamer binding motifs are in uppercase, and the spacer is in lowercase. Mutated nucleotides are underlined.

| Name     | Motif                  | $K_s$ nM |
|----------|------------------------|----------|
| Wild-type| 5'-GGCTCTttcAGTTCT-3'  | 251 >1000|
| -4 T-A   | 5'-'GGAGCttcAGTTCT-3'  | 104 144  |
| -4, -2 T-A| 5'-'GGAGCttcAGTTCT-3' | 29 148   |
| +2 A-T   | 5'-'GGCTCTttcAGTTCT-3' | 33 222   |
| -4 T-A; +2 A-T | 5'-'GGAGCttcAGTTCT-3' | 31 71    |

Point Mutations in the Left Half Site of sc-ARE1.2

To exclude possible effects of the sequences flanking the sc-ARE1.2 motif in the enhancer or its three-nucleotide spacer on receptor binding, we introduced the two hexameric half-sites of the slp-HRE2, the PB-ARE2, and the C3(1) ARE in the context of sc-ARE1.2 and evaluated AR- and GR-DBD binding to these elements in vitro (Fig. 3B). No significant differences could be found in $K_s$ values for AR- or GR-DBD binding to the wild-type motifs and the same motifs in the context of sc-ARE1.2 (data not shown).

Point Mutations in the Left Half Site of sc-ARE1.2 Differentially Influence Receptor Binding in Vitro and Alter the in Vivo Steroid Specificity of the sc ARU—To determine the sequence requirements for AR-specific binding and transcriptional regulation, we have investigated the effects of point mutations in the sc-ARE1.2 on AR- and GR-DBD binding in vitro as well as on the functionality of the sc ARU. In view of the similarities between the three AR-specific motifs described to date, we chose to mutate the sc-ARE1.2 element at positions +2, -2, and -4 relative to the central nucleotide in the three-nucleotide spacer. The mutation of the A at position +2 to a T creates a perfect 5'-TGTCTT-3' right half-site motif, common to all high affinity steroid receptor binding elements. Both mutations in

The introduction of an A at position -4 in the sc-ARE1.2 increases the affinity of the AR-DBD approximately 2-fold relative to the wild-type element (Table I and Fig. 4A). Although the GR-DBD shows no affinity at all for the wild-type sc-ARE1.2, a $K_s$ value of 144 nM was calculated for GR-DBD binding to the -4 T-A mutant. The affinity of the AR-DBD for the element is augmented an additional 3-fold by a subsequent T to A mutation at position -2, whereas GR-DBD binding to the element was not influenced by this mutation. The A to T mutation at position +2 in the sc-ARE1.2 dramatically increases the affinity of both the AR-DBD ($K_s$: 33 nM) and GR-DBD ($K_s$: 222 nM). The A to T mutation at position +2, combined with the T to A mutation at position -4, does not result in a further increase of the affinity of the AR-DBD ($K_s$: 31 nM), but the affinity of the GR-DBD is increased an additional 3-fold ($K_s$: 71 nM).

We have introduced the same mutations in the sc-ARE1.2 motif in the pSC-TATA-Luc reporter vector and tested the androgen and glucocorticoid responsiveness of these constructs in transient transfection experiments (Fig. 4B). Transactivation by the AR was not influenced by any of the tested mutations: induction factors are 40, 35, 23.5, 26, and 27.5 for the reporters constructs containing the wild-type and the -4 T-A, the -4, -2 T-A, the +2 A-T, and the -4 T-A; +2 A-T mutated sc-ARE1.2 motifs, respectively. Absolute transcriptional activities in the presence of androgens increased only mildly in the mutated constructs (up to a maximum of 2.6-fold in the -4 T-A; +2 A-T mutant).

The same mutations, however, drastically change the glucocorticoid responses of the sc enhancer: dexamethasone induction factors increase approximately 2- and 4-fold for the +2 A-T (IF: 16) and the -4 T-A (IF: 24) mutations, respectively, and approximately 10-fold in the reporter constructs carrying the -4, -2 T-A and the -4 T-A; +2 A-T mutations (IFs are 75 and 65, respectively). Accordingly, absolute transcriptional activities of the mutated constructs are augmented dramatically relative to the wild-type construct (up to approximately 20-fold in the -4, -2 T-A mutant).

Similar Mutations in the slp-HRE2 Influence GR-DBD Binding in Vitro and Alter GR-mediated but Not AR-mediated Transactivation by the slp Enhancer—We next extended our experiments to the slp-HRE2 and its functionality within the slp enhancer. We introduced a T to A change at position -4 and the combined mutation of the T at position -4 (to an A) and the A at position +2 (to a T) and investigated their effects on AR- and GR-DBD binding in in vitro binding assays (Table II and Fig. 5A). The affinity of the AR-DBD was augmented approximately 5-fold by the T-A mutation at position -4 and an additional 3-fold by the subsequent A to T change at position +2 ($K_s$...
values were 166, 30, and 13 nM for the wild-type and the two mutated motifs, respectively). Whereas the GR DBD does not interact with the wild-type slp-HRE2, relatively high affinity binding was demonstrated to the −4 T-A and −4 T-A+2 A-T mutated elements (K_s values were 74 and 67 nM, respectively). Next, the −4 T-A and −4 T-A+2 A-T mutations were introduced in the slp-HRE2 element in the pSLP-TATA-Luc reporter construct and their effects on androgen and glucocorticoid responsiveness of the reporter construct were investigated (Fig. 5B). Similar to the sc upstream enhancer, AR-mediated transactivation was not dramatically changed by the mutations: induction factors were 32.5, 19.1, and 17.5 for the wild-type, the −4 T-A and the −4 T-A+2 A-T mutated reporter constructs, respectively. In the same way, absolute transcriptional activities in the presence of androgens did not change significantly relative to the wild-type construct. Glucocorticoid induction factors increased 10-fold in the −4 T-A mutant and an additional 1.5-fold by the supplementary +2 A-T mutation (IFs are 30.5 and 44.3, respectively). Absolute transcriptional activities of both mutant reporter constructs in the presence of glucocorticoids are an average of 12-fold higher than the activity of the wild-type pSLP-TATA-Luc.

**DISCUSSION**

The ARUs described in the SC, SLP, and PB genes are all androgen-specific in co-transfection experiments in HeLa cells.
We demonstrate that essential response elements within the sc and slp enhancers show specificity for the androgen receptor, both in vitro binding assays and in transient transfection experiments (Fig. 2). We have investigated by mutational analysis the functionality of the sc-ARE1.2 and slp-HRE2 motifs, both in the contexts of their respective enhancers and as isolated motifs. In the accompanying paper by Schoенmakers et al. (28), we further characterize the interaction of the AR- and GR-DBD with the C3(1) ARE and the PB-ARE2 and investigate the effects of mutations and/or deletions of the receptor DBDs on in vitro binding to, and transactivation through, both elements.

The Left Half-site Motif of the sc-ARE1.2 Determines the Androgen Specificity of the sc Enhancer—The sc-ARE1.2 motif (5’-GGCTCTtcttGTTCTT-3’) determines the androgen specificity of the sc upstream ARU because replacing it with the C3(1) ARE motif abolishes the functional specificity of the enhancer, whereas substitution with either the PB-ARE2 or the slp-HRE2 AR-specific motifs does not (Fig. 3A). This correlates with the in vitro affinities of the purified receptor DBDs for the respective motifs (Figs. 2A and 3B). For the right half-site of the ARE, it is known that nucleotides at the positions +2, +3, +4, and +6 relative to the central nucleotide in the threenucleotide spacer are highly constrained for high affinity AR and GR binding and transactivation (4, 5). The SR-DBD is proposed to first contact the high affinity right-half site of the HRE, which is subsequently followed by a less stringent, cooperative binding of the dimer partner to the DNA/protein surface (29). Binding of the dimer partner in a head-to-head conformation is cooperative and requires a dimerization interface (the D-box), located within the second Zn-finger of the DBD. For high affinity dimeric GR-DBD binding to, and transactivation through, a glucocorticoid response element (GRE), the nucleotides at positions –3 and –4 must be C and A, respectively (4). Dimeric GR-DBD binding in vitro to the TAT-GRE II (5′-TGTAAGGAgGTTCTT-3’) e.g. was found to be abolished by an A to T mutation at position –4 (30). The same rules were found to apply to AR binding to motifs that were isolated by a DNA-binding site selection (5). Simple sequence comparison of the PB-ARE2 and the sc-ARE1.2 motifs (Table III) reveals that they differ from the consensus high affinity ARE/GRE (5′-GGTACAnnTGGTTCT-3’; Ref. 31) at three positions: the thymine residues at position –4 and –2 and the adenine residue at position +2. Based on our initial studies (14), as well as on the

| Name          | Sequence       | Ref. | Specificity |
|---------------|----------------|------|-------------|
| C3(1) ARE     | 5′-GTGAAGGAgGTTCTT-3’ | 26, 14 | Nonspecific |
| MMTV-HRE1     | 5′-GGTACAnnTGGTTCT-3’ | 3, 14 | Nonspecific |
| slp-HRE3      | 5′-GAAGGAgGTTCTT-3’ | 12   | Nonspecific |
| TAT-GRE II    | 5′-TGTAAGGAgGTTCTT-3’ | 18   | Nonspecific |
| Consensus     | 5′-GGTACAnnTGGTTCT-3’ | 14   | Nonspecific |
| PB-ARE2       | 5′-GGTACAggAGTTCT-3’ | 14, 19 | AR-specific |
| slp-HRE2      | 5′-GGTACAggAGTTCT-3’ | 16   | AR-specific |
| slp-HRE2      | 5′-TGTTACAggAGTTCT-3’ | This report | |
| Consensus     | 5′-KNNGTTACAnnTGGTTCT-3’ | 7     | AR-specific |

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![Fig. 6. AR- and GR-mediated transactivation through wild-type and mutated sc-ARE1.2 and slp-HRE2 as isolated motifs. TK minimal promoter driven luciferase reporter constructions containing four copies of the motifs indicated on the left were transiently transfected into HeLa cells and incubated for 24 h in the absence or presence of hormones. Bars represent the induction factors (calculated as in Fig. 1) for androgen (open bars) and glucocorticoid (filled bars) stimulation + S.E. values. Mutant nucleotides are underlined. The empty pTK-TATA-Luc vector was used as a negative control for hormone induction.](http://www.jbc.org/)

The effects of the introduction of point mutations in the slp-HRE2 on both AR- and GR-DBD binding in vitro and AR- or GR-mediated transactivation through the enhancer are identical to those observed the ARE1.2 element in the sc ARU (Table II and Fig. 5B). Briefly, changing the nature of the repeat from direct to inverted results in a change of specificity of both of the isolated HRE2 element and of the slp enhancer fragment. Again, as for the sc ARU, AR-mediated transactivation through the enhancer remains unaltered.
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The Isolated sc-ARE1.2 and slp-HRE2 Elements Are Sufficient for the Maintenance of Androgen Specificity—As depicted in Figs. 2B and 6, four copies of the isolated sc-ARE1.2, as well as of slp-HRE2, confer an androgen-specific transcriptional response to the TK minimal promoter. Functional analysis of point mutations that increase the in vitro affinity of both receptor DBDs reveals an additional functional difference between the AR and GR (Fig. 6). Indeed, transcriptional activation mediated by the AR through elements for which both receptor DBDs show comparable in vitro affinities is always significantly lower as compared with that of the GR. This is obvious when we compare, for example, the androgen and glucocorticoid inductions of the reporter constructs containing the −4 T-A sc-ARE1.2 or the −4 T-A slp-HRE2. This 10-fold difference in induction might be a reflection of a difference in intrinsic transactivation capacities between both receptors, as has been reported previously (32), or of the fact that the GR shows a higher degree of cooperativity on multimerized elements. The difference in the transactivation potential between AR and GR may be a reflection of a difference in the strength of their AF1 and/or AF2 activation functions (33) and/or a receptor-dependent differential recruitment of co-activators (9, 10).

All results presented here are in agreement with the hypothesis that the AR is able to recognize directly repeated hexamer motifs with a three-nucleotide spacer. It is intriguing to consider the possibility that the AR, as opposed to the GR, might “have a choice” between head-to-head and head-to-tail binding. Different binding conformations could possibly result in differences in the mechanisms of transcriptional activation or a different recruitment of co-activators, leading to a different overall functionality of the receptor. The data presented in the accompanying paper by Schoenmakers et al. (28) strongly suggest that, in the case of the PB-ARE-2, actual head-to-tail binding by the AR-DBD is responsible for the specificity. The fact that both AR- and GR-DBD binding is enhanced to approximately the same extent by the A to T mutation in the right half sites of the sc-ARE1.2 and the slp-HRE2, creating the consensus SR interaction motif (see Figs. 4A and 5A and Tables I and II) is also indicative for the fact that AR- and GR-DBD monomers are probably equally stringent in the recognition of their half-site binding motifs.

Binding of the AR to Low Affinity, Specific DNA Motifs as a General Mechanism Involved in Androgen-specific Transcriptional Regulation—AR-specific binding to a single, nonconventional, low affinity response element seems to be the key factor in AR-specific transactivation, not only through the sc upstream ARU but also the slp enhancer, and thus might be a functional mechanism in other androgen-specific regulatory regions. AR-mediated transcriptional activation through cooperative binding to relatively low affinity binding sites has been described for the rat pb promoter and the human prostate-specific antigen promoter and upstream enhancer (21, 34, 35). Our findings are not only in agreement with these results but also indicate that AR-mediated transactivation through such low affinity binding sites is already at its maximal level and cannot be increased by raising the affinity of the AR for the binding sites.

Androgen specificity of the C′Δ9 fragment of the slp enhancer has been attributed to a cooperative or stimulatory interaction of the enhancer-bound AR, with accessory, nonreceptor proteins binding to different regions within the enhancer (23). The primary interaction site for the AR (and GR) would be the high affinity, nonspecific HRE3 (5′-GAAACAgcctGTGTCT-3′). An NFκB-like protein and an octamer transcription factor have been proposed to play a key role in the androgen specificity of the enhancer (13, 25). Recently, binding of AML3/CBFα1 to two sites within the slp enhancer was suggested to play a crucial role in the androgen specificity of the enhancer (11). Interaction of AML3/CBFα1 to the downstream site, overlapping HRE2, would repress the GR, but not the AR, bound to HRE3. We have now identified HRE2 as a functional interaction site for the AR but not the GR. The AR might, therefore, compete with AML3/CBFα1 for binding to this site, thus preventing putative repression of HRE3-bound AR. In the same way, the introduction of the different point mutations in HRE2, all changing the 5-TGTGGT-3′ AML3/CBFα1 interaction site to 5′-TGTTGGA-3′, might prevent AML3/CBFα1 from binding and thus from repression of the HRE3-bound GR. Alternatively, these mutations could allow the GR to compete with AML3/CBFα1 for binding, thereby causing a relief of AML3/CBFα1 repression of HRE3-bound GR. Our experiments with oligonucleotide reporter constructs (Figs. 2B and 6), however, demonstrate that AR specificity of transcriptional response is dependent solely on the sequence of the hexamer motifs of the ARE and not on whether or not other transcription factor binding sites are present, because the sc-ARE1.2 as well as the PB-ARE-2 (see Ref. 28), are sufficient to generate an androgen-specific transcriptional response. In agreement with our results, Scheller et al. (36) concluded from co-transfection experiments using chimeric proteins consisting of different AR and GR domains that the AR-DBD plays a crucial role in the androgen specificity of the slp-C′Δ9 fragment.

The presence of high and low affinity receptor interaction sites adjacent to each other within the same enhancer does not necessarily imply that the higher affinity sites are functionally dominant over the low affinity elements. Firstly, the low affinity binding site might overlap with an interaction site for a repressor protein. Whether or not a steroid receptor can selectively compete with this repressor for the low affinity site would determine functional specificity of the enhancer. Secondly, the orientation of a binding element on the nucleosomal surface determines whether a motif can be recognized by its cognate protein (37). It is therefore conceivable that the orientation of two adjacent HREs on a nucleosomal surface might be such that the low affinity binding element can be bound by the receptor preferentially to the high affinity HRE simply because it has the correct orientation on the nucleosomal surface. Indeed, HREs 2 and 3 in the slp C′Δ9 fragment are separated by 16 base pairs, i.e. 1.5 turns of the DNA double helix, indicating that when one element is accessible for receptor binding in a nucleosomal structure, the other would be inaccessible directed toward the histone surface. Transiently transfected DNA has been proposed to adopt a conformation consisting of nucleosome arrays (38). We would like to propose here a model of transcriptional activation by steroid receptors based on the two-step model of transcription activation that is generally accepted to date (24, 38). Initial binding of an androgen receptor dimer to its binding site on the nucleosomal surface would initiate the recruitment of chromatin remodeling activities and integrator proteins and would cause the first “opening” of the chromatin structure over the enhancer. This would result in the unmasking of additional binding sites for receptors or other transcription factors. The data we present here suggest that, at least for the sc upstream ARU and the slp enhancer, subtle characteristics of the motifs that are initially recognized by the receptor can determine whether or not transcriptional activation would be hormone-specific. Our findings indicate that the combination of intrinsic DNA binding characteristics and transactivation functions of the AR and GR can impose androgen versus glucocorticoid specificity upon enhancer function without the need for additional receptor- or enhancer-specific co-factors.
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