Many of the aspects involved in steroid-specific transcriptional regulation are still unsolved to date. We describe here the detailed characterization of the mouse sex-limited protein enhancer as a paradigm for androgen-specific control of gene expression. By deletion analysis, we delineate the minimal enhancer region displaying androgen sensitivity and specificity. We also show that each of the three hormone response elements (HRE), which constitute this minimal enhancer region, is essential but not sufficient for its functionality. When investigated as isolated elements, HRE1 is inactive and HRE3 is a potent androgen response element as well as GRE. Only the non-canonical HRE2 (5-TGGAAGAGTTCT-3') is capable of conferring an androgen-specific transcriptional response to a heterologous promoter. This finding is correlated with the fact that HRE2 is recognized in binding assays in vitro by the DNA-binding domain (DBD) of the androgen but not the glucocorticoid receptor, while HRE3 is recognized by both DBDs. Differential binding of the androgen receptor to HRE2 in the context of the enhancer was analyzed in more detail in footprinting assays in vitro. In transient transfection experiments using chimeric receptors, the inability of the glucocorticoid receptor to transactivate via the slp-ARU as well as the isolated slp-HRE2 was rescued by the replacement of its DNA-binding domain with that of the androgen receptor. Our data suggest that the functional interplay between the weak, but highly androgen-specific HRE2 and the adjacent strong, but non-selective HRE3 is the major determinant in the generation of androgen specificity of transcriptional response via the sex-limited protein enhancer.

The fact that the androgen (AR), glucocorticoid (GR), progesterone (PR), and mineralocorticoid (MR) receptors (forming the PR group of the steroid hormone receptors (SR)), essentially recognize identical DNA motifs (1, 2), has long since posed the problem of how steroid-specific transcriptional regulation is achieved in the living cell. The DNA-binding domains (DBD) of the human PR group members are 71% identical and 84% homologous. The DBD of an SR is located in the central part of the receptor and is structured in two zinc fingers (3, 4). From the crystal structure of the GR-DBD complexed with an HRE (4), it is known that the amino-terminal zinc-finger contains an α-helical region that is inserted in the major groove of the DNA. This region (also called P-box) contains the amino acid residues that make base-specific contacts with the core hexamer SR recognition motif. The P-box residues are 100% conserved within the PR group of steroid hormone receptors, which all recognize and bind DNA motifs that are comprised of a three nucleotide spaced, partial inverted repeat of a 5′-TGTCTTCT-3′ motif. From the GR-DBD-DNA-bound co-crystal structure, it is known that the carboxyl-terminal zinc-finger of the DBD is involved in the cooperative “head-to-head” dimerization of two receptor DBDs on a palindromic repeat of the monomer binding motif. By analogy, this mode of interaction has been assumed to hold true for the other steroid hormone receptors as well. Indeed, all members of the PR group of steroid hormone receptors can activate transcription by interacting with a partial palindrome of the 5′-TGTCTTCT-3′ hexamer (2, 3, 5). We and others (6–11), however, have recently identified and characterized motifs that are recognized only by the AR and not by the other members of the PR group. Contrary to the classic hormone response elements, these motifs can be considered partial direct repeats of the canonical monomer binding motif and are found in most, if not all, of the enhancers or promoters that have been described to date to be androgen-specific in transient transfection assays (e.g. the rat probasin gene promoter, the mouse sex-limited protein, and human secretory component upstream enhancers, the mouse pem gene promoter). In our current hypothesis, the direct repeat nature of these elements is the main determinant involved in their specific action. The AR-DBD is the only SR-DBD that is able to cooperatively bind to such partially directly repeated elements, possibly by dimerizing on the DNA in a head-to-tail conformation, similar to the members of the all-trans-retinoic acid receptor9-cis-retinoic acid receptor group of nuclear receptors (12). A more thorough analysis of the specific AR-, as opposed to GR-, DBD interaction to the ARE2 motif in the rat probasin promoter (13, 14) has strengthened this hypothesis. Taken together, this alternative mechanism of DNA binding by the AR could constitute an important factor in the generation of androgen specificity of transcriptional control.
The mouse sex-limited protein upstream androgen responsive unit (ARU) confers androgen responsiveness to a heterologous promoter in transient transfection assays (15). Its androgen (versus glucocorticoid) specificity was reported in a later publication by the same research group (16) and has since served as a paradigm of an androgen-specific response unit (e.g. Refs. 17 and 18). From the three putative HRE motifs identified within the enhancer (HREs 1, 2, and 3), only HRE3 was considered a genuine functional SR interaction site (19). The mechanism responsible for the androgen specificity of the enhancer was, therefore, attributed to the interaction of other transcription factors (e.g. OCT or AML/CFBα3) to the ARU and their cooperativity with HRE3-bound AR (18, 20, 21) or their putative inhibitory influence on GR action (21).

In this report, we describe the detailed characterization of the slp-ARU and the interpretation of the obtained results in the light of our hypothesis of differential DNA recognition by the AR as a possible mechanism involved in specific transcriptional responses. By testing hormonal responsiveness of sequential deletions from the 5’- and 3’-ends of the enhancer, we delineate the minimal enhancer region necessary for full functionality and specificity. We also investigate the role of each of the HREs in the functionality of the enhancer by introducing a point mutation that destroys SR-DBD interaction. Of each of these motifs, we study the affinity for the AR- and GR-DBD in vitro DNA binding assays, as well as their functionality and specificity as isolated elements in transient transfection assays. In addition, we study AR- and GR-DBD interaction with the different HREs in their native context in the enhancer, by in vitro footprinting experiments.

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction and modifying enzymes were obtained from Invitrogen, Amersham Biosciences, Promega Corp. (Madison, WI), Roche Molecular Biochemicals, and MBI Fermentas GMBH. Dexamethasone and methylthionone were purchased from Sigma-Aldrich. The pGEM15Z(-) cloning vector and the pGL3 basic luciferase reporter vector were obtained from Promega Corp. [α-32P]dCTP was purchased from Amersham Biosciences. Oligonucleotides were purchased from Eurogentec (Seraing, Belgium) or MWG Biotech GMBH. Oligonucleotides containing the rTAT-GRE motif were radiolabeled at the 3′-end by a fill-in reaction using the Klenow fragment of DNA polymerase I. The resulting fragments were cloned as EcoRI/BamHI fragments in pGEM15Z(-) and from the resulting construct as XbaI/SstI fragments in the correct orientation in the pTK-TATA-Luc vector. For the sequential deletions from the 5′- and 3′-ends of the enhancer fragment, shorter oligonucleotides were used as 5′- or 3′-parts of the enhancer, as appropriate, and hybridized with the full-size, complementary oligonucleotide. For the 5′ deletions, oligonucleotide primers start at positions −1971, −1961, −1951, and −1941. For the 3′ deletions, oligonucleotides start at positions −1922 and −1932. These primer combinations resulted in the enhancer fragments described below in Fig. 1. Oligonucleotides containing the HRE1, HRE2, HRE2–4T-A, HRE2–4T-A–2A-T, HRE3, and rTAT-GRE motif (22), in their original contexts (i.e. including the four nucleotides flanking the hexamer half sites and the three-nucleotide spacer), and as they were used in band shift assays, were cloned in the XbaI-digested pTK-TATA-Luc vector, resulting in the insertion of two or four copies of the motif with alternating orientations, upstream of the minimal TK-TATA-box. All reporter plasmids used in this study were checked by sequence analysis on the ALF express sequencer using the Promega Dye Primer Kit (Amersham Biosciences). The numbering of the nucleotides within the 15-bp HRE sequence is according to Verrijdt et al. (23).

The central spacer nucleotide is given position 0, the positions of the other nucleotides are indicated as − (upstream of the central nucleotide) or + (downstream of the central nucleotide). The rTAT-GRE-E1b-luciferase reporter contains two motifs of the rTAT-GRE, controlling the E1b minimal promoter and was a gift from Dr. Jenster (24).

**Cell Culture and Transfection Assays—**HeLa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium containing 1000 mg/liter glucose, supplemented with penicillin (100 IU/ml), streptomycin (50 μg/ml), and 10% fetal calf serum (Invitrogen) at 37 °C in 5% CO2. On the first day of each transfection experiment, the appropriate receptor expression plasmid was cotransfected with the pGEM15Z(-) plasmid containing the wild-type or mutated slp enhancer, was radiolabeled at the 3′-end by a fill-in using the Klenow fragment of DNA polymerase I in the presence of [α-32P]dCTP. In each footprinting reaction, 20,000–30,000 cpm of radiolabeled probe was used. Footprinting reactions were performed essentially according
to Lemaigre et al. (26), except that no poly(dI-dC) was included in the binding reactions. G and AG chemical cleavage reactions, according to Maxam and Gilbert (27), were performed on the same DNA fragment and were used as a reference in each footprinting gel. GST-AR- and GST-GR-DBD fragments were used as DBD fragments. Competition experiments consisted of the inclusion of 10 pmol (an approximate 400-fold excess) of cold competitor oligonucleotide containing the rTAT-GRE motif, in the binding mixture.

RESULTS

Delineation of the Minimal Enhancer Fragment—The 80-bp full-size slp enhancer fragment, as used in Ref. 23, was stepwise shortened from the 5' and 3'-ends (Fig. 1A), and the resulting reporter constructs were tested in transient transfection assays (Fig. 1B). A deletion up to 30 bp from the 5'-end of the full-size enhancer has a minor effect on its androgen responsiveness, whereas the glucocorticoid response remains low. This 50-bp fragment (from nt −1902 to −1951) lacks three 5'-nucleotides of HRE1. The additional deletion of 10 bp from its 5'-end silences the enhancer. This deletion removes the entire HRE1, indicating the dependence of enhancer function on this DNA segment and that the joint action of the HREs 2 and 3 on themselves is not sufficient for the androgen response. A 20-bp deletion from the 3'-end of the ARU (del5 in Fig. 1) removes HRE3 and has a dramatic effect on the androgen responsiveness. The low, residual androgen sensitivity is lost upon the additional deletion of 10 bp, covering HRE2, from its 3'-end (del6). The minimal enhancer region necessary for functionality and specificity consists, therefore, merely of the HREs 2 and 3 and the downstream half of HRE1 (del3 in Fig. 1). As a positive control experiment for hormone induction, a promoter construct containing two copies of the rTAT-GRE was included in each experiment. Its activities relative to the androgen-stimulated reference construct are depicted in the right panel.
slp-ARU, the guanine at position 3 of the right half sites of the hexamer motif was replaced by a thymine (Fig. 2A), a mutation that is known to destroy high affinity interaction of any SR-DBD to the element. The hormone responsiveness of the corresponding reporter constructs was tested in transient transfection assays in CV-1 cells (Fig. 2B).

The results show clearly that each mutation significantly affects the function of the enhancer, indicative of the crucial role of each motif, although the HRE1-mutated enhancer is still androgen-responsive. Its androgen-induced activity still rises up to almost 50% of that of the wild-type enhancer fragment and is more than 2-fold higher compared with the enhancer fragments containing the HRE2 or HRE3 mutant. Strikingly, the HRE2- and HRE3-mutated enhancer fragments display a similar decrease in hormone responsiveness and a comparable residual androgen sensitivity (androgen-mediated induction factors are 5.4 and 5.9, respectively).

In Vitro Binding of AR- and GR-DBD to the Isolated slp-HREs—Radiolabeled probes containing the HRE1, -2, or -3 motifs were analyzed in band shift experiments in vitro using recombinant AR- or GR-DBD fragments. As a positive control for AR- and GR-DBD binding, radiolabeled rat tyrosine aminotransferase (rTAT) GRE (5'-TGTACGGA-TGTCTC-3'), which has been previously described as a classic, nonspecific HRE (2, 22, 24), was used. For each of the four motifs, binding curves were generated, and best fits were calculated to a plot with four-parameter allosteric Hill kinetics (Fig. 3).

In the negative control experiment, the empty promoter vector displays low activity that is not increased by hormone stimulation (empty vector in panels A and B of Fig. 4). Similar results were obtained in CV-1 cells (data not shown). In the negative control experiment, the empty promoter vector displays low activity that is not increased by hormone stimulation (empty vector in panels A and B of Fig. 4). As a positive control for hormone induction, in each experiment a promoter construct was included containing two copies of the rTAT-GRE.

The reporter construct containing two copies of HRE1 is not responsive to either androgens or glucocorticoids, and four copies of this element mediate a very weak 1.8- and 5-fold induction of transcriptional activity by androgens and glucocorticoids, respectively. Both two and four copies of the slp-HRE2 display functional androgen specificity: the reporter construct containing two copies is induced 3.6-fold by androgens and not...
(induction factor is 1.0) by glucocorticoids. Four copies of slp-HRE2 mediate a 41-fold induction by androgens, whereas glucocorticoid stimulation results in only a 5.3-fold stimulation of transcriptional activity.

The reporter construct containing two copies of the high affinity HRE3 mediates a 26- and 10-fold increase of transcriptional activity upon androgen- and glucocorticoid stimulation, respectively. This correlates with the approximate 3-fold higher affinity of the AR-, versus the GR-, DBD for this element in the band shift experiments. Four copies of this element, however, stimulate transcription up to 160- and 170-fold upon androgen and glucocorticoid administration, respectively.

Site-directed Mutagenesis of the slp-HRE2—To confirm the essential role of HRE2, not only in the activity, but also in the androgen specificity of the slp-ARU, we tested the effect of mutations in HRE2 on both the functionality of the isolated motif and the full-size enhancer (Fig. 5). In the first mutation, the thymine at position −4 was substituted for an adenine. This essentially changes the nature of the half site repeat from partially direct to partially palindromic (see also under “Discussion”). The specificity of the slp-ARU is lowered upon the introduction of this mutation in HRE2 (androgen induction remains 32, whereas glucocorticoid induction increases to 8.8). In the reporter constructs containing two copies of the slp-HRE2, the functional androgen specificity of the wild-type element is lost upon the substitution of the thymine at position −4 with an adenine. The -fold induction by androgens is 10, whereas glucocorticoids cause an 8-fold stimulation of activity. In the four-copy construct induction increased up to 90- and 260-fold for androgen and glucocorticoid stimulation, respectively.

In a double mutant of HRE2, the adenine at position −2 was additionally changed to a thymine to match the consensus 5′-TGTTCT-3′. In the context of the full-size ARU, the double mutant becomes highly sensitive to glucocorticoid stimulation (−fold induction is 32), whereas its androgen responsiveness increases only 50% compared with the wild-type ARU. As an isolated motif, the −4T-A;+2A-T mutant of HRE2 becomes even more responsive to glucocorticoids compared with androgens. The androgen and glucocorticoid mediated induction factors are 9- and 32-fold, respectively, for the construct containing two copies of this double mutant and 67- and 160-fold for the four-copy construct. These findings are in perfect agreement with the loss of specificity caused by the same mutation(s) in the slp-HRE2 in in vitro DNA binding assays using AR- and GR-DBD protein fragments (Fig. 5).

In Vitro Footprinting of the AR- and GR-DBD on the Wild-type and Mutated slp Enhancer—The three HRE motifs in the slp-ARU are separated from each other by only a single base pair (Fig. 1B). It is therefore conceivable that receptor dimer binding to one site influences binding of a second dimer to the adjacent motif. We therefore investigated the binding of receptor DBDs to the HREs in their original context in the full-size

![In vitro binding curves of the AR- and GR-DBD to the slp-HREs.](image-url)
slp enhancer. First, as a positive control for the footprinting reaction, a DNA fragment comprised of a tandem repeat of the rTAT-GRE element was incubated with increasing amounts of receptor DBD. A gradual protection of the GREs is seen when using both AR- and GR-DBD, and, as expected, there are no apparent differences in their affinities for the element (Fig. 6A). Binding of the receptor fragments to the motifs could be competed for by adding an excess of non-radiolabeled rTAT-GRE (lanes 6 and 11) but not by the addition of a nonspecific competitor (not shown).

When using the wild-type slp-ARU as a radiolabeled probe, a protection of HRE3 by both AR- and GR-DBD is observed (Fig. 6, B and C, compare lanes 1 with lanes 6). This protection is competed for by the addition of an excess of the rTAT-GRE motif (compare lanes 6 and 7). A hypersensitive site is present 4 nt upstream of HRE3 (the thymine at position +5 of HRE2, Fig. 6B, filled triangle) when using the AR-DBD (left panel) but not the GR-DBD (right panel, empty triangle). It already appears when using 20 ng of protein, whereas it is never seen with any of the amounts of GR-DBD that were used.

The slp-HRE2 motif is protected against DNase I degradation only by the AR- and not by the GR-DBD. Protection of its left half site (5’-TGGTCA-3’) is clear, a hypersensitive band appears 4 nt upstream of slp-HRE2 (the upper black triangle in the left panel of Fig. 6B). AR-DBD binding to the right half site of HRE2 is less evident due to the band intensities in the DNase I degradation in the absence of protein, which are extremely low for most bands, or extremely high (for the guanine in the 5’-GCC-3’ spacer of HRE2, which does not completely disappear). No protection of HRE2 was observed when using the GR-DBD fragment (Fig. 6B). This confirms the specific interaction of the AR-DBD to this motif as seen in the band shift experiments. No binding of either AR- or GR-DBD to the HRE1 motif, or to any other region within the slp-ARU, could be demonstrated.

To have a better insight in the changes in receptor DNA binding in the “change-of-specificity mutation” of the slp ARU, we also tested the enhancer fragment carrying the double mutant of the HRE2 motif (Fig. 6C). In this DNA fragment the mutated HRE2 element is bound by both the AR- and GR-DBD to a similar extent. Surprisingly, however, protection of HRE3 is less evident. The footprint window caused by AR and GR-DBD extends 4–5 nt downstream of HRE2 and seems to prevent DBD interaction to HRE3. As for AR-DBD interaction to HRE3 in the wild-type enhancer, AR-DBD interaction to the mutated HRE2 causes the appearance of a hypersensitive site 4 nucleotides upstream of the mutated HRE2 (indicated by the filled triangle in Fig. 6C). This hypersensitive site, however, is now also present when the GR-DBD is used (Fig. 6C, right panel).

The AR-DBD Is Essential for the Functional Specificity of the slp-ARU—To investigate the role of the DBDs in the hormone stimulation of transcription through the slp enhancer, we replaced the DNA-binding domain of the GR for that of the AR (Fig. 7). The wild-type GR and the chimeric receptor are equally active when we used the reporter vector containing two copies of the rTAT-GRE (Fig. 7A). When testing the slp oligonucleotide reporter constructs (Fig. 7B), the wild-type slp-HRE2 clearly distinguishes between the AR and GR. The inactivity of the GR is, however, significantly relieved by the replacement of its DBD by that of the AR, because the same reporter construct is activated by the GAG chimer to a 17-fold higher level than the GR in the left panel of Fig. 7B, compare wtGR with GAG). The double mutant of slp-HRE2 behaves similarly to the rTAT-GRE, in the sense that the activities of the AR, GR, and the GAG chimer are comparable. Similar observations were made for the full-size slp-ARU (Fig. 7C), albeit that the inactivity of the GR is not completely rescued by the DBD switch. The increase in glucocorticoid-induced transcriptional activity is more than 2-fold compared with that of the GR (in the left panel of Fig. 7C, compare wtGR with GAG). The GAG-induced transcriptional level is, however, still 3-fold lower compared with the AR-induced level of transcription (compare wtAR with GAG). Similar to the rTAT-GRE-E1b con-
trol, the ARU containing the double mutant slp-HRE2 is equally responsive to AR, GR, and GAG. These findings might indicate that, next to the absolute necessity of the AR-DBD, other receptor domains are also required for the proper functioning of the enhancer.

**DISCUSSION**

The high homologies between the DNA-binding domains of the steroid hormone receptors and the fact that they seem to recognize identical response elements has long since posed the problem of how steroid specificity is achieved in the organism. Several mechanisms can account for differential steroid effects on gene expression, like e.g. differential receptor expression and/or hormone metabolism (28), a differential recruitment of co-activators (29, 30), and the additional binding of specific accessory transcription factors to steroid-responsive DNA regions (17). Most probably, it is a combination of several of these mechanisms that will eventually lead to a specific hormonal response.

Ever since the cloning and characterization of the steroid hormone receptors (31–35) and the description of a GR-DBD dimer bound to an HRE (4), the mode of DNA interaction of the different steroid hormone receptors has been considered to be analogous to that of the GR. We and others have, however, recently provided evidence that subtle differences in DNA recognition between the steroid receptors might be an important factor in the generation of steroid-specific hormonal action. Over the last few years, at least five different DNA motifs have been found to discriminate between the AR and GR (6–10, 23, 36). All these motifs form part of enhancer or promoter regions that are functionally androgen specific both in vivo and in transient transfection experiments. Mutational analysis sug-
Fig. 6. In vitro footprinting experiments on wild-type and mutated slp-ARU. A, a PstI/MluI DNA fragment from the rTAT-GRE-E1b luciferase reporter vector, a positive control in transfection experiments, was used as a radiolabeled probe. Lanes G and AG are Maxam-Gilbert sequencing reactions. DNase I degradation was performed in the absence (lane 1) or the presence of 20 (lane 2) to 250 ng (lanes 5 and 6) of GST-AR-DBD and 20 (lane 7) to 250 ng (lanes 10 and 11) of GST-GR-DBD. In lanes 6 and 11, an approximate 400-fold excess of a cold specific competitor oligonucleotide (the rTAT-GRE) was added. White boxes to the right indicate the presence of footprinting windows; arrows to the left indicate the location of HRE half site motifs and their orientations. B, an EcoRI/MluI fragment containing the entire wild-type slp-ARU was used as a radiolabeled probe. G and AG are Maxam-Gilbert sequencing reactions. In lanes 1, no protein was added. In lanes 2, 10 ng; lanes 3, 20 ng; lanes 4, 40 ng; lanes 5, 100 ng; and lanes 6 and 7, 200 ng of GST-AR-DBD (left panel) or GST-GR-DBD (right panel) DBD was used. In lane 7, a 400-fold excess of a cold specific competitor (the rTAT-GRE) was included in the binding mixture. To the left are indicated the positions of the HREs 1, 2, and 3. Arrows depict the orientation of the half sites of each HRE. The filled triangles depict hypersensitive sites that appear as a consequence of protein binding. The empty triangle in the right panel depicts the location of the hypersensitive site that is present in the left but absent in the right panel. White boxes to the right of each gel indicate the presence of clearly protected regions within the enhancer. C, an EcoRI/MluI fragment encompassing the entire slp-ARU carrying the −4T-A:+2A-T mutation in HRE2 was used as a radiolabeled probe. Experiments were performed and are presented as in B and 100 ng of GST-AR-DBD (left panel) or GR-DBD (right panel) was used. Filled triangles depict the hypersensitive sites that are induced upon DBD interaction to the mutant HRE2.
gested that it is their direct repeat nature that determines their differential action. The higher affinity of the AR for these direct repeat motifs is dependent on its DNA-binding domain. Mutational analysis of the AR- and GR-DBDs in in vitro binding assays suggested that the dimerization interface used by the AR-DBD when interacting with a selective motif, is different from that used when an SR-DBD interacts with a classic palindromic repeat element (14). In particular, residues in the second zinc-finger and the carboxyl-terminal extension (CTE) of the DBD seem to be involved. This prompted us to suggest the AR-DBD might be the only steroid hormone receptor that is able to dimerize in a head-to-tail conformation on these elements, similar to e.g. the vitamin D receptor (12, 14). The inability of the GR-, PR-, or MR-DBD to interact with these selective motifs as a homodimer (13) is most likely due to a strongly disfavored head-to-head dimerization of the second dimer partner on a directly repeated element as a result of the strong bias against the binding of an SR-DBD to a motif containing an adenine instead of a thymine, next to the guanine (5'-TGATCT-3') of the hexamer (37).

**Fig. 7. Transactivation via the slp-ARU depends on the AR-DBD.** Reporter constructs containing two copies of the rTAT-GRE (A), four copies of the slp-HRE2 (B), or the full-size slp-ARU (C) were co-transfected in HeLa cells with expression vectors for the wt AR, wt GR, or the GAG chimer, as indicated below. Samples co-transfected with AR were stimulated with 1 nM R1881; samples co-transfected with GR or GAG were stimulated with 10 nM dexamethasone. The experiments using the rTAT-GRE-E1b reporter vector were used as a control for receptor function. For all experiments using the same reporter vector, all activities are reported relative to the activity of the androgen-stimulated samples, which were set at 100% in each experiment. In B are compared the activities of the reporter containing four copies of the wild-type slp-HRE2 (to the left) with those of the vector containing four copies of the −4T-A:+2A-T mutated slp-HRE2 (to the right). In C are compared the activities of the reporter containing the wild-type slp-ARU (to the left) with those of the reporter vector containing the slp-ARU in which the HRE2 was mutated (to the right). White bars are the relative activities in the absence of hormone; black bars are the relative activities of the indicated reporters in the presence of hormone.

The Essential Role of HRE2 in the Functionality and Specificity of the slp-ARU—The slp-HRE2 (5'-TGTCAGgccGTTCT-3') is bound by the AR and can confer an androgen-specific hormonal response to a heterologous promoter (23). The mutational analysis of the slp-ARU reported here reveals that HRE2, as well as HRE3, are essential for the proper functioning of the enhancer, because a destructive point mutation of either of these motifs strongly diminishes its androgen responsiveness (Fig. 2). Mutation or partial deletion of HRE1 also decreases the activity of the enhancer, albeit to a somewhat lesser extent.
The minimal active and specific slp enhancer fragment is comprised of the HRE2 and -3 and the downstream half of HRE1 (Fig. 1) and, therefore, lacks 30 bp from the 5'-end of the fragment that was defined in earlier experiments (23). Within this 30-bp fragment, interaction sites for several transcription factors have previously been proposed to play a role in the androgen-specific action of the ARU (20, 21). These factors were proposed to either cooperate with the AR or have an inhibitory effect on GR, both of which are bound to the high affinity HRE3 element.

In the minimal enhancer fragment, which we have found to be active and selective (del3 in Fig. 1), however, the OCT core binding element, as well as the upstream AML/CFB3 binding motif, are deleted. These factors are therefore most probably not involved in the androgen selectivity of the enhancer. Another putative AML/CFB3 binding motif partially overlaps with HRE2 and is still present in the shorter, inactive, 40-bp enhancer fragment, indicating that this element is either not functional or not sufficient for full activity of the enhancer. The +3G-T mutation of HRE2 lies outside the 5'-TGTGCGT-3' core AML/CFB3 binding motif (see also Fig. 1A) and therefore theoretically does not affect binding of this factor. The fact, however, that this mutation does severely decrease the enhancer function again indicates that the combined action of HRE3-bound AR together with AML/CFB3 is not sufficient to generate androgen responsiveness.

According to our deletion analysis, essential nucleotides for the interaction of a transcription factor involved in the functionality of the enhancer would reside in the 10-bp fragment (from nt -1951 to -1941; 5’-ATTATCTGTT-3’) that is deleted in the shortest, inactive fragment. An obvious possibility is that this region contains essential nucleotides for the interaction of an additional AR dimer. However, no evident AR-DBD interaction to the HRE1 motif is observed in band shift or footprint assays, nor can AR transactivate via two or four copies of this motif, making it unlikely that this DNA fragment functions as an AR interaction site. A search of the Transfac data base (38) revealed that the deleted sequence is a perfect match to the GATA transcription factor consensus binding site (5’-TTATC- WNYKY-3’). GATA-2 and -3 have been described to be required for an optimal androgen stimulation of prostate-specific antigen promoter/enhancer constructs in transfected LNCaP cells, which depend on the presence of multiple GATA binding sites within the enhancer (39). Whether or not GATA transcription factors are indeed involved in the function of the slp-ARU remains to be determined.

The AR-DBD as a Crucial Element in Androgen-specific Transactivation—From the co-transfection experiments using a chimera of the GR containing the DBD of the AR, it is clear that the inability of the GR to transactivate via four copies of the slp-HRE2 is relieved by replacing its DBD with that of the AR. Hormone-mediated induction of transcriptional activity and induction factors of the slp-HRE2 reporter construct are 16-fold higher compared with wild-type GR (Fig. 7B). This indicates that androgen-specific transcriptional response can be dependent solely on the AR-DBD. The same is not entirely true for the full-size slp-ARU: the GAG chimeric receptor is only 2.6-fold more active compared with the wild-type GR. Glucocorticoid responsiveness of both the oligonucleotide and the full-size ARU reporter constructs containing the double mutant of HRE2 was equal to or surpasses their response to androgens, irrespective of whether the wtGR or the chimera were used (Fig. 7, B and C, right panels).

An earlier publication by Scheller et al. (25) reports the ability of the same chimeric receptor to mediate glucocorticoid responsiveness via the wild-type slp-ARU, up to 100% of the activity of the AR. In fact, in the same study, again in agreement with the hypothesis proposed here, none of the wild-type or chimeric receptors containing the GR-DBD could transactivate the enhancer, whereas all but one receptor containing the AR-DBD did.

AR-specific Cooperation of the slp-HREs 2 and 3—The in vitro DNA binding assays as well as the transfection experiments have shown that the slp-HRE1 is probably not a functional HRE (Figs. 3 and 4). The slp-HRE2 is bound by the AR-DBD with relatively high affinity (K_d is 186 nM) and specificity (K_d for GR-DBD interaction is 775 nM, Fig. 3). As an isolated element it displays functional androgen specificity (Fig. 4). This is already evident when investigating two copies of the element, but becomes much more striking in the reporter constructs containing four copies. Compared with HRE2, the HRE3 element has a higher in vitro affinity for both the AR-and GR-DBD (Fig. 3). Its affinity for the AR-DBD is 3.5-fold higher than that for the GR-DBD, which is correlated with the difference in responsiveness of the two-copy reporter construct (Fig. 4). The four-copy construct, however, is equally responsive to androgens and glucocorticoids, indicating that the GR is probably able to overcome a lower binding affinity for a response element when multiple copies are present. When comparing the results from the DNA binding assays using HRE3 and the wild-type and mutated slp-HRE2 motif with the results from the functional assays, we find that the calculated K_d values correlate remarkably well with the hormone responsiveness of the reporter constructs containing two copies of the respective motif. This correlation is much less clear when considering the results of functional assays using the four-copy-containing reporters. The GR is a more potent activator than the AR (e.g. compare the K_d values for AR- and GR-DBD binding to the double mutant of HRE2 with the results of the functional assays in Fig. 5), and this seems to allow the GR to overcome a lower DNA-binding affinity when multiple copies of the response element are present (compare the K_d values and functional data on the single mutant of HRE2 in Fig. 5).

The in vitro footprinting experiments confirm the absence of receptor DBD interaction with the HRE1 motif, the specific interaction of the AR- but not the GR-DBD to HRE2, and the binding of both AR- and GR-DBD to HRE3. In the enhancer fragment containing the double mutant of HRE2, HRE2 is protected to the same extent by both the AR- and GR-DBD, correlating with the androgen and glucocorticoid responsiveness of this enhancer fragment in transient transfection assays. Surprisingly, protection of HRE3 is much less evident in the mutated enhancer (Fig. 6C), which might indicate that binding of a receptor DBD to both HREs is, to some extent, mutually exclusive.

We therefore hypothesize that AR-DBD interaction to the DNA fragment containing slp-HRE2 and -3 is only possible when HRE2 is recognized as a direct repeat element. The appearance of a hypersensitive site upon AR-DBD binding, 4 nt upstream of HRE3, might demonstrate a bending of the DNA that could facilitate interaction of two AR homodimers to the tightly clustered HREs 2 and 3. This bending would create a sterical conformation that could favor head-to-tail AR-DBD dimerization to the adjacent HRE2, whereas HRE3 can be bound in the classic head-to-head conformation. In the mutated enhancer fragment, the affinity of the double mutant of HRE2 for both AR- and GR-DBD is higher compared with HRE3 (Figs. 3C and 5A). Both DBDs, therefore, first interact with the mutant HRE2, which, however, seems to exclude DBD interaction to the HRE3, probably because both motifs are classic, palindromic repeats. The fact that no receptor interaction can be demonstrated to HRE3 in the context of the mutant en-
hancer, which is clearly androgen- and glucocorticoid-responsive, links enhancer activity to occupation of HRE2 but not necessarily HRE3. This therefore suggests that HRE2 has the optimal spatial and/or rotational positioning relative to a yet unidentified additional factor, to mediate androgen responsiveness via the slp enhancer. The fact that HRE3 is, however, indispensable for optimal enhancer function, suggests two possible roles for this element: 1) As a high affinity SRE, it might “attract” activated receptors to the enhancer, thereby creating a higher local receptor concentration and creating the optimal steric conformation to allow the AR, but not the GR, to interact to the specific, but low affinity HRE2. 2) HRE3 might also function as a high affinity “amplifier” of the highly androgen-selective action of HRE2.

A differential protection of HRE2, by baculovirus produced AR and not GR, was observed previously (40). In these experiments, however, the differential binding of HRE2 by AR was attributed to steric hindrance of GR but not AR when interacting with HRE3. The fact that we observe the same phenomenon using the receptor’s DBDs, however, probably excludes differential steric hindrance and suggests that intrinsic differences in sequence recognition and/or DNA binding conformation between both receptors are also involved.

General Conclusion—From our findings in the DNA binding assays, as well as the functional assays, we propose that the functional interplay of at least three different mechanisms accounts for the observed androgen specificity of the slp-ARU. First, HRE2, which is essential for enhancer function, is intrinsically androgen-specific. Second, the relative specificity of HRE2 could add to the specificity of HRE2, its high affinity probably also adds to the overall level of androgen stimulation by either “attracting” activated receptors or “amplifying” the weak but selective HRE2 action. Third, DNA bending induced by AR- and not GR-DBD binding to HRE3 could favor additional direct repeat interaction with HRE2.

Taken together our results strongly suggest that the functional interplay between HRE2 and HRE3, each displaying clearly distinct receptor binding and functional characteristics as isolated elements, is the main mechanism involved in the generation of androgen-specific transcriptional control via the slp-ARU. Moreover, we believe the linkage of the slp enhancer action to the occupation of the selective HRE2, and not necessarily of HRE3, constitutes additional and convincing evidence that androgen specificity of DNA binding is a major determinant in the generation of the androgen-specific response of this enhancer. To what extent differential DNA interaction is involved in androgen specificity of transcriptional regulation in general remains to be elucidated.

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REFERENCES

1. Roy, A. K., and Chatterjee, B. (1995) Crit. Rev. Eukaryot. Gene 5, 157–176
2. Verrijdt, G., Haelens, A., Schoenmakers, E., Rombouts, W., and Claessens, F. (2002) Biochem. J. 361, 97–103
3. Tsai, M.-J., and O’Malley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486
4. Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991) Nature 352, 497–505
5. Khorasanizadeh, S., and Rastinejad, F. (2001) Trends Biochem. Sci. 26, 384–390
6. Rennie, P. S. Bruchovsky, N., Leco, J. K., Sheppard, P. C., McQueen, S. A., Cheng, H., Snoek, R., Hamel, A., Bock, M. E., MacDonald, B. S., Nickel, B. E., Chang, C., Liao, S., Cattini, P. A., and Matusik, R. (1993) Mol. Endocrinol. 7, 23–36
7. Claessens, F., Alen, P., Devos, A., Peeters, B., Verhoeven, G., and Rombouts, W. (1996) J. Biol. Chem. 271, 19013–19016
8. Verrijdt, G., Schoenmakers, E., Alen, P., Haelens, A., Peeters, B., Rombouts, W., and Claessens, F. (1999) Mol. Endocrinol. 13, 1558–1570
9. Haelens, A., Verrijdt, G., Schoenmakers, E., Alen, P., Rombouts, W., and Claessens, F. (1999) J. Biol. Chem. 275, 12290–12297
10. Loreni, F., Stavenhagen, J., Kalff, M., and Robins, D. M. (1988) Mol. Cell. Biol. 8, 2350–2360
11. Adler, A. J., Danielsen, M., and Robins, D. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11660–11663
12. Robins, D. M., Scheller, A., and Adler, A. J. (1994) J. Steroid Biochem. Mol. Biol. 51, 295–251
13. Scheller, A. C., Scheller, A. Thompson, E., and Robins, D. M. (1997) DNA Cell Biol. 16, 45–57
14. Adler, A. J., Scheller, A., Hoffman, Y., and Robins, D. M. (1991) Mol. Endocrinol. 5, 1587–1596
15. González, M. I., and Robins, D. M. (2001) J. Biol. Chem. 276, 6429–6428
16. Adler, A. J., Danielsen, M., and Robins, D. M. (1999) J. Biol. Chem. 274, 30624–30630
17. Jantzen, H.-M., Strahle, U., Glass, B., Stewart, F., Schmid, W., Boshart, M., Mikaeci, R., and Schütz G. (1987) Cell 49, 29–38
18. Verrijdt, G., Schoenmakers, E., Alen, P., Peeters, B., Verhoeven, G., Rombouts, W., and Claessens, F. (2000) J. Biol. Chem. 275, 12298–12305
19. Sui, X., Bramlett, K. S., Jorge, M. C., Swanson, D. A., von Eschenbach, A. C., Jenster, G. (1999) C. Schütte, J., Schleuning, W. D., and Haendler, B. (2001) Mol. Endocrinol. 15, 1803–1816
20. Khorasanizadeh, S., and Rastinejad, F. (2001) Trends Biochem. Sci. 26, 384–390
21. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
22. Funder, J. W. (1993) Mol. Cell. Endocrinol. 65, 499–560
23. Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987) Science 237, 268–275
24. Sui, X., Bramlett, K. S., Jorge, M. C., Swanson, D. A., von Eschenbach, A. C., Jenster, G. (1999) C. Schütte, J., Schleuning, W. D., and Haendler, B. (2001) Mol. Endocrinol. 15, 1803–1816
25. Scheller, A., Scheller, A., Thompson, E., and Robins, D. M. (1997) DNA Cell Biol. 16, 45–57
26. Muller, J. M., Isele, U., Metzger, E., Rempel, A., Moser, M., Fischer, A., Breyer, T., Holubarsch, C., Buettner, R., and Schiehe, C. (1999) EMBO J. 18, 3589–3595
27. Trappman, J. T., Klaassen, P., Kuiper, G. G., vor der Korput, J. A., Faber, P. W., van Rooij, H. C., Geurts van Kessel, A., Voorhorst, M. M., Mulder, E., and Brinkmann, A. O. (1998) Biochem. Biophys. Res. Commun. 253, 241–248
28. Misrahi, M., Ager, M., d’Auriol, L., Loosfelt, H., Meriel, C., Fridlansky, F., Guichon-Mantel, A., Galibert, F., and Milgrom, E. (1987) Biochem. Biophys. Res. Commun. 148, 740–744
29. Miesfeld, R., Okret, S., Wilkstrom, A. C., Wrang, O., Gustafsson, J. A., and Yamamoto, K. R. (1984) Nature 312, 779–781
30. Zilliacus, J., Wright, A. P. H., Carlstedt-Duke, J., and Gustafsson, J. A. (1995) Mol. Endocrinol. 9, 241–248
31. Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruhl, M., Reuter, I., and Schacherer, F. (2000) Nucleic Acids Res. 28, 316–319
32. Porco, Stable, C. M., de las Pozas, A., and Roos, B. A. (2000) Mol. Cell. Endocrinol. 167, 43–53
33. Scheller, A., Scheinman, R. I., Thompson, E., Scarlett, C. O., and Robins, D. M. (1996) Mol. Cell. Endocrinol. 121, 75–86