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Androgen-specific gene activation via a consensus glucocorticoid response element is determined by interaction with nonreceptor factors

(steroid receptors/*Slp* gene/transcriptional specificity)

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ABSTRACT **A fundamental issue in steroid hormone regulation is the question of how specific transcription is attained *in vivo* when several receptors can bind the same DNA sequence *in vitro*. We report an enhancer of the mouse sex-limited protein (*Slp*) gene that, unlike previously characterized enhancers, is activated by androgens but not by glucocorticoids or progestins. Potent androgen induction requires both a consensus glucocorticoid (hormone) response element and auxiliary elements also present within a 120-base-pair DNA fragment. Cotransfection assays with wild-type and mutant receptors reveal that glucocorticoid receptor can bind, but not transactivate from, the hormone response element within the enhancer. The positive effect of androgen and the null effect of glucocorticoid appear to require the amino-terminal domains of the respective receptors. Thus, exclusive transcriptional response to androgens, and lack of response to glucocorticoids, derives from factor interactions that are determined by the context of the receptor binding site rather than by its distinct sequence.**

Glucocorticoid, progestin, and androgen hormones function in distinct biological programs via their cognate receptors, which are ligand-activated transcription factors (1, 2). Given the dissimilar effects of these steroids, it is a paradox that their receptors recognize a common DNA binding site (3, 4). The derived consensus glucocorticoid response element (GRE) sequence (GGTACAnnnTGTTCT) can function as a response element for all steroid classes except estrogens, and so this sequence is also called a hormone response element (HRE) (2). Essential transcriptional specificity *in vivo* could be enforced by subtle sequence differences in response elements, but this has not been demonstrated. Alternatively, accessory factors could selectively interact with the receptors to determine precise gene activation.

Slp is a duplicated complement *C4* gene (5, 6) whose expression in several tissues is androgen-dependent due to the influence of an inserted provirus (7, 8). A DNA fragment within the 5′ proviral long terminal repeat, 2 kilobases (kb) upstream of *Slp*, functions as a hormone-dependent enhancer and shows multiple nuclear protein binding sites (9). One element within this fragment that is necessary, but not sufficient, for strong induction is a consensus HRE (HRE-3). This is the only element within the enhancer that shows androgen receptor (AR) binding *in vitro* as well as some hormonal response by itself (9). Induction by the HRE is greatly augmented, or diminished, by multiple accessory elements within the enhancer that bind nonreceptor factors.

The complexity of the *Slp* enhancer suggested that the additional factors may be required to strengthen an intrinsically weak AR activity. Further, these factors might elicit specific response to AR from DNA sites also recognizable by the glucocorticoid and progesterone receptors (GR and PR). We examined the specificity of the hormonal response of *Slp* reporter constructs coexpressed with wild-type and mutant steroid receptors. Our findings indicate that specificity is conferred by differential factor interactions and is not inherent to the receptor DNA binding site.

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MATERIALS AND METHODS

Reporter and Receptor Expression Plasmids. The C′Δ2, C′Δ9, HRE-3, 2×HRE-3, and 3×HRE-3 plasmids have been described (9); they contain *Slp* enhancer sequences, either as restriction fragments or synthetic oligonucleotides of HRE-3, inserted before a reporter gene consisting of the herpes virus thymidine kinase promoter fused to the coding sequence of bacterial chloramphenicol acetyltransferase (tkCAT). To generate the plasmid Bst-3, the 5′ terminus of the C′Δ9 *Slp* insert was isolated as a 39-base-pair (bp) *Bst*NI fragment, filled in to blunt the ends, and inserted at the *Sma* I site before the single HRE in HRE-3.

The mouse AR (mAR) expression vector (10) was from D. Tindall; the rat GR expression vector (11) and mutant derivatives VAX556 (12), VAC500Y (13), and N556 (14) were from K. Yamamoto; and the human PR expression vector was the gift of B. O'Malley (Baylor College of Medicine, Houston). The chimera, C, was constructed with mouse GR (mGR) (15) and mAR, by ligating three DNA fragments: (*i*) a 1.3-kb *Bgl* II–*Hin*dIII (partial) fragment from pSV2Wrec (15) that encodes the N-terminal domain of mGR; (*ii*) a 1.2-kb *Hin*dIII–*Xba* I fragment from pCMV-mAR (10) that encodes the DNA- and hormone-binding domain of mAR; (*iii*) a 7.1-kb *Bam*HI–*Xba* I fragment that contains the pCDNA1neo vector (Invitrogen).

Cells and Transfection. CV-1 cells were grown and were transfected by the DEAE method, as before (9), except that after transfection cells were incubated in medium containing 3.5% charcoal-stripped NuSerum (Collaborative Research). CAT activity was determined by liquid scintillation counting of modified and unmodified forms of chloramphenicol after either chromatography (16) or liquid-phase extraction (17); results of the two assays were comparable.

RESULTS

A Fragment of the *Slp* Enhancer Confers Androgen-Specific Response. To compare effects of different hormones in acti-

Abbreviations: AR, androgen receptor; mAR, mouse AR; GR, glucocorticoid receptor; mGR, mouse GR; PR, progesterone receptor; GRE, glucocorticoid response element; HRE, hormone response element; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase.

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vation of the *Slp* enhancer, we transfected receptor expression plasmids into CV-1 cells, which lack endogenous steroid responses (Fig. 1). A 120-bp enhancer fragment that contained a HRE, C′Δ9, conferred almost 20-fold induction upon a tkCAT reporter gene with cotransfected AR but, remarkably, showed no response to GR or PR. This selectivity was not intrinsic to the receptor binding site, as two copies of HRE-3 placed upstream of the tk promoter (2×HRE-3) gave strong induction with each receptor. Multimerization was used to accentuate weak induction obtained with the single HRE-3 site (about 2-fold; data not shown), presumably by allowing cooperation of receptor dimers (19). A 160-bp *Slp* fragment, C′Δ2, contains 40 bp of additional sequence contiguous to the 5′ end of the C′Δ9 fragment. C′Δ2 showed significant induction with each receptor, unlike C′Δ9. This indicated that nonspecific factors could augment response to GR and PR, as well as to AR, via binding sites not present in the AR-specific DNA fragment, effectively overriding specificity (see *Discussion*).

GR Can Bind but Not Transactivate the Androgen-Specific Enhancer. The fragment C′Δ9 contained sufficient information for androgen-specific enhancement, which is not shown by the HRE alone. The failure of C′Δ9 to respond to glucocorticoid could derive from the inability of GR to bind HRE-3 in the context of C′Δ9 sequence, perhaps due to interference of proteins bound at adjacent sites. Alternatively, GR might bind to C′Δ9 but be unable to transactivate in conjunction with factors that are competent for AR response. This latter possibility was interesting, since GR seems to cooperate promiscuously with many transcription factors (20). To examine this further, we first asked whether GR could inhibit the AR induction of C′Δ9 (Fig. 2). 5α-Dihydrotestosterone-induced CAT activity of C′Δ9 was diminished by increasing amounts of GR plasmid. Full inhibition required dexamethasone (data not shown).

GR's inhibition of AR induction could be due to direct competition for a DNA binding site, from which AR but not GR could activate transcription, or indirect competition for limiting factors in transactivation. The latter mechanism

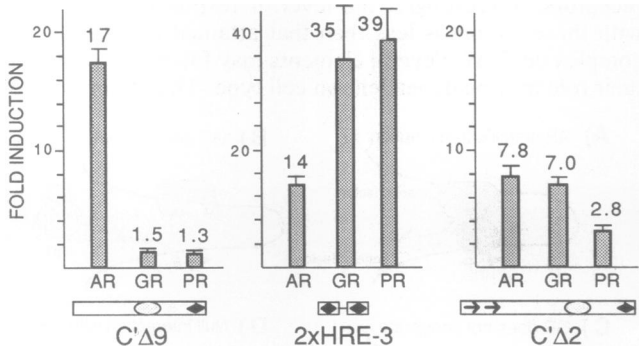

FIG. 1. Androgen-specific response of the *Slp* enhancer fragment in C′Δ9. mAR (10), rat GR (11), or human PR (from B. O'Malley) expression plasmids were cotransfected into CV-1 cells with reporter constructs containing *Slp* sequences upstream of a tkCAT gene. C′Δ9, a 120-bp fragment, is drawn with a diamond marking HRE-3 and an oval marking a ubiquitous protein binding site. The sequence of HRE-3 (GAAACAgccTGTTCT) differs from the consensus GRE at two positions that do not affect hormonal response (18). 2×HRE-3 has two copies of a HRE-3 oligonucleotide. C′Δ2 has 40 bp more *Slp* sequence 5′ to the C′Δ9 fragment, including a 17-bp repeat (arrows). Transfections were done with 6 μg of reporter plasmid and 6 μg of receptor plasmid. Subsequent treatment was for 40–44 hr with no hormone or 1 μM 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), dexamethasone, or progesterone, as per receptor. Inductions are the average of at least five assays for AR and GR and three for PR; SEMs are indicated. Basal expression levels of reporter plasmids were similar, with sufficient CAT to acetylate 1% of input radioactive chloramphenicol.

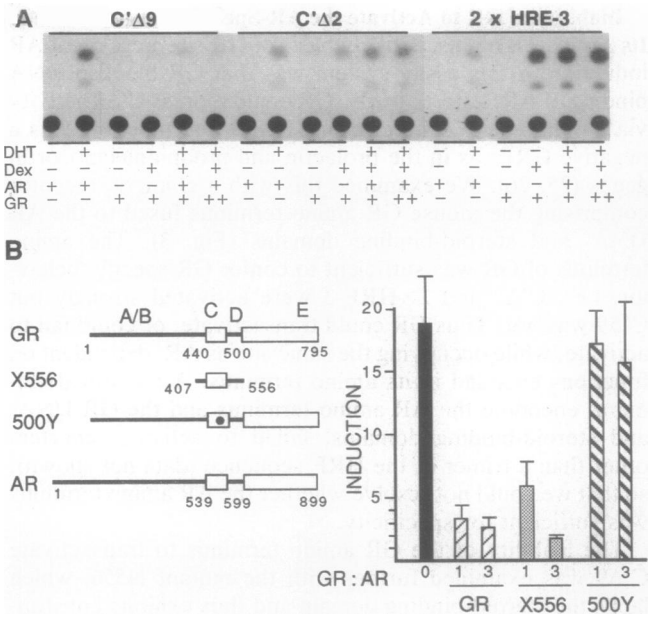

FIG. 2. GR blocks androgen specific induction of C′Δ9 by binding DNA. Expression plasmids encoding AR and various forms of GR were cotransfected; all plates received 3 μg of reporter and 2.5 μg of AR plasmid, with GR plasmid and/or vector alone to an equivalent DNA amount. (*A*) Effect of hormone plus AR, GR, or both together is shown for C′Δ9, which is androgen-specific, and for C′Δ2 and 2×HRE-3, which respond to both 5α-dihydrotestosterone (DHT) and dexamethasone (Dex). The chromatogram is representative of several assays; GR points have 2.5 μg (+) or 5 μg (++) of expression plasmid. (*B*) Mutant GRs VAX556 (X556) (12) and VAC500Y (500Y) (13) are compared with wild-type GR and AR; numbers signify amino acid position. Region C includes the zinc finger DNA-binding domain (amino acids 440–500 for GR, 539–599 for AR), E the steroid-binding domain, and the A/B amino terminus much of the transactivation function. A black dot in 500Y represents the Cys → Tyr mutation in region C. Averages of five transfections are plotted with SEMs. A GR/AR ratio of 1 indicates that 2.5 μg of each receptor DNA was used; a ratio of 3 indicates 7.5 μg of GR plasmid.

("squelching") occurs for steroid receptors and other transcription factors and does not require DNA binding (21–24). In our case, squelching did not seem to account for repression of C′Δ9 activity, because C′Δ2 and the HRE-3 dimer gave full induction with both receptors present (Fig. 2*A*). Thus, an antagonistic effect of GR was exerted only for the DNA target that showed specificity for AR.

To discern the basis of GR antagonism, expression plasmids encoding mutant GRs were tested in this assay system. A plasmid encompassing the DNA-binding domain of GR (VAX556, encoding amino acids 407–556) shows constitutive activity 10% that of wild-type GR plus hormone on other promoters, implying that the zinc finger domain can localize to the nucleus, bind DNA, and contact the transcriptional machinery (12). When cotransfected with AR and C′Δ9, this GR fragment was as effective as full-length GR in depressing AR induction (Fig. 2*B*). Thus, DNA binding of GR may be sufficient to repress AR activation of the *Slp* enhancer.

To prove that DNA binding was necessary, we tested the GR point mutant VAC500Y, in which a cysteine of the second zinc finger is altered to a tyrosine (13). This receptor accumulates equivalently to wild type, translocates to the nucleus with hormone, and has domains that could contact other factors, but it cannot bind DNA and therefore confers no activity through a GRE. When this mutant GR was expressed in CV-1 cells with AR, there was no repression of the androgen induction of C′Δ9 (Fig. 2*B*). Therefore, competition with AR required that GR bind DNA.

Inability of GR to Activate the AR-Specific Target Is Due to Its Amino Terminus. A likely basis of GR's repression of AR induction in this assay system was that GR blocked DNA binding of AR. Alternatively, GR could repress C′Δ9 activity via a binding site distinct from HRE-3 that functioned as a negative GRE, as in the prolactin and proopiomelanocortin genes (25, 26). We examined this with a chimeric receptor comprising the mouse GR amino terminus fused to the AR DNA- and steroid-binding domains (Fig. 3). The amino terminus of GR was sufficient to confer GR-specific behavior; i.e., C′Δ2 and 2×HRE-3 were activated strongly but C′Δ9 was not. Thus GR could transactivate, or could fail to activate, while occupying the same site as AR, dependent on functions encoded in its amino terminus. A reciprocal chimera, encoding the AR amino terminus and the GR DNA- and steroid-binding domains, failed to activate reporters other than a trimer of the HRE sequence (data not shown), so that we could not resolve whether the AR amino terminus was sufficient for specificity.

The inability of the GR amino terminus to transactivate C′Δ9 was examined further with the mutant N556, which lacks the steroid-binding domain and thus exhibits constitutive transactivation (14). While HRE-3 alone is a weak response element for wild-type GR, N556 provoked high activity from this plasmid (Fig. 4). Similarly, C′Δ2 expressed CAT severalfold better with N556 than with GR plus hormone. C′Δ9, in contrast, showed no response to N556, suggesting that elements in the reporter depressed the activity that HRE-3 alone could produce. N556 thus exaggerated the behavior of GR with respect to specificity. That other elements affected N556 activity was also evidenced by the construct Bst-3, an internal deletion mutant of C′Δ9 that retained the HRE-3 site. Sequences in Bst-3 contained information sufficient to depress N556 activity on the HRE (but insufficient to elicit significant androgen response; data not shown).

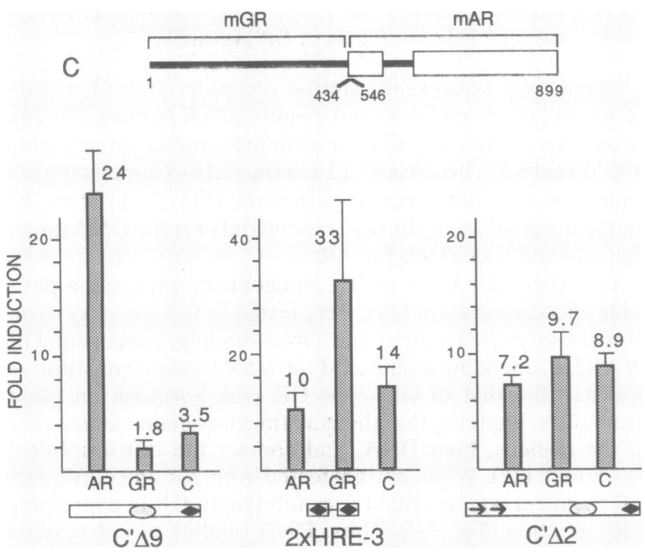

FIG. 3. Amino-terminal regions of receptor are involved in specificity. A GR–AR chimeric expression plasmid, C (shown at top), was made by fusing sequences from mouse GR (15) encoding the amino terminus until amino acid 434 to the mouse AR (10) from amino acid 546 to the carboxyl terminus, using a conserved *Hin*dIII site. The fusion point is 1 amino acid after the second cysteine of the DNA-binding domain. Activation by the chimeric receptor C was compared with activation of AR and GR as in Fig. 1; cells receiving GR were treated with dexamethasone, and those receiving AR or C were treated with 5α-dihydrotestosterone. Inductions are the averages of at least three assays, with SEMs indicated.

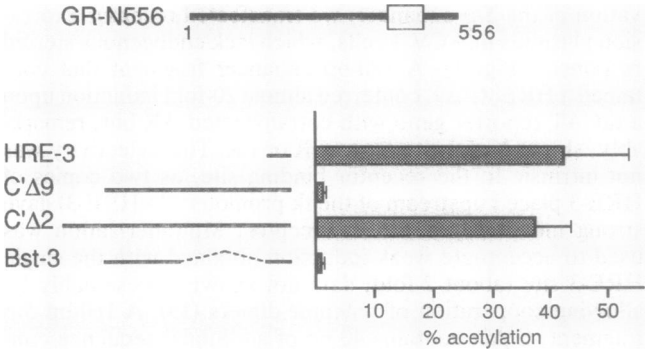

FIG. 4. Transactivation by the amino terminus of GR may be inhibited by interaction with nonreceptor factors. The expression vector for the GR mutant N556 (14) (shown at top) encodes the rat GR amino terminus and DNA-binding domain. Reporter plasmids on the left are aligned to show sequences in common; the dashed line in Bst-3 indicates sequences deleted. These reporters were cotransfected with N556 (6 μg each); CAT activity is shown as % acetylation rather than induction, because N556 does not bind hormone. For comparison, GR plus dexamethasone resulted in acetylation of about 2% of input chloramphenicol with HRE-3 and 20% with C′Δ2. Bars represent the average of three to five assays for each construct, with SEM indicated.

DISCUSSION

These results demonstrate that the *Slp* hormone-dependent enhancer, C′Δ9, is specifically regulated by androgens and not by glucocorticoids or progestins. This stringent hormonal control does not reside in the AR binding site itself but in adjacent sequences (Fig. 5). Presumably, factors bind these sites to form a transcription complex that is responsive only to androgens. Recent data (A.J.A. and D.M.R., unpublished work) indicate that multiple protein binding sites may be required for C′Δ9 function. Furthermore, C′Δ9 itself is sensitive to context, since specificity is lost in the longer fragment C′Δ2. Factors bound to sequences of C′Δ2 that are not present in C′Δ9 can apparently interact with several receptors; interestingly, the level of response to androgen with these factors is less than that attained by the specific complex on C′Δ9. Several elements may function *in vivo* and their role may be dependent on cell type. The importance of

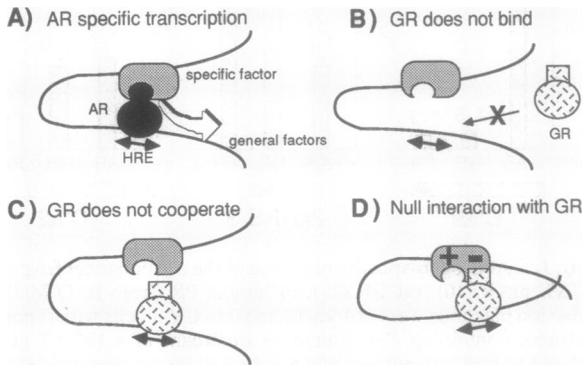

FIG. 5. Specificity of hormonal response. (*A*) Androgen-specific activation occurs when AR (black) binds the consensus site HRE-3 (double arrow) and interacts with a specific factor(s) (shaded) on C′Δ9 to enhance transcription. Specificity includes the failure of GR to activate transcription for one of several possible reasons. (*B*) GR may not bind the consensus site in the AR-specific enhancer. (*C*) GR does not interact with the AR-specific complex and induction of HRE-3 alone may be negligible. (*D*) GR may interact nonproductively with the androgen-specific complex. Results discussed in the text favor *B* least; *C* would suffice as an explanation, but some evidence favoring *D* shows a suppressive interaction between GR and the AR-specific complex.

cell-specific nonreceptor factors in hormonal activation is supported by the fact that C'Δ9 responds strongly to AR in CV-1 (monkey kidney-derived) cells but not in T-47D human mammary cells (9). This reflects *in vivo* regulation of *Slp*, which is expressed in kidney but not in mammary gland (8), despite the presence of AR in both. Therefore, C'Δ9 provides a simple model for analysis of androgen-specific gene activation, although additional elements may be required for completely appropriate regulation *in vivo*.

That GR fails to activate C'Δ9 could be explained by several possible mechanisms. GR could be unable to bind the consensus HRE in the context of C'Δ9 sequences (Fig. 5*B*). This is unlikely since our studies show that GR can compete with AR for DNA binding at the HRE. GR could be inactive despite binding if it cannot cooperate with androgen-specific factors to augment the otherwise weak response of HRE-3 (Fig. 5*C*). Alternatively, GR may interact, but nonproductively, with the androgen-specific complex (Fig. 5*D*). This last model gains indirect support from the behavior of the C-terminal deletion mutant of GR, N556, which can activate a single HRE strongly but not in the context of C'Δ9. Unlike negative glucocorticoid regulation, which can arise from GR binding at distinct sites or from prevention of GR binding (25–28), these studies suggest an intriguing null regulation, in which the potency of GR transactivation from a consensus GRE may be dictated by interactions with adjacent elements. Null may differ from negative regulation, in that there is little or no increase in expression, rather than decreased expression from an appreciable level.

GR inhibition of AR induction may not occur *in vivo* as it does experimentally, but serves to accentuate an underlying dilemma. That is, GR is ubiquitous and abundant relative to AR. Therefore, for androgen-specific genes it may be as problematic to remain inactive in the presence of glucocorticoids as it is to become active when androgens increase. Because AR response depends on sequences that can also function as GREs, an efficient specificity mechanism may be simultaneously positive for AR and null for GR, if only to prevent leaky GR activation. To allow expression, accessory factors need only favor interaction of AR over GR. Therefore it is these specific cohorts, and not the receptor binding site, that orchestrate the precision of hormonal response attained *in vivo*. Interaction may prove to be a fundamental means of specific regulation for families of transcription factors that bind similar sequences.

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