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Analysis of Glucocorticoid and Androgen Receptor Gene Fusions Delineates Domains Required for Transcriptional Specificity

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Androgen receptor (AR) and glucocorticoid receptor (GR) influence distinct physiologic responses in steroid-responsive cells despite their shared ability to selectively bind in vitro to the same canonical DNA sequence (TGTTCT). While the DNA-binding domains (DBDs) of these receptors are highly conserved, the amino N-terminal domain (NTD) and hormone-binding domain (HBD) are evolutionarily divergent. To determine the relative contribution of these functional domains to steroid-specific effects in vivo, we constructed a panel of AR/GR gene fusions by interchanging the NTD, DBD, and HBD regions of each receptor and measured transcriptional regulatory activities in transfected kidney and prostate cell lines. We found that GR was approximately 10-fold more active than AR when tested with the mouse mammary tumor virus promoter, and that this difference in activity was primarily owing to sequence divergence in the NTDs. We also tested transcriptional activation of the androgen-dependent rat probasin promoter, and in this case, AR was at least twofold more active than GR. Analysis of the chimeric receptors revealed that this difference mapped to the DBD region of the two receptors. Transcriptional repression functions of the wild-type and chimeric receptors were measured using an activator protein 1 (AP-1) transrepression assay and identified the GR HBD as a more potent transrepressor of AP-1 transcriptional activation than the AR HBD. Taken together, our analyses reveal that evolutionary sequence divergence between AR and GR functional domains results in unique promoter-specific activities within biologic systems in which both AR and GR are normally expressed.

Key Words: Glucocorticoid receptor; androgen receptor; steroid-regulated gene expression; transrepression; evolutionary divergence.

Introduction

Steroid hormone receptors are a group of ligand-dependent transcription factors that direct a diverse array of developmental and physiologic processes in response to their cognate hormones. The androgen receptor (AR) and glucocorticoid receptor (GR) have been placed in the same subfamily of steroid hormone receptors based on conservation of amino acid sequence in their DNA-binding domains (DBDs) *(1)*. It is not surprising, therefore, that these two receptors can be shown to bind to the same DNA sequences using in vitro binding assays *(2)*. By contrast, amino acid residues in the N-terminal domain (NTD) are less well conserved between AR and GR (<15% similarity), which may explain in part why these closely related receptors elicit distinct physiologic responses in vivo. The moderate degree of conservation between the hormone-binding domains (HBDs) of GR and AR (51% sequence similarity) is consistent with the observed ligand-binding specificities of the two receptors *(3)*.

Steroid hormone receptors are thought to activate transcription by binding to target DNA sites and recruiting protein factors to the transcription initiation complex that modulate chromatin structure and RNA polymerase II activity *(4)*. Since kidney and prostate cells are exposed to both androgens and glucocorticoids during development, and these cells express appreciable levels of both AR and GR, it has been proposed that amino acid differences between these two receptors account for observed steroid-specific effects. Although DNA-binding specificity has been shown to contribute to the target gene specificity of AR and GR *(2)*, this activity alone is not sufficient to explain selective steroid responses in stimulated cells *(5–7)*. Another mechanism of steroid specificity could be selective interaction of hormone receptors with transcriptional coactivator proteins *(8,9)*. For example, the interaction between RIP140 and GR inhibits receptor function, including transactivation, whereas an interaction between RIP140 and AR increases transactivation by the receptor *(10,11)*.

Steroid-selective effects can also be the result of different transcriptional repression specificities. It has been established that GR and AR are capable of repressing the activation of some transcription factors, such as activator protein 1 (AP-1) *(12–14)*. Although the mechanism of transrepression is not

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completely understood, it is likely to involve direct or indirect interference with signaling pathways through protein-protein interactions. AR transrepression appears to be partially owing to competitive binding to the CREB binding protein (CBP) transcriptional coregulator *(13,15,16)*, which leads to a net loss of free CBP to activate target promoters. More important, the requirement for CBP protein-protein interactions to mediate transrepression is dissimilar for AR and GR *(16)*.

To better understand how differences in the amino acid sequence of AR and GR specify cell- and promoter-selective effects, we constructed gene fusions using the three primary functional domains (NTD, DBD, HBD) of AR and GR and tested these chimeric receptors in transfected kidney and prostate cells. Using both transcriptional activation and repression assays, we found that the potency of each receptor is determined by distinct functional domains and that these regions of the receptors do not contribute equally to the combined function. We propose that sequence divergence between AR and GR within these three primary functional domains permits steroid-specific responses in cells that express both receptors and are chronically exposed to androgens and glucocorticoids.

Results

Differences in Activity Between AR and GR on Mouse Mammary Tumor Virus Map to the NTD

We constructed a panel of AR/GR chimeric receptors to investigate the contribution of each functional domain to the activity of its cognate receptor. To facilitate the exchange of functional domains between AR and GR, two unique restriction sites flanking the DBDs were engineered into the cDNAs of each receptor (Fig. 1A). These two sites introduced three amino acid changes into the coding regions of each altered receptor but did not lead to a change in activation ability on mouse mammary tumor virus-chloramphenicol acetyltransferse (MMTV-CAT) (Fig. 1B). The modified receptors, AR-AB (AAA) and GR-AB (GGG), were used to construct six distinct chimeric fusion genes. The chimeric receptors were named according to the source of each of the three domains, beginning with the NTD. For example, fusing the AR NTD with the GR DBD and GR HBD results in the chimeric receptor AGG.

The AR/GR chimeric receptors were first tested for their relative abilities to activate transcription from MMTV-CAT reporter gene in CV-1 monkey kidney cells. GR was found to be 10-fold more active than AR in this cell and promoter context, as shown in Fig. 2 comparing GGG/AAA (10.1-fold). To determine whether the three receptor functional domains contribute equally to this difference, we tested individual chimeric receptors by swapping out one domain at a time. In the context of this assay, the largest difference in activity was found to be between the NTDs. Comparing **G**AA/**A**AA (fivefold) and **G**GG/**A**GG (fivefold), it is apparent that the GR NTD contributes to the observed higher activation attributable to GR. The difference between **G**AG and **A**AG was only about fourfold, and **A**GA is twofold less active than **G**GA, suggesting that interdomain functions may also be important to receptor activity *(17–19)*. Radiolabeling of protein extracts with ^{3}H-dexamethasone (dex) or ^{3}H-R1881 confirmed that differences in transactivation potential of chimeric receptors were not owing to differences in level of protein expression (data not shown).

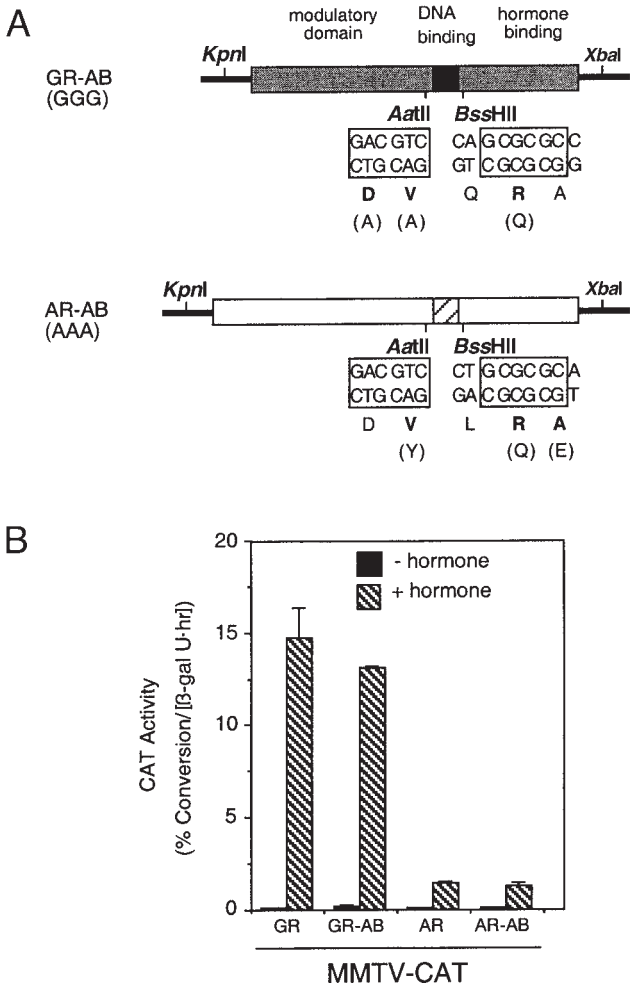

Fig. 1. Site-directed mutagenesis of GR and AR cDNAs and differences in transactivation function. (**A**) Unique restriction sites flanking the DBDs were introduced into GR and AR as described in Materials and Methods. The nucleic acid sequences of the *Aat*II and *Bss*HII sites are boxed. The resulting receptors, GR-AB (GGG) and AR-AB (AAA), each contained three amino acid changes (shown in bold) from the wild-type sequence (shown in parentheses). (**B**) Results from transient transfection assays comparing the activity of the modified receptors (GR-AB and AR-AB) with that of GR and AR, respectively, in CV-1 cells.

The data in Fig. 2 also can be used to analyze the relative contributions of the DBD and HBD to MMTV activation in CV-1 cells. First, there is roughly a 2-fold enhancement in activation by the GR DBD when making the direct comparisons of A**G**A/A**A**A (2.5-fold) and G**G**G/G**A**G (1.7-fold), although this is not consistently seen in all DBD swaps (G**G**A/

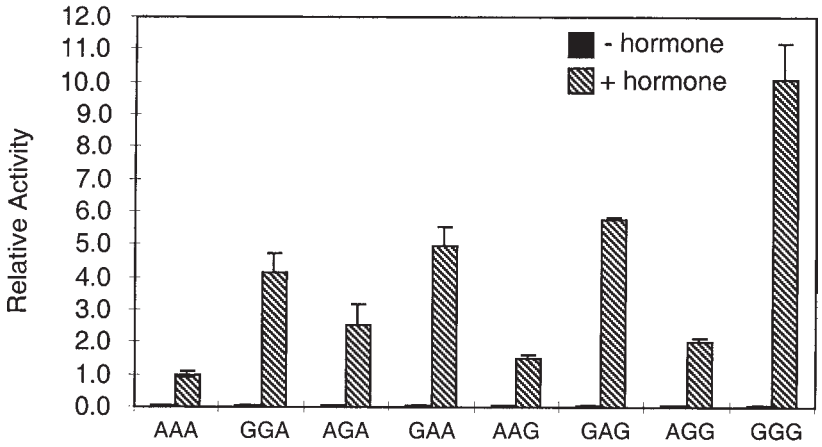

Fig. 2. Transactivation potential of the GR/AR chimeric receptors on the MMTV reporter plasmid. Results from transient co-transfection assays in CV-1 cells using the eight chimeric receptors (pcDNA1/neo expression vector) are presented. Solid and hatched bars correspond to CAT activities measured in the absence or presence of dex (AAG, GAG, AGG, GGG) or DHT (AAA, GGA, AGA, GAA), respectively. The CAT activity is normalized to AAA activity and error bars represent SEM.

G**A**A, 0.8-fold; A**G**G/A**A**G, 1.4-fold). Second, the HBD seems not to play a role in MMTV activation in CV-1 cells if one compares AA**G**/AA**A** (1.5-fold), GA**G**/GA**A** (1.2-fold), and GG**G**/GG**A** (2.4-fold); however, GGG is more than twice as active as GGA.

AR Is a More Active Inducer of Prostate-Specific Probasin Promoter Than GR

To study a transactivation context in which AR is more active than GR, we constructed two probasin reporter plasmids using a minimal segment of the rat probasin promoter. As shown in Fig. 3A, ProbTK contains nucleotides –298 through –70 of the promoter region, including androgen response element-1 (ARE-1) and ARE-2 *(20)*, fused to the herpes simplex virus (HSV) thymidine kinase (tk) promoter upstream of the CAT gene. The Prob reporter gene contains nucleotides –298 through 32, which include the CAAT and TATA elements of the rat probasin promoter.

Figure 3B shows the results of transient cotransfections of the ProbTK and Prob reporter genes into C1.1, a rat prostate cell line immortalized with SV40 large-T antigen *(21)*. In this assay, AR was found to be more active than GR on both probasin reporter constructs, whereas GR was more active than AR when using the MMTV reporter gene in these cells. More important, AR was nearly threefold more active than GR on ProbTK, and nearly fivefold more active on Prob.

We next tested the AR/GR chimeric receptors in C1.1 cells using the Prob reporter gene. The data in Fig. 4 show that the G**A**G chimera was the most active in this assay, suggesting that the AR DBD is responsible for the difference in AR/GR activity on the Prob promoter. The other AR/GR comparisons support this conclusion, as can be seen by A**A**A/A**G**A (4.0-fold), G**A**A/G**G**A (not applicable; GGA is inactive on Prob), and A**A**G/A**G**G (2.7-fold). Consistent with these results, Rennie et al. *(20)* found that DNA-binding experiments with bacterially expressed DBDs revealed that AR has a higher affinity for the ARE-2 of the probasin promoter than a canonical GRE.

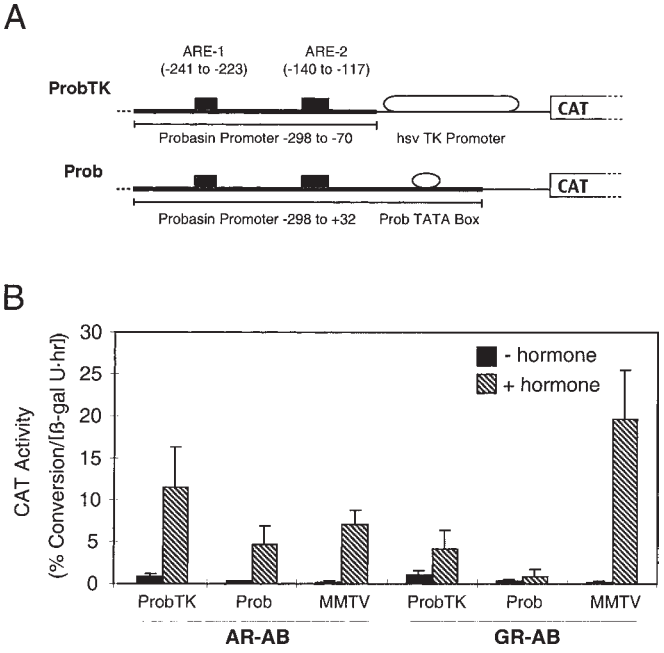

Fig. 3. Features and transactivation from the Prob-driven CAT reporter constructs. (**A**) Diagrammatic representation of the ProbTK-CAT and Prob-CAT reporter constructs. The shaded boxes represent ARE-1 and ARE-2, while the ovals represent the HSV TK promoter and Prob TATA box in ProbTK and Prob, respectively. (**B**) Transactivation by AR -AB and GR-AB (p6R expression vector) from ProbTK, Prob, and MMTV in C1.1 cells. Results from transient transfection assays are presented as CAT activity (described in Materials and Methods) in the absence (■) and presence (⫽) of DHT (AR) and dex (GR), including SEM.

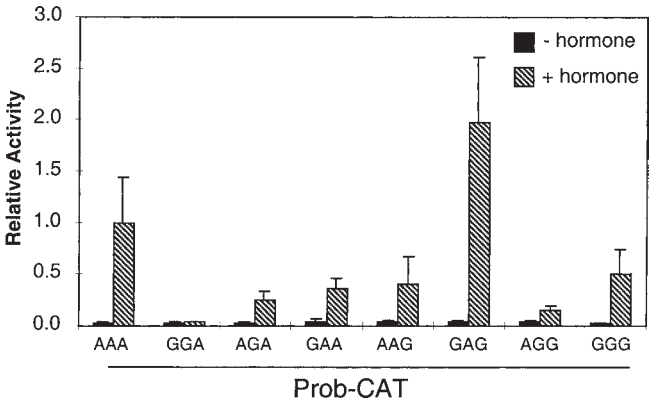

Fig. 4. Transactivation potential of GR/AR chimeric receptors (pcDNA1/neo expression vector) on the Prob reporter plasmid. Results from transient cotransfection assays in C1.1 cells using the eight chimeric receptors are presented. Solid and hatched bars correspond to CAT activities measured in the absence or presence of dex (AAG, GAG, AGG, GGG) or DHT (AAA, GGA, AGA, GAA), respectively. The CAT activity is normalized to AAA and error bars represent SEM.

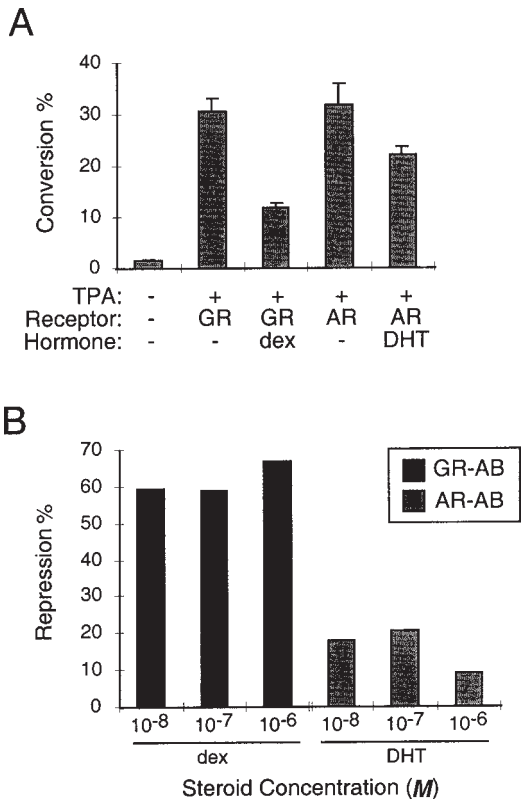

Fig. 5. Repression of AP-1 activity by AR and GR. (**A**) C1.1 cells were transfected with 5xTRE-CAT and either AR-AB or GR-AB expression plasmids (p6R). Activity of AP-1 as assayed by transactivation of the 5xTRE-CAT reporter plasmid is presented as percentage of conversion of CAT, including SEM. (**B**) Results of a dose-response curve for dex (black bars) and DHT (gray bars) initiating repression by GR and AR, respectively, are presented as percentage of repression of AP-1 activity.

GR Is a More Potent Repressor of AP-1 Activity Than AR

GR is capable of inhibiting AP-1 activity through a mechanism referred to as transrepression *(12,22,23)*. Studies have also shown that AR is able to repress AP-1 function *(24)*. The mechanism of steroid receptor–mediated transrepression is thought to involve competition for limiting cellular factors such as CBP *(13,15,16)*. To first determine whether there are quantitative differences in AR and GR transrepression functions, we stimulated AP-1 activity in CV-1 cells by treatment with the phorbol ester TPA, and then we measured the effect of steroids in cells cotransfected with an AP-1 reporter gene (5XTRE-CAT) and AR or GR expression plasmids.

The data in Fig. 5A show that when CV-1 cells were transfected with a GR expression plasmid and grown in the presence or absence of 100 n*M* dex prior to TPA stimulation, we found a 61% dex-dependent inhibition of AP-1 activity. However, when the same assay was performed using the analogous AR expression plasmid in cells treated with 5α-dihydrotestosterone (DHT), AP-1 activity was only inhibited by 26%. To confirm that this difference in AP-1 transrepression by AR and GR was not owing to differences in hormone potency, we performed the transrepression assay using 10 n*M*, 100 n*M*, or 1 μ*M* dex or DHT. The results shown in Fig. 5B indicate that under all three conditions, GR has a greater ability to transrepress AP-1 activity than does AR.

Interactions between GR and c-fos have been mapped to the DBD *(25)*, whereas, CBP appears to bind to the HBD of steroid and nuclear receptors *(13)*. To determine whether the enhanced ability of GR to transrepress AP-1 activity could be mapped to any one of the three major domains, we tested the entire set of AR/GR chimeric receptors in the transrepression assay. The data from these experiments are shown in Fig. 6. Using the GGG and AAA receptors in the pcDNA1/neo expression vector (see Materials and Methods), we confirmed our previous results (Fig. 5) by finding that GGG is significantly more potent in AP-1 transrepression than is AAA (58 and 24%, respectively; Fig. 6). Replacement of the NTDs did not have a notable effect on transrepression. Although **A**GG was less active than **G**GG, the **A**AA and **G**AA chimeras had comparable activities. Additional NTD pairwise comparisons (**G**AG/**A**AG and **G**GA/**A**GA) revealed that chimeric receptors containing the GR NTD have transrepression function substantially similar to those that have the AR NTD in that position. By contrast, pairwise comparisons of the DBD chimerics (G**G**G/GA**G**, A**G**A/AA**A**, G**G**A/GA**A**, and A**G**G/AA**G**) showed no enhancement of transrepression function when the GR DBD was present.

The most consistent effect on transrepression function was seen when the GR HBD replaced the AR HBD (Fig. 6). In all four pairwise comparisons of the HBD chimerics (GG**G**/GG**A**, AA**G**/AA**A**, AG**G**/AG**A,** and GA**G**/GA**A**) we found that transrepression function was markedly higher when the GR HBD was present. Taken together, these results indicate

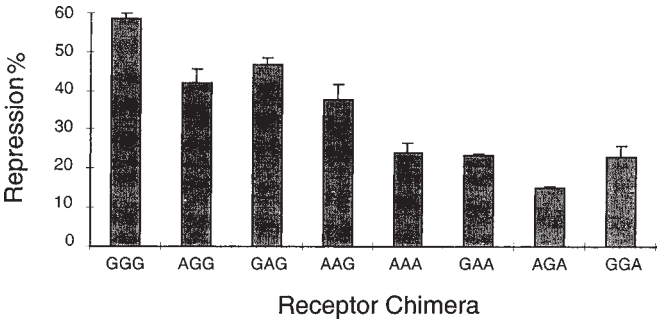

Fig. 6. Transrepression potential of GR/AR chimeric receptors acting on AP-1. Results from transient cotransfection assays in C1.1 cells using the eight chimeric receptors (pcDNA1/neo expression vector) are presented. Bars correspond to percentage of hormone-dependent repression of AP-1 activity (5xTRE-CAT) by dex (AAG, GAG, AGG, GGG) or DHT (AAA, GGA, AGA, GAA), including SEM.

that the ability of GR and AR to inhibit AP-1 activity is primarily owing to sequence differences in the GR and AR HBDs and, to a lesser extent, to sequence divergence in the NTDs.

Discussion

Steroid hormone receptor specificity has been investigated at the level of DNA binding *(5)*, chromatin remodeling *(26)*, protein-protein interactions *(27,28)*, and subdomain functions *(29,30)*. Previous studies have shown that GR has a greater potential to activate transcription than AR *(31)* in transient transfections of monkey kidney cells (CV-1) from a promoter containing a hormone response element from the MMTV promoter. However, it has also been shown that AR is a more potent transcriptional activator than GR when tested with the prostate-specific probasin (Prob) promoter in prostate cells *(20)*. These observations allow for a comparison between AR and GR activity in two different contexts. In one context (MMTV/kidney cell), GR is more active, whereas in another context (Prob/prostate cell), the reverse is true. Despite their highly conserved DBDs and similar DNA-binding specificities, AR and GR direct distinct cellular responses through binding of their cognate hormones. We investigated the contribution made by each of the three major domains of AR and GR to the activity of each receptor in both transactivation and transrepression functions to elucidate how steroid hormone receptor specificity is achieved.

Consistent with other studies *(29,30)*, we found that the GR and AR subdomains are utilized differently by the two receptors. Moreover, our studies showed that the differences in subdomain utilization were dependent on the function and context of the assay. In both transactivation contexts, the NTD and the DBD were most responsible for differences in activation between AR and GR. The greater activation potential of GR on the MMTV promoter mapped largely to the GR NTD, whereas the higher activation potential of AR on the probasin promoter mapped almost exclusively to the DBD. Taken together, these results suggest that, although DNA-binding specificity is involved in receptor selection at hormone-responsive promoters *(5–7,32,33)*, other domains and functions are also utilized *(26)*.

This finding supports results reported by others that DNA-binding affinity does not directly correlate with activation potential by AR and GR *(7)*. The notion that DNA binding serves to do more than simply tether transcription activation domains to a specific site on DNA is not new *(34,35)*. The DNA topology of the MMTV promoter may affect the conformation of bound receptors in a way that favors the GR NTD in making important protein-protein contacts with the transcription machinery. Alternatively, the GR NTD could interact more efficiently with other factors bound to the promoter or could escape putative negative interactions imposed on the AR domain by MMTV-binding factors *(28,29,36)*. In either case, these and other results demonstrate that there is more than one strategy for creating promoter specificity; rather, promoters and receptors have evolved to take advantage of the manifold differences to derive selectivity.

The GR tau1 core domain, defined as amino acids 187–227 of human GR, has been shown to be responsible for transactivation by the NTD *(37,38)*. Similarly, we have previously mapped two transactivation regions within the AR NTD called AF-1a and AF-1b, which span amino acids 154–167 and 295–359, respectively *(39)* (corresponding to amino acids 173–186 and 297–361 of the AR Mutation Database *[40]*). The amino acid sequence of the GR tau1 and AR AF-1a and AF-1b regions are totally distinct, consistent with our finding that the AR and GR NTDs possess differential activities. Even though these minimal transactivation regions of GR and AR appear unrelated based on amino acid sequence, it is possible that other regions of the GR and AR NTDs may share some sequence homology based on the overall relatedness of these receptors in the DBD and HBD regions.

To test this idea, we took advantage of the numerous GR and AR sequences in the GenBank database that have been obtained from a large number of vertebrate species and searched for any conserved residues between the GR and AR NTDs. We began by building a separate consensus sequence among all species for each of GR tau1 and AR AF-1a and AF-1b. We then attempted to align the GR tau1 consensus sequence with the entire AR NTD and the AR AF-1a and AF-1b consensus sequences with the entire GR NTD. Interestingly, we found a short stretch of amino acids in the GR tau1 core sequence that shares homology with a region in the AR NTD that is outside the AF-1a and AF-1b region (Fig. 7). In the region of similarity, 8 of 15 amino acids are functionally similar, of which 4 amino acids are identical. One of the absolutely conserved amino acids is a tryptophan residue in the first position of the shared sequence that has been shown in GR to be particularly important for

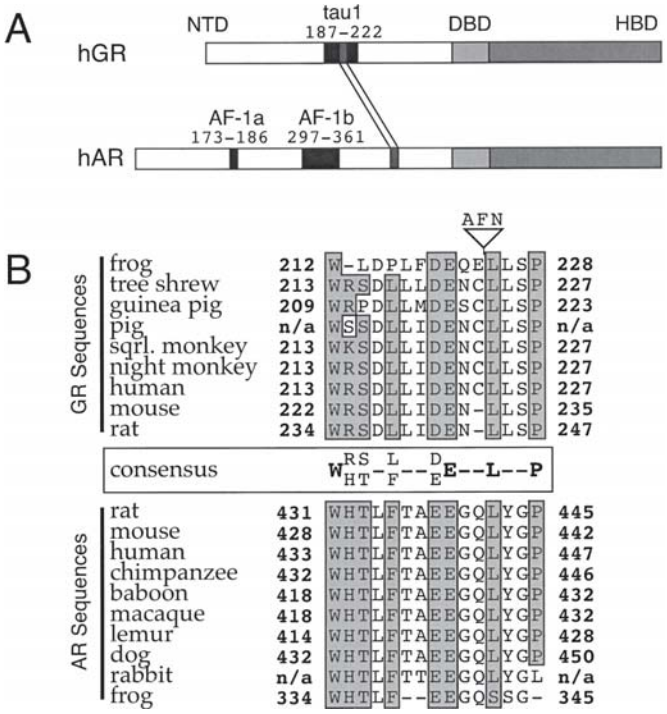

Fig. 7. A highly conserved region of AR is similar to the GR tau1. (**A**) Previously defined regions required for activation are indicated by labeled black boxes, and the relative position of this conserved sequence in GR and AR is shown. Amino acid boundaries for the activation domains are provided for human GR and human AR. The AR amino acid numbers are consistent with those used in the AR Gene Mutation Database curated by Dr. Bruce Gottlieb (http://www.mcgill.ca/androgendb/). (**B**) Amino acid sequence of the conserved GR and AR region in vertebrates. The first and last amino acids of the listed sequence are indicated according to the sequence of the appropriate species. Functionally similar amino acids conserved between both receptors are boxed, and sites of consensus are indicated in bold. Note the three amino acid insertion in frog GR indicated by a flag with the three inserted residues.

the activation function *(41)* and proposed to play a role in binding transcriptional cofactors *(42)*.

The corresponding sequence in AR (rat amino acids 431–445) is part of the TAU-5 region, implicated in transactivation by a constitutively active AR lacking the HBD *(43)*. Despite being identified in an HBD-deleted AR, TAU-5 has been implicated in NTD:HBD interactions and in SRC1 coactivator binding *(44,45)*. Although not as precisely defined, this same region may determine AR specificity in the mouse *Slp* enhancer *(29)*. More recently, the first five amino acids of this conserved stretch in AR, WHTLF, were shown to play a prominent role in the interaction between the AR NTD and HBD *(46)*. We suggest that this region of conservation between the AR and GR NTDs may be indicative of a predecessor sequence that evolved to play different roles.

In addition to observed transactivation differences, we found that GR was a more potent hormone-dependent transrepressor of AP-1 activity than AR. This greater repression potential was mostly dependent on differences in the HBDs of the two receptors. Initially, the ability of GR to transrepress AP-1 activation was shown to require protein-protein contacts between the DBDs of GR and c-Jun *(47)*, but later studies indicated that competition for CBP is more likely the mechanism of cross-repression between AP-1 and GR *(13)*. Furthermore, the interaction between GR and CBP was mapped to the HBD of the receptor *(13)*. The greater AP-1 transrepression potency exhibited by GR could be the result of more efficient interactions between CBP and the GR HBD compared with CBP interactions with AR.

The traditional view of steroid hormone receptors being modular proteins in which each domain acts independently of the others is no longer considered to be accurate. The question now is, How do the domains function together to direct the diverse functions attributed to them? We have presented data that add to the growing story of how the domains of the holoreceptors act cooperatively to affect precise transcriptional regulation in accordance with the physiologic specificity of their cognate hormones. Furthermore, the domains of the receptors do not merely act cooperatively toward this end; rather, in some cases they seem to act synergistically.

Materials and Methods

Construction of Receptor Expression Plasmids and Reporter Genes

Unique restriction sites flanking the DBDs of rat AR and GR were introduced by site-directed mutagenesis (Oligonucleotide Directed In Vitro Mutagenesis System v. 2.1; Amersham). The upstream *Aat*II sites were located at amino acids 533–534 and 431–432, and the downstream *Bss*HII sites were at amino acids 623–624 and 521–522 in AR and GR, respectively (see Fig. 1). For AR, these sites correspond to AR Mutation Database amino acid numbers 550–551 and 640–641 for *Aat*II and *Bss*HII, respectively. All constructs were verified by double-stranded dideoxy-DNA sequencing (Sequenase kit v. 2.0; United States Biochemical) and subcloned into the eukaryotic expression vector pcDNAI/neo (Invitrogen). Additionally, the AR and GR constructs containing the engineered restriction sites were subcloned into p6R *(48)*, used to produce the data in Figs. 3B and 5. The reporter plasmid pMMTV-CAT contains 1.4 kb of the MMTV long terminal repeat upstream of the CAT gene *(31)*. The 5XTRE-CAT reporter gene contains five copies of a consensus AP-1 binding site upstream of a minimal promoter linked to CAT *(12)*. The reporter plasmid pProbTK-CAT contains 369 bp of the probasin promoter *(20)*, which was obtained by polymerase chain reaction (PCR) amplification from the rat genomic DNA of the C1.1 cell line *(21)*, using the primers PBXba (5'-GCGCTCTAGAAGAATGGGACAGGCATTGGG-3') and PBBam (5'-CCGGGGATCCCAGTTGGCAGTTGTACACAG-3'), incorporating terminal *Xba*I and *Bam*HI restriction sites,

respectively. The sequence of this PCR product was confirmed by sequencing and cloned into the *Xba*I and *Bam*HI restriction sites of pBLCAT2 *(49)*. The reporter plasmid pProb-CAT was made similarly, using the PBXba and PBXho (5'-CCGGCTCGAGAGCTCTGTAGGTATCTG-GACC-3') primers, incorporating terminal *Xba*I and *Xho*I restriction sites, respectively, and cloned into the *Xba*I and *Xho*I sites of pBLCAT3 *(49)*.

Cell Culture, Transfections, and CAT Assays

CV-1, COS-7, and C1.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum (HyClone, Logan, UT) or 10% charcoal-stripped, heat-inactivated calf bovine serum. For transient cotransfection assays, 1×10^6 cells were transfected by the calcium phosphate method or by cationic lipid (Avanti Polar Lipids, Alabaster, AL) *(50)*. Transcription activation assays used equimolar receptor expression plasmid (10 μg for AR), 2 μg of reporter plasmid, and 5 μg of β-galactosidase (β-gal) expression plasmid pEQ176 *(48)* for each 10-cm^2 plate. After transfection, DMEM containing 10% charcoal-stripped, heat-inactivated calf bovine serum plus 1 μ*M* DHT, the synthetic glucocorticoid dex, or carrier control was added to the cells. Forty to 48 h after transfection, the cells were harvested and assayed for CAT activity *(31)* following normalization for transfection efficiency using β-gal activity. The percentage of conversion of [^{14}C]chloramphenicol to the acetylated forms was quantitated by a phosphorimager (Molecular Dynamics, Sunnyvale, CA). AP-1 transrepression assays were done in a similar manner except that equimolar receptor plasmid (2 μg for AR-AB/AAA), 2 μg of 5xTRE-CAT, and 60 ng/mL of phorbol 12-myristate 13-acetate (Sigma), a tumor-promoting agent, were added to each culture 16 h prior to harvesting. CAT activity is expressed as percentage of conversion/(β-gal units·hour) of CAT assay. β-Gal units are defined as A410/(milligrams of protein·minute) determined by the β-gal assay, multiplied by the micrograms of protein in the CAT assay.

Gene Sequences and Comparisons

All AR and GR sequences were retrieved from GenBank. AR sequences were as follows: *Canis familiaris* (accession no. AF197950), *Eulemur fulvus collaris* (accession no. U94178) *(51)*, *Homo sapiens* (accession no. M20132) *(52)*, *Macaca fascicularis* (accession no. U94179) *(51)*, *Mus musculus* (accession no. X59592) *(53)*, *Oryctolagus cuniculus* (accession no. U16366) *(54)*, *Pan troglodytes* (accession no. U94177) *(51)*, *Papio hamadryas* (accession no. U94176) *(51)*, *Rattus norvegicus* (accession no. M23264) *(55)*, and *Xenopus laevis* (accession no. U67129) *(56,57)*. GR sequences were as follows: *Aotus nancymaae* (accession no. U87952) *(58)*, *Cavia porcellus* (accession no. L13196) *(59)*, *Homo sapiens* (accession no. M10901) *(60)*, *Mus musculus* (accession no. NM_008173) *(61)*, *Rattus norvegicus* (accession no. M14053) *(62)*, *Saimiri sciureus* (accession no. AF041834), *Sus scrofa* (accession no. AF141371), *Tupaia belangeri* (accession no. Z75079), and *Xenopus laevis* (accession no. X72211) *(63)*. Sequence manipulations and alignments were performed with MacVector (Oxford Molecular Group).

Acknowledgments

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