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Symposia

Specific Steroid Response from a Nonspecific DNA Element

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A fundamental dilemma of steroid hormone regulation is how specific transcription is attained *in vivo* when several receptors recognize the same DNA sequence *in vitro*. We have identified an enhancer of the mouse sex-limited protein (*Slp*) gene that is activated by androgens but not by glucocorticoids in transfection. Induction requires a consensus hormone response element (HRE) and multiple auxiliary elements within 120 base pairs. Androgen specificity relies on a dual function to augment androgen but prevent glucocorticoid action from a site that both receptors can bind. The nonreceptor factors are the dominant force in transcriptional specificity, although HRE sequence variations can affect the stringency and magnitude of hormonal response. The effect of HRE variations suggests that receptor position is altered relative to the other factors. Thus protein interactions that elicit specific gene regulation are established by the array of DNA elements in a complex enhancer and can be modulated by subtle sequence differences that may influence precise protein contacts.

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INTRODUCTION

Steroid hormones function in precise biological programs via their specific receptors, which are ligand-activated transcription factors [1, 2]. These proteins thus link extracellular cues for reproduction, development, and homeostasis directly to gene regulation. In contrast to the evolutionarily conserved structure of the nuclear receptor family, its members exhibit impressive functional diversity. Intriguingly, this diversity is not reflected in the response elements of their target genes. How is specific hormonal regulation of gene expression attained when multiple receptors recognize similar DNA binding sizes?

Transcriptional specificity is a fundamental problem for several transcription factor families but is especially evident for the steroid receptors. The glucocorticoid response element (GRE) consensus is a palindrome of TGTTCT half-sites separated by 3 bases. In gene transfer, this sequence can also mediate induction by androgen, progesterone and mineralocorticoid receptors (AR, PR and MR) [1]. Further, purified or recombinant receptors can bind this sequence *in vitro* [3, 4]. Therefore, the consensus is referred to as a hormone response element (HRE) to indicate its generality. While there may be some overlap in sets of genes regulated by these hormones, their physiological functions are generally distinct. Requisite transcriptional specificity could be enforced by subtle sequence differences in response elements, or by accessory factors that selectively interact with receptors to determine precise gene activation.

While the HRE mediates induction in transfection by several receptors, naturally occurring enhancers of GR-responsive genes are often comprised of multiple imperfect consensus elements that show cooperative effects with each other and with sites for various other factors [5, 6]. AR-dependent genes face a greater challenge for specific expression if DNA sites are recognized by multiple receptors, because in most target organs GR is also present and in greater abundance. Some AR-dependent genes that express in prostate (C3, prostate specific antigen, and probasin)

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have complex response elements [7–9], further suggesting that an androgen-specific response requires more than just the HRE sequence.

Since many natural enhancers may be composed of multiple elements, a likely mechanism for imposing specific transcription is through differential protein–protein interactions. In this way, the context of an HRE may have as much impact as its sequence. As an example, mutations in nonHRE sequences of MMTV show differential response to GR, PR and AR [10]. The existence of hormonal specificity factors may be inferred from the promoter- and cell type-dependent activities of some receptors [11]. Receptor structure suggests protein interaction is a major determinant of activation, because while there is extraordinary conservation of the DNA-binding domains, there is little similarity in the large amino termini that encode transactivation functions and presumably contact components of the transcriptional machinery [2].

ANDROGEN-SPECIFIC RESPONSE

We have been studying the androgen regulation of mouse sex-limited protein (*Slp*). *Slp* arose from a duplicated complement component gene that acquired androgen dependence from a proviral insertion [12]. The transcriptional enhancer of *Slp*, located within the proviral long terminal repeat, contains a near-perfect HRE (HRE-3) that is necessary but not sufficient for hormonal induction [13]. Multiple interacting sites for auxiliary factors are required to augment weak AR activity. We asked whether these factors might also elicit specific response to AR from DNA sites recognized by GR and PR, by cotransfection with steroid receptors into receptor-deficient CV-1 cells [14].

The 120 bp enhancer fragment, C′Δ9, conferred almost 20-fold androgen response upon tkCAT but, remarkably, showed no response to glucocorticoid or progesterone (Fig. 1). This selectivity was not intrinsic to the receptor binding site, as two copies of HRE-3 (2 × HRE-3) before tkCAT induced strongly with each receptor. That specific response of C′Δ9 is due to nonreceptor factors rather than to the HRE sequence is also demonstrated by the fact that it is host cell-dependent. That is, C′Δ9 does not show selective response in T47D cells but is inducible by GR as well as by AR [15]. C′Δ9 thus provides a model element to address the mechanism of specificity *in vitro*.

With the specific enhancer, we could ask why GR does not activate C′Δ9 from HRE-3. Possibly GR was unable to bind HRE-3 in the context of C′Δ9, perhaps due to interference of proteins bound at adjacent sites. Alternatively, GR might bind but be unable to transactivate in conjunction with factors competent for AR response. This latter possibility is especially intriguing since GR cooperates promiscuously with many transcription factors [6].

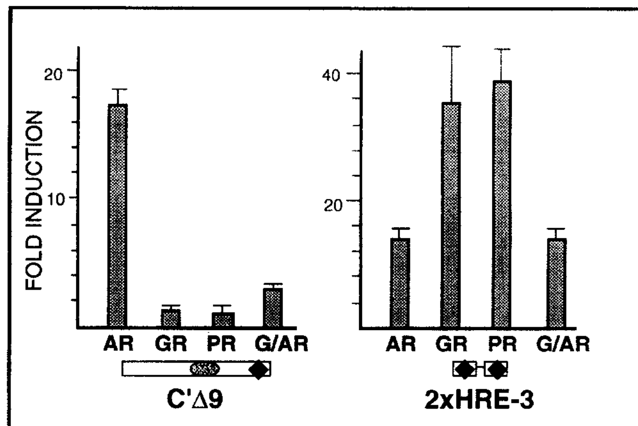

Fig. 1. Androgen-specific response of the *Slp* enhancer. Reporter tkCAT plasmids with either the C′Δ9 fragment (HRE-3 is shown as a closed diamond and a binding site for a ubiquitous protein as an oval) or two copies of just the HRE (2 × HRE-3) were cotransfected into CV-1 cells with receptor expression vectors for AR, GR, PR, or the chimera G/AR, which is the GR amino terminus fused to the AR DNA and ligand binding domains. Transfection and CAT assays were performed as in Adler *et al.* [14]. Fold inductions with the appropriate hormone are the mean of at least 3 experiments, with SEM indicated.

GR CAN BIND BUT NOT ACTIVATE THE AR-SPECIFIC ENHANCER

To understand GR's failure to activate C′Δ9, we first asked whether GR could inhibit AR induction, as an indication of GR's ability to bind HRE-3 in the enhancer context. In fact, induction was greatly diminished when GR and AR were coexpressed (Fig. 2). This was not indirect competition for other rate-limiting factors, because 2 × HRE-3 was fully inducible in the presence of both receptors [14]. Thus the antagonistic effect of GR was target-specific.

Using mutant GRs, we showed that GR DNA binding was both necessary and sufficient to block AR

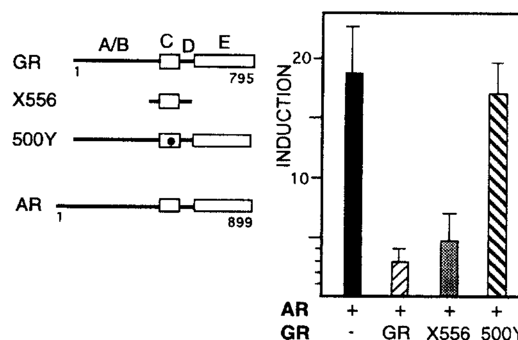

Fig. 2. The GR DNA binding domain blocks AR induction of C′Δ9. CV-1 cells were transfected with C′Δ9, AR expression vector, and no, wildtype or mutant GR plasmids, as indicated below the histogram (GR plasmids were present in a 2-fold excess to AR). Receptors are diagrammed to the left; the dot in 500Y is the cys to tyr mutation. Fold inductions are the average of 5 transfections, with SEM indicated. See Adler *et al.* [14].

action on C′Δ9. The GR plasmid X556 encodes 150 amino acids around the zinc fingers, sufficient for nuclear localization, DNA binding and weak constitutive activity [16]. X556 was as effective as intact GR in repressing AR induction of C′Δ9. In contrast, GR mutant 500Y, in which a zinc finger cysteine was converted to tyrosine, can localize to the nucleus but cannot bind DNA [17]. Coexpression of 500Y had no effect on C′Δ9's response to AR.

To confirm that GR repressed AR action by blocking AR binding to HRE-3, as opposed to GR binding a distinct negative GRE, we used a chimeric receptor that fused the amino terminus of GR to the DNA and steroid binding domains of AR. This allowed simultaneous testing of whether the GR domain containing the major transactivation function was sufficient for GR-specific behavior, i.e. the ability to activate 2 × HRE-3, but not C′Δ9. The chimeric receptor G/AR behaved essentially like GR and activated the general but not specific reporter (Fig. 1). This showed that while tethered to HRE-3 via the AR DNA binding domain, GR's amino terminus could not transactivate with the AR-specific complex. Further, this suggests that the AR NH_2-terminus is required for AR-specific induction of C′Δ9.

That the AR-specific complex contains factors that repress GR activity, as opposed to simply do not cooperate with GR, is suggested by two experiments. First, a constitutive GR mutant lacking the ligand binding domain strongly activates a single HRE-3 before tkCAT, yet cannot activate this site in the context of C′Δ9. Further, when HRE-3 is mutated in C′Δ9 and reinserted at various distances in the plasmid, GR can function, evidently due to escaping repression by juxtaposed nonreceptor factors [15].

GR's inhibition of AR induction may not occur *in vivo* as it does experimentally, but accentuates an underlying dilemma. 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 to express 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, to prevent leaky GR activation (Fig. 3). To allow expression, accessory factors need only favor interaction of AR over GR. Therefore the specific cohorts, and not the receptor binding site, orchestrate precise hormonal response *in vivo*.

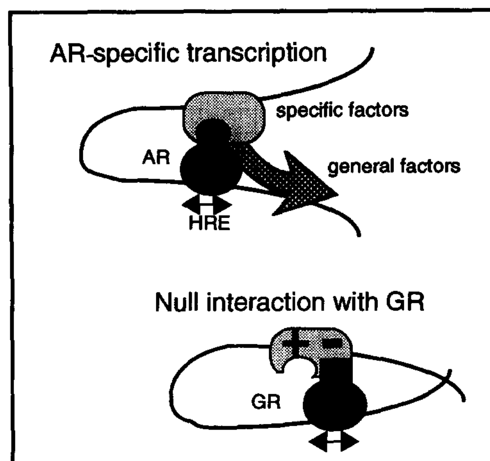

Fig. 3. Model for specific hormonal response. Androgen-specific induction is attained by interaction of AR (directly and/or indirectly) with a variety of accessory transcription factors. When GR binds to the common receptor binding site (HRE), it is prevented from transactivating by some components of the androgen-specific complex.

HRE SEQUENCE AFFECTS RECEPTOR INTERACTIONS

To further characterize elements of specific hormonal response, we introduced clustered point mutations across C′Δ9 with PCR site-directed mutagenesis [15]. Many mutations affected but did not abolish androgen response, suggesting that the enhancer was composed of multiple, partially redundant elements. No mutation allowed response to GR, suggesting that while each mutation had quantitative effects on androgen response, no single mutation qualitatively altered specificity. The behavior of the mutants suggests that HRE-3 mediates a hormone response whose magnitude over a broad range depends on interaction with several other elements.

While HRE-3 was nonspecific in steroid response and gained specificity from interaction with nonreceptor elements, we had not tested an influence of the precise sequence of the HRE. This was at issue since simply distancing the HRE had dramatic effects on GR activity; i.e. allowing half the induction elicited by AR compared to no response on C′Δ9 [15]. Therefore, we replaced by mutagenesis the *Slp* HRE-3 with one from MMTV that is also a good match to the consensus and responds to AR, GR and PR [18]. MTV-HRE differs from HRE-3 at 2 bp in the imperfect half-site and 2 in the spacer (Fig. 4). The half-site differences are not expected to affect response, whereas a GC-rich spacer was shown to reduce GR induction several-fold [19]. Converting HRE-3 to the MTV sequence nearly doubled AR response. More surprisingly, GR response increased 10-fold (Fig. 4). Thus, stringent hormonal specificity was lost in C′Δ9-MTV, although androgen preference was retained. With other mutants, both half-site and spacer sequences were shown to contribute to specificity and/or magnitude of hormonal response.

Given the striking effect on specificity of HRE sequence differences, we examined the orientation of the HRE in the enhancer, since HRE-3, like most natural elements, is asymmetric. When we inverted HRE-3 so that the perfect half-site was proximal to the tk promoter, androgen response was unchanged, but

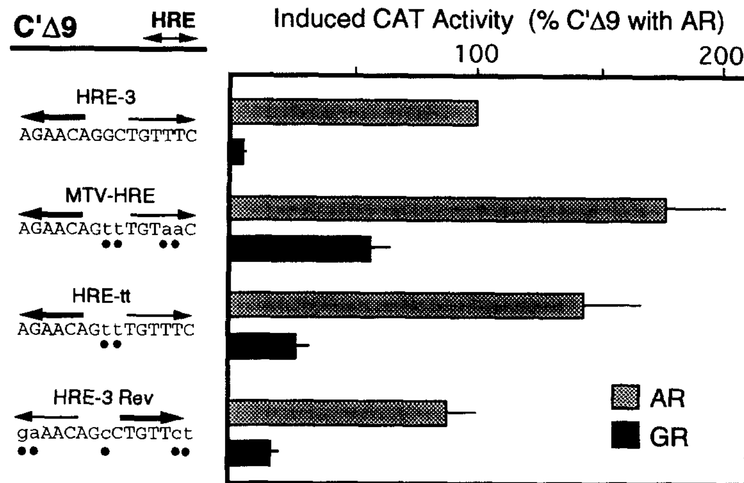

Fig. 4. HRE base changes within the context of C′Δ9 affect differential hormone response. Specific bases in HRE-3 were mutagenized in C′Δ9 to the sequences shown and response of these plasmids to AR and GR was compared by cotransfection into CV-1 cells as before. Dots highlight bases differing from HRE-3 in its natural orientation. See Adler *et al.* [15] for details.

glucocorticoid response tripled (Fig. 4). Together, the effects of the HRE mutations imply that HRE location, sequence and orientation modulate the stringency of specificity, without altering the overall preferential response of the enhancer to androgen.

A component of response of the HRE mutants could be differential receptor affinities. To correlate inducibility with binding site preference, full-length baculovirus-expressed AR [20] or GR [21] (from E. M. Wilson and G. B. Thompson, respectively) was bound to HRE-3 DNA. The amount of each HRE shown in Fig. 4 required to compete 50% of the receptor–HRE-3 complex was used as an assay of relative affinity. The complex of AR and HRE-3 diminished equivalently with each sequence, indicating that AR showed little ability to distinguish among these HREs. GR, however, bound 3-fold better to the MTV-HRE than to HRE-3 [15]. The affinity differences of GR corresponded qualitatively with the response of C′Δ9-HRE mutants. However, multimerized HREs do not confer inductions reflecting their relative affinities, since all respond strongly to both receptors. Therefore, differential affinity may be a component of activation that is aggrandized or diminished dependent on interaction with other factors. Further, androgen specificity may take advantage of receptor binding sites that are less preferred by GR.

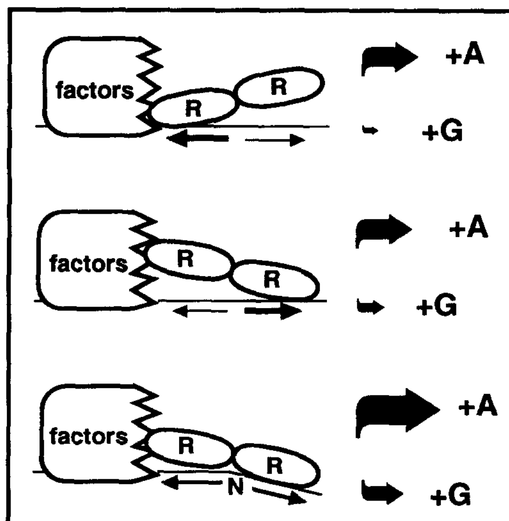

Fig. 5. Receptor binding sites affect differential interactions with nonreceptor factors. In the natural C′Δ9 configuration (top), AR interacts well with neighboring factors but GR activity is abrogated. When the HRE is inverted (middle), GR can function to some extent. When the HRE sequence is altered, both receptors induce more strongly, but only in the context of the enhancer.

CONCLUSION

These studies reveal an interplay between the precise sequence of the response element and the array of accessory sites, which may allow gene expression *in vivo* to vary with hormone, concentration, and cell type. Receptor affinity within the enhancer may reflect affinity to nearby factors as well as to the response element (Fig. 5). For example, inverting the HRE alters receptor contacts to DNA as well as to neighboring proteins, which has little effect on AR activity but notably enhances GR (Fig. 5, middle). Changes within the HRE increase the action of both receptors, due to higher DNA affinity or better contacts with other components (Fig. 5, bottom). For all HRE arrangements in this enhancer context, however, AR response is greater than GR, revealing that the nonreceptor factors have a dominant influence to the response element in steroid-specific induction. Protein–protein interaction may prove to be a fundamental means of specific regulation for families of transcription factors that bind similar sequences.

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