A natural sequence consisting of overlapping glucocorticoid-responsive elements mediates glucocorticoid, but not androgen, regulation of gene expression

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Cytosolic aspartate aminotransferase (cAspAT) is regulated by glucocorticoids in rat liver and kidney. Part of this regulation is mediated by an unusual glucocorticoid-responsive element (GRE)-like sequence called GRE A. GRE A is composed of two overlapping imperfect GREs, each comprising a conserved half-site (half-sites 1 and 4 respectively) and a poorly conserved half-site (half-sites 2 and 3 respectively). The sequence binds co-operatively two dimers of the glucocorticoid receptor (GR) and mediates efficient glucocorticoid regulation of gene expression. Analysis of deletions of the cAspAT gene promoter and subcloning of GRE A upstream of the thymidine kinase promoter indicate that this sequence is responsive to glucocorticoids, but not to androgens. Electrophoretic mobility shift assays indicate that the GRE A unit does not bind the androgen receptor (AR). The modification of three nucleotides in the poorly conserved half-sites 2 and 3, converting GRE A into two overlapping high-affinity GREs (ov-cGRE), resulted in co-operative binding of the AR. Furthermore, ov-cGRE efficiently mediated androgen regulation of the thymidine kinase promoter. A single base modification in half-site 2 or 3 in GRE A allowed the binding of the AR as one or two dimers respectively, and restored transcriptional activation by androgens only in the latter case. Thus the poor affinity of the AR for half-sites 2 and 3 prevented its binding to GRE A, indicating that the overlapping GRE A sequence of the cAspAT gene promoter discriminates a glucocorticoid-mediated from an androgen-mediated response.

Key words: androgen receptor, glucocorticoid receptor, overlapping GREs, tetramer, transcription.

INTRODUCTION

The glucocorticoid receptor (GR) and the androgen receptor (AR) are members of the steroid/nuclear receptor superfamily [1], which also comprises the progesterone receptor (PR), the mineralocorticoid receptor (MR), the oestrogen receptor (ER), the thyroid receptor and the retinoid receptors. These nuclear receptors are transcription factors that mediate the effects of a variety of hormones. Upon ligand binding, the receptor can activate transcription by interacting with specific DNA sequences located within or in the vicinity of gene promoters.

All members of the first subfamily, which includes the AR, PR, GR and MR, have sequence similarity in their DNA-binding domains [2]. This group of receptors bind tightly to the same consensus partially palindromic sequence, called the glucocorticoid-responsive element (GRE). The GRE is an inverted repeat in which the half-sites are separated by 3 bp: 5′ GGTACA NNN TGTTCT 3′ [3]. In *in vitro* transcription assays, this responsive element is not receptor-specific, which contrasts with the observation that different steroids have different specific effects in different cell types [4]. It therefore remains unclear how a single element can mediate distinct physiological activities of different hormones. Potential mechanisms that could account for these specificities include cell-specific regulation of receptors levels, tissue-specific ligand metabolism, such as the metabolism of cortisol by 11β-hydroxysteroid dehydrogenase [5], receptor-specific interactions with transcription factors [6] and/or specific interactions with variant GRE sequences. However, in the case of many genes that are specifically regulated by a single or a few steroid hormones, this question remains unanswered.

Cytosolic aspartate aminotransferase (cAspAT) is regulated by glucocorticoids in rat liver and kidney [7,8]. We have identified in the promoter of the cAspAT gene an unusual GRE-like sequence (GRE A) composed of two overlapping GREs, each comprising both a highly conserved and a poorly conserved half-site [9] (see Figure 1). This sequence binds co-operatively a tetramer of the GR, which mediates the glucocorticoid regulation of this gene. Modification of the poorly conserved half-sites to increase their affinity for GR gave a DNA sequence that also bound a GR tetramer in a co-operative manner. This unit, consisting of two overlapping consensus GREs (ov-cGRE, formerly called GRE Aup [9]), mediated glucocorticoid induction with a 4-fold higher efficiency than did the consensus GRE (cGRE). Other members of the steroid hormone receptor family, such as the ER, can also form tetramers on an overlapping response element. The interaction of the ER with two overlapping oestrogen-responsive elements was less co-operative than that of the GR with ov-cGRE [10]. This probably results from the ER bending DNA to a greater extent than does the GR [11].

It was shown previously that, in the rat hepatoma cell line Fao, in which the glucocorticoid induction of cAspAT was studied, testosterone was inefficient [7]. However, the AR status of those

Abbreviations used: AR, androgen receptor; cAspAT, cytosolic aspartate aminotransferase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; ER, oestrogen receptor; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; cGRE, consensus GRE; ov-cGRE, unit consisting of two overlapping consensus GREs; MR, mineralocorticoid receptor; PR, progesterone receptor; TK, thymidine kinase.

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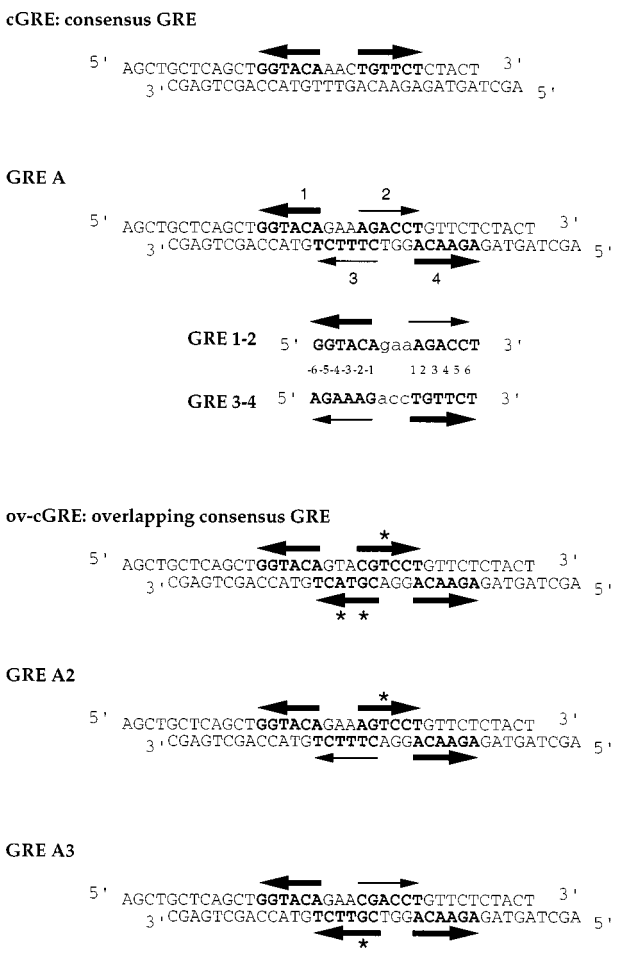

Figure 1 DNA sequences of the various GREs used in the study

Thick arrows indicate conserved half-sites. Thin arrows indicate poorly conserved half-sites. Modified nucleotides are indicated with a star.

cells was not established. In other studies that have been conducted in epithelial rat prostate cells, which are known to respond to androgens, it was shown that cAspAT was not regulated by these hormones, despite the presence of a functional AR [12,13]. Those studies suggested that the hormonal specificity of cAspAT gene regulation could relate to an intrinsic property of the cAspAT gene promoter. The aim of the present study was to determine whether the structure of GRE A accounts for the glucocorticoid-specific regulation of the cAspAT gene. We show that GRE A does not bind the AR. Furthermore, this sequence is not responsive to testosterone in its promoter context or in the context of the heterologous thymidine kinase (TK) promoter because of the low-affinity half-sites in it. Thus GRE A discriminates glucocorticoid from androgen induction, and provides a novel mechanism for the specificity of gene regulation by steroid hormones.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line HepG2 [14] and the monkey kidney cell line Cos7 were maintained in Dulbecco's minimal essential medium supplemented with 10 % (v/v) fetal calf serum (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin (Diamant) and 0.5 μg/ml fungizone (Squibb).

Plasmids

The expression vectors for the human GR and the human AR were gifts from Dr R. Evans (The Sal Institute, La Jolla, CA, U.S.A.) and Dr H. Klocker (University of Innsbruck, Austria) respectively. Deletion constructs of cAspAT were described previously by Aggerbeck et al. [8]. GRE oligonucleotides were subcloned into the *Hin*dIII site of the TK-CAT plasmid (a gift from Dr C. Forest, CNRS, Meudon, France). The double-stranded oligomers (cGRE, ov-cGRE, GRE A, GRE A2 and GRE A3) (Figure 1) have 5′ extensions that are compatible with a *Hin*dIII site. However, the restriction site is lost in the recombinant plasmid. The luciferase plasmid, RSV-Luc, was purchased from Promega.

Transfection experiments

Transfection experiments were performed as described by Massaad et al. [15]. At 1 day before transfection, HepG2 cells (10^6 cells/10 cm dish) were seeded in Dulbecco's minimal essential medium containing 10 % (v/v) fetal calf serum. A portion of 10 ml of fresh medium containing 10 % (v/v) charcoal-treated serum was added to the cells 2–3 h before transfection. The chloramphenicol acetyltransferase (CAT) plasmids (5 μg), the human AR or human GR expression vectors (as indicated), and the luciferase expression vector (1 μg) were introduced into the cells by the calcium phosphate co-precipitation technique. Following a glycerol shock, 10 ml of fresh medium containing 5 % (v/v) charcoal-treated serum was added to the cells. After 16 h, serum-free medium was added and cells were treated with the various hormones or drugs tested. After an additional 24 h incubation, cells were homogenized for CAT and luciferase assays.

Luciferase assay

Luciferase activity was used to normalize transfection efficiency [16] and was assayed according to the manufacturer's instructions (Promega). Briefly, transfected cells were washed twice with 5 ml of calcium- and magnesium-free PBS, and lysed in 500 μl of Reporter Lysis Buffer 1 × (Promega) for 15 min. After centrifugation at 10000 ***g*** for 5 min, 20 μl of the supernatant was mixed with 100 μl of luciferase assay reagent at room temperature. Luciferase activity was measured using a luminometer 30 s after addition of the assay reagent.

CAT assay

CAT activity was determined using the two-phase assay developed by Neumann et al. [17]. Briefly, 60 μl of cellular extract, heated at 65 °C for 10 min, was incubated with 1 mM chloramphenicol, 0.5 mM acetyl-CoA and 0.5 μCi of [^{3}H]acetyl-CoA (NEN product no. NET-290 L) at 37 °C for 30 min. The solution was transferred to a mini-vial and layered with 4 ml of Econofluor (NEN product no. NEF 969). After vigorous mixing, the two phases were allowed to separate for at least 15 min, and the radioactivity was determined by scintillation counting. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not unreacted acetyl-CoA, diffuses into the Econofluor phase. For these experiments, blanks were obtained

by assaying CAT activity in cells that had undergone the same treatment in the absence of a CAT plasmid. Blank values for luciferase and CAT assays were obtained by assaying lysates from non-transfected cells. Transcriptional activation was obtained by determining the ratio of CAT activity over luciferase activity.

Preparation of cell extracts

Cos7 cells were transfected as described by Ishikawa et al. [18]. Briefly, cells were plated at 2×10^6 cells/10 cm dish. After 24 h the cells were washed twice with PBS, and then 500 μl of trypsin solution was added. Cells were incubated for 10 min at room temperature, and then 20 μg of AR expression vector, 400 μg of DEAE-dextran and 0.1 mM chloroquine were added. The cells were incubated for 4 h, followed by a DMSO shock for 2 min. At 1 h before harvesting, testosterone (0.1 μM) was added to the cells. Cells were washed twice with chilled PBS, collected in the binding buffer [20 mM Tris/HCl (pH 7.5), 2 mM dithiothreitol, 20 % (v/v) glycerol and 0.4 M KCl for AR binding assays, or 20 mM Tris/HCl (pH 7.5), 2 mM dithiothreitol, 20 % (v/v) glycerol and 0.55 M KCl for GR binding assays]. Whole-cell extracts were prepared by freezing the cells at -80 °C, thawing them over ice and centrifuging at 10000 ***g*** for 15 min at 0 °C in a Sigma centrifuge. The supernatant was stored at -80 °C.

AR- or GR-enriched cells infected with baculovirus were prepared as described previously [19–21].

Electrophoretic mobility shift assay (EMSA)

GRE oligonucleotides (see Figure 1) were hybridized and ^{32}P-labelled using the Klenow fragment of DNA polymerase I, essentially as described by Cao et al. [22]. Binding reactions were carried out in 20 μl of buffer containing 20 mM Tris/HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA, 10 % (v/v) glycerol, 3 μg/μl BSA, 100 mM NaCl, 0.3 ng of radiolabelled purified DNA probe and 2 μg of dI·dC. AR or GR, in the amounts indicated in the Figure legends, was then added. After incubation at room temperature for 15 min, the reaction mixtures were loaded on a pre-electrophoresed (100 V/12 cm; 30 min) 4.5 % (w/v) acrylamide gel (acrylamide/bisacrylamide, 29:1, w/w) containing 0.25× Tris/borate/EDTA, and electrophoresis was continued for 90 min (200 V/12 cm). Gels were dried and autoradiographed. In order to quantify the retarded complexes, the bands were excised from the gel and radioactivity was counted in a scintillation counter. Alternatively, the densitometric studies were performed using NIH Image software.

RESULTS

Lack of activation of the cAspAT gene promoter by testosterone

We compared the effects of androgens and glucocorticoids on the cAspAT gene promoter. We co-transfected either the GR or AR expression vector into the HepG2 cells along with p-2405/$-$26CAT, a plasmid that contains a 2.4 kb fragment of the cAspAT gene promoter upstream of the CAT gene. As expected, 0.1 μM dexamethasone activated by 9-fold the $-2405/-26$ promoter fragment when co-transfected with 0.05 μg of GR expression vector [8] (Figure 2). In contrast, 0.1 μM testosterone did not or only weakly activated this promoter fragment in the presence of 0.25 μg or 2.5 μg of AR expression vector respectively.

The use of several deletion fragments of the promoter has suggested that glucocorticoids regulate the cAspAT gene promoter through two sites [8,23]. Upon deletion of the distal site ($-1983/-1718$ [8]), part of the glucocorticoid effect was conserved. The proximal effect of glucocorticoids is mediated by the

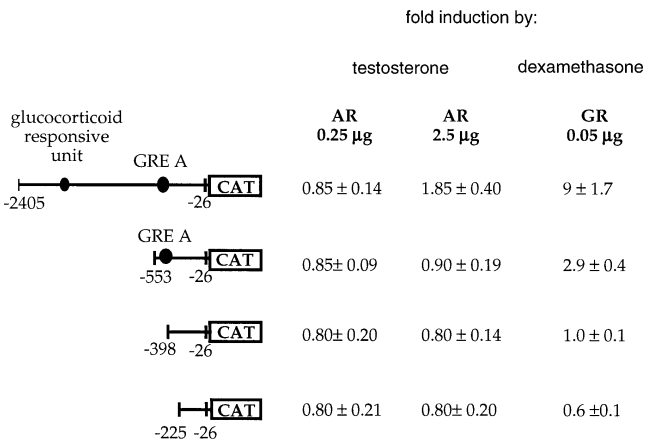

Figure 2 Effects of androgens and glucocorticoids on cAspAT promoter fragments

HepG2 cells were transfected with plasmids containing deletion fragments of the cAspAT gene promoter and with either the GR expression vector (0.05 μg) or the AR expression vector (0.25 μg and 2.5 μg). Testosterone (0.1 μM) or dexamethasone (0.1 μM) was added for 24 h. The fold activation of the promoters by either testosterone in the presence of AR or dexamethasone in the presence of GR was calculated. The results are means ± S.E.M. of four independent experiments.

GRE A-containing sequence (compare the $-553/-26$ and the $-398/-26$ fragments [9]). Interestingly, the $-553/-26$ promoter fragment, which includes GRE A, is also not able to mediate the testosterone response, nor are the other smaller promoter fragments. These results indicate that this GRE A-containing cAspAT gene promoter fragment is regulated by dexamethasone, but not by testosterone, in HepG2 cells.

To determine whether the inability of the AR to activate the $-553/-26$ cAspAT promoter fragment was due to the promoter context or to the intrinsic properties of the GRE A, we subcloned the GRE A upstream of the heterologous TK promoter. The effects of androgens and glucocorticoids on the GRE A–TK and cGRE–TK promoters were compared. As shown in Figure 3(A), 0.1 μM testosterone activated the cGRE–TK promoter as a function of the amount of transfected AR expression vector, in agreement with the cGRE being a known target of the AR. In the case of the GRE A, no increase was observed with 0.5 or 2.5 μg of AR expression vector in the presence of 0.1 μM testosterone. These results suggest that the GRE A is not active as an androgen response element either in the context of the natural cAspAT promoter or when fused to a heterologous promoter. Similar experiments performed using a GR expression vector resulted in activation of both cGRE- and GRE A-containing promoters, by 7- and 5-fold respectively, in the presence of 0.5 μg of transfected GR expression vector and 0.1 μM dexamethasone (Figure 3B).

We next investigated whether the lack of GRE A-mediated transcriptional activation by the AR could be due to the inability of the AR to activate promoters containing overlapping GREs. A sequence consisting of two overlapping cGREs (ov-cGRE, formerly GRE Aup [9]), which is derived from GRE A, was created by transforming half-sites 2 and 3 into high-affinity half-sites (Figure 1). As shown in Figure 3(A), testosterone efficiently activated the ov-cGRE–TK promoter in the presence of 0.5 and 2.5 μg of transfected AR expression vector, and this activation was 3–4-fold greater than that of cGRE–TK, suggesting synergistic activation from two overlapping cGREs. Transcriptional

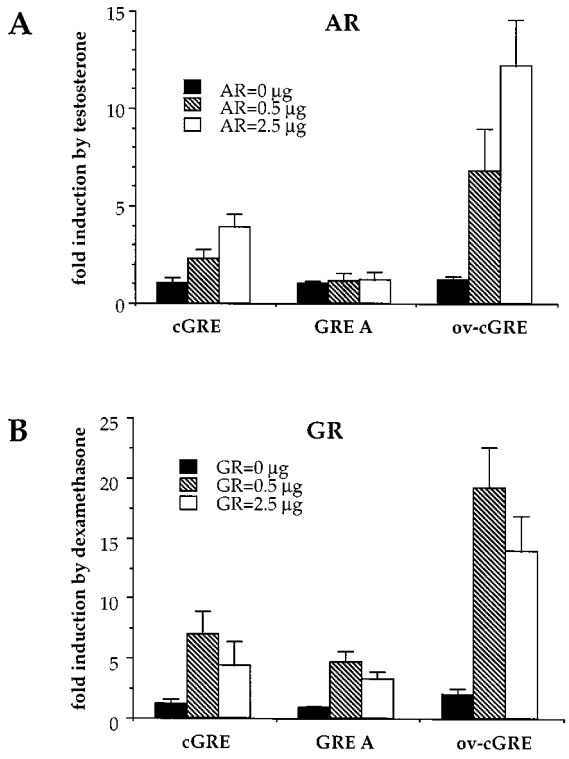

Figure 3 Functionality of GRE A

HepG2 cells were transfected with the indicated amounts of the AR (**A**) or the GR (**B**) expression vector, together with cGRE–TK–CAT, GRE A–TK–CAT or ov-cGRE–TK–CAT. After 24 h, cells were treated with testosterone (0.1 μM) (**A**) or dexamethasone (0.1 μM) (**B**). The fold activation of the promoters by either testosterone in the presence of AR or dexamethasone in the presence of GR was calculated. The results are means $\pm$ S.E.M. of four independent determinations.

activation elicited by the GR on the ov-cGRE–TK–CAT construct was 3 times that with cGRE–TK–CAT (Figure 3B).

Receptor-binding specificity of GRE A

We compared the ability of the AR and the GR to bind to the GRE A using extracts from cells overexpressing the receptors (baculovirus-infected Sf9 cells or transfected Cos7 cells). In order to verify whether the amounts of GR and AR in Sf9 cell extracts were comparable, Scatchard analysis was carried out using the cGRE probe and EMSA (results not shown). We determined the amount of functional receptors in each extract, and we found that, under the experimental conditions used, the affinity of the GR for the cGRE oligonucleotide was similar to that of the AR (K_d approx. 2 nM; two independent experiments). We next compared the ability of the AR and the GR to bind to the GRE A, cGRE and ov-cGRE probes using these extracts (approx. 12 fmol of each receptor in each assay mixture). In the case of cGRE, a band of similar intensity was observed with both the GR and the AR, corresponding to dimeric GR–GRE and AR–GRE complexes respectively (Figure 4, lanes 2 and 5). In the case of GRE A, a slower-migrating band was observed with the GR, which corresponded to the tetrameric GR–GRE A complex reported previously by Garlatti et al. [9] (Figure 4, lane 1). In contrast, no complex was observed with the AR (Figure 4, lane 4). In additional experiments, we were unable to observe any complex-formation between GRE A and the AR, even when the amounts of the AR and of the GRE A probe were increased by

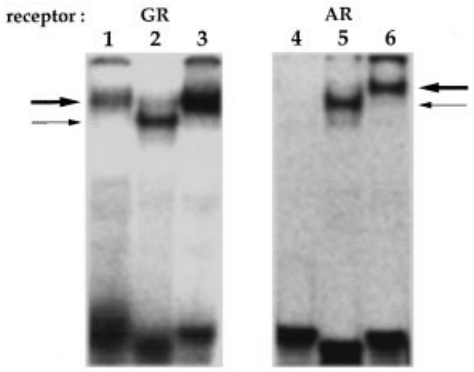

Figure 4 Receptor-binding specificity of GRE A

EMSA experiments were performed by incubating Sf9 extracts containing approx. 12 fmol of GR (lanes 1–3) or AR (lanes 4–6) with 0.2 ng (50000 c.p.m.) of radiolabelled GRE A (lanes 1 and 4), cGRE (lanes 2 and 5) or ov-cGRE (lanes 3 and 6) probe, and the complexes were revealed by EMSA. The upper thick arrow indicates the putative tetrameric complex, and the lower thin arrow indicates the dimeric complex. This experiment was repeated three times with essentially identical results.

up to 10-fold (results not shown). The inability of the AR to bind GRE A is likely to account for the lack of regulation of the cAspAT gene by testosterone. In the case of ov-cGRE, slow-migrating complexes were observed with both the GR (lane 3) and the AR (lane 6), and could correspond to tetrameric receptor–DNA complexes. Similar results were obtained with all these probes using AR-enriched extracts from Cos7-transfected cells (see Figure 5). These data indicate that the sequence of GRE A, rather than the presence of overlapping GREs, prohibits AR binding, since the AR as well as the GR could form a tetrameric complex with consensus overlapping sequences.

DNA sequence requirement for AR DNA binding

The GRE A consists of two overlapping GREs, GRE 1–2 and GRE 3–4 (Figure 1). Each GRE consists of a conserved half-site (1 and 4) and a poorly conserved half-site (2 and 3). In the case of the GR, each GRE is inactive, but the ability of the GR to bind GRE A stems from co-operative binding of GR dimers to overlapping elements [9].

In binding assays, the AR present in extracts from baculovirus-infected cells (Figure 4) or in Cos7-transfected cells (Figure 5) was not able to bind the GRE A significantly. In contrast, the receptor present in either one of the two extracts bound the ov-cGRE sequence and formed an abundant slow-migrating complex that could correspond to an AR tetramer. Only a small amount of the dimer complex was formed, even at low receptor concentrations, suggesting strong co-operative binding. To investigate further the contribution of each half-site, GRE A2 and GRE A3 were synthesized, in which the sequences of GRE A half-sites 2 and 3 respectively were modified and made as close as possible to the consensus sequence (Figure 1). GRE A2 bound predominantly an AR dimer at high AR concentration, while GRE A3 formed predominantly a tetrameric complex (Figure 5). We conclude that AR dimers can bind co-operatively to overlapping response elements provided that the half-sites are of sufficient high affinity. In the case of the GRE A, the sequences of half-sites 2 and 3 account for the inability of the AR to bind and transactivate.

We have tested the functionality of these constructs in transient transfection experiments. HepG2 cells were transiently trans-

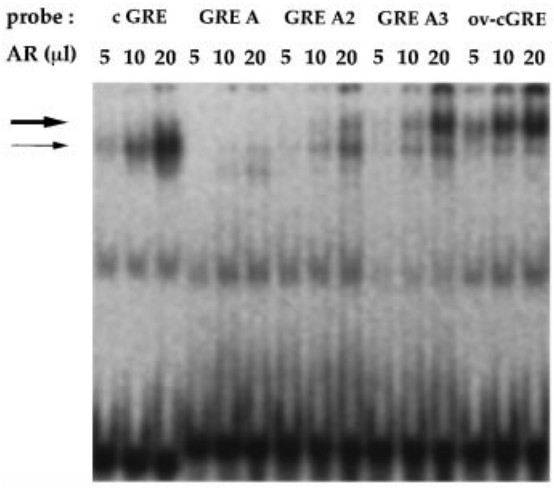

Figure 5 Effects of mutations in the poorly conserved half-sites of GRE A on AR binding

EMSAs were performed with increasing amounts of AR-enriched Cos7 extracts (5 μl, 10 μl and 20 μl) and with the GRE A, cGRE, GRE A2, GRE A3 or ov-cGRE probe (see Figure 1 for sequences). The upper thick arrow indicates the putative tetrameric form of the AR, and the lower thin arrow indicates the dimeric complex. The experiment was repeated three times with identical results.

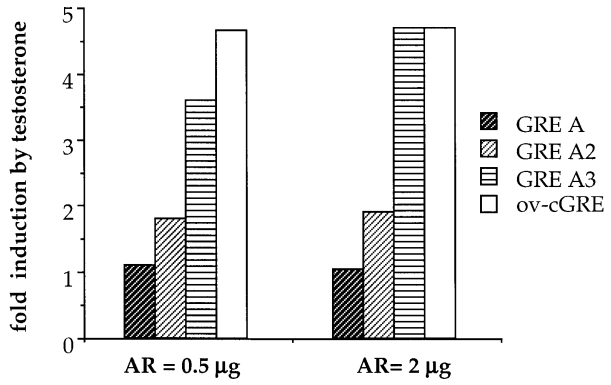

Figure 6 Functionality of the GRE A2 and GRE A3 sequences

HepG2 cells were transiently transfected with increasing amounts of AR expression vector (0.5 and 2 μg) and with one of the following plasmids: GRE A–TK–CAT, GRE A2–TK–CAT, GRE A3–TK–CAT or ov-cGRE–TK–CAT. After 24 h, cells were treated with testosterone (0.1 μM) for an additional 24 h. These results are the means of results from two independent experiments that differed by less than 20%.

fected with increasing amounts of AR expression vector (0.5 and 2 μg) and with one of the following plasmids: GRE A–TK–CAT, GRE A2–TK–CAT, GRE A3–TK–CAT and ov-cGRE–TK–CAT. As shown in Figure 6, testosterone (0.1 μM) did not activate transcription of the CAT gene from the GRE A plasmid, while it poorly activated (2-fold) transcription from the GRE A2-containing promoter. The GRE A3 construct was active in mediating transcription in the presence of 0.5 μg of transfected AR expression vector. Furthermore, the GRE A3–TK and ov-cGRE–TK constructs both activated by 5-fold the transcription of the CAT gene in the presence of 2 μg of transfected AR expression vector. The results observed in transfection experiments are in accordance with those of the gel-retardation assays. Similar experiments were performed using the various promoters and various concentrations of a GR expression vector. In this case all promoters were responsive to dexamethasone addition; GRE A–TK was the least sensitive, while both GRE A2–TK and GRE A3–TK were as efficient as the ov-cGRE–TK promoter (results not shown).

DISCUSSION

Members of the subfamily of steroid hormone receptors, which includes the GR, MR, PR and AR, bind to the same consensus response element, the GRE, and yet tissue- and cell-specific effects are observed *in vivo*. This is probably accounted for, in part, by differential tissue-specific expression of the steroid receptors. However, in cell types that co-express multiple receptors of the subfamily, the molecular mechanisms underlying these specific steroid responses are of particular interest. Such specificity could be explained by regulatory units containing not only GREs, but also other transcription factor binding sites that display selective interactions. It could also be due to differential binding of these receptors to divergent GREs, as shown in the present study.

Androgen response elements present in natural promoters can be classified into two groups. The first consists of GRE-like sequences that bind GRs, PRs, MRs and ARs, and includes the MMTV (murine mammary tumour virus) 5′ GRE, the C3-A and C3-B sites of the C3 subunit of the rat prostatein gene, and the GRE in the human growth hormone gene promoter [24]. A second class consists of sequences that discriminate GR and AR binding and transactivation. The probasin element ARE-2 displays a higher affinity for the AR than for the GR [25] because the left half-site (5′-GGTTCT-3′) excludes GR binding. Few other sequences discriminate androgen from glucocorticoid effects *in vitro*. Binding-site selection from a pool of degenerate double-stranded oligonucleotides identified GREs with increased AR or GR specificity. In one study, oligonucleotides formed of overlapping direct repeats (DR-1) or a complex arrangement of repeats (two half-sites are overlapping direct repeats and one half-site is inverted) were shown to bind exclusively the AR, but not the GR [26]. The mouse sex-limited protein (*Slp*) gene is activated exclusively by androgens, but not by glucocorticoids, in transient expression studies. This discrimination is due to combinatorial functions of receptor and non-receptor binding site sequences [27,28]. Recently, Scheller et al. [29] reported that the differential action of glucocorticoids and androgens on *Slp* regulation was also due to intrinsic properties of the AR. In this case, interaction of the N-terminal subdomain and the ligand-binding domain contribute to the androgen-specific gene activation. In other studies, it was shown that these interactions could be influenced by nuclear receptor co-activators [30,31]. It is also possible that the activity of the AR could be modulated by interference with other transcription factors, such as RelA [32].

Little is known about natural sequences displaying regulation by glucocorticoids but not by androgens. In the present report we show that the cAspAT promoter is regulated by dexamethasone, but not by testosterone, even in the presence of the AR. This regulation is mediated by two sites: one in the distal region of the cAspAT gene promoter and the second in the proximal region of the promoter. The second site, GRE A, is composed of two imperfect overlapping GREs [9] which, when subcloned in a heterologous promoter, mediates a glucocorticoid response but not an androgen response. This was not due to the inability of the AR to mediate transcriptional regulation from overlapping GREs, since the ov-cGRE unit, which comprises two overlapping high-affinity GREs, elicited synergistic activation of transcription by both glucocorticoids and androgens.

The lack of binding of the AR to GRE A may have resulted from its inability to form a tetramer, due to steric hindrance. In fact, the AR is a larger protein than the GR (919 and 777 amino acids respectively). Thus binding of the first AR dimer may prevent binding of a second AR dimer on the opposite side of the DNA double helix. This hypothesis is unlikely, however, because the ov-cGRE unit binds co-operatively either two dimers or a tetramer of the AR. Even at low AR concentrations, ov-cGRE forms a single low-mobility complex with AR, suggesting AR co-operative binding.

A second possibility for the inability of the AR to bind GRE A is a low binding affinity. GRE A is formed of two GREs: GRE 1–2 (5′-GGTACA gaa AGACCT-3′) and GRE 3–4 (5′-AGAAAG acc TGTTCT-3′). Zhou et al. [26], using *in vitro* selection, and Marschke et al. [33] have shown that the half-site 5′-TGTTCT-3′ is required for AR binding. This motif is not conserved in GRE 1–2, which probably accounts for the lack of detectable AR binding. Changing A to T in position 3 of GRE 1–2 (GRE A2; Figure 1) allows the binding of the AR predominantly as a dimer. One difference between this modified sequence and cGRE is the presence of an A in position 1. It should be noted, however, that the probasin ARE2 also has an A at this position, and is able to bind the AR efficiently.

The 5′-TGTTCT-3′ motif is present in GRE 3–4, but the left half-site of GRE 3–4 is not conserved (5′-AGAAAG-3′), which probably excludes AR binding. Modification of a single base (A to C at position −3) in this half-site results in AR binding as two dimers or a tetramer (GRE A3), although the binding was less co-operative than with the conserved ov-cGRE. It is noteworthy that half-site 3 in GRE A3 (5′-AGAACG-3′) is similar to one half-site of the C3-C-responsive element present in the prostatein C3 subunit gene, which exhibits similar AR and GR binding. Thus the presence of a C at position −3 is important for AR binding. Although both the A2 and A3 modifications improved the binding of the AR, they resulted in different binding patterns. In the case of GRE A3, a low-mobility complex migrating as a tetramer was observed, which was not the case with GRE A2. While these observations suggest a critical effect of the modification of half-site 3, other points have to be taken into consideration. First, the A3 modification yields a sequence that is closer to the consensus than does the A2 modification. Secondly, the modification of a strategic position in half-site 3 (−2) resulted in a modification of half-site 2 at position +1. Although unlikely, the latter modification could contribute to the increased affinity of the sequence for the AR and to the formation of a tetramer. The data are best accounted for by the following model: in GRE A, the sequence of half-site 3 prevents AR binding. Once this sequence is modified by the A3 mutation, the binding of the AR dimer to GRE 3–4 facilitates the binding of a second dimer on GRE 1–2, probably through a co-operative interaction.

In conclusion, we have shown that the imperfect GRE overlapping sequences of GRE A, present in the cAspAT gene promoter, discriminate glucocorticoid and androgen effects. However, the co-operative binding of the AR to overlapping GREs required more conserved response elements. This could be an intrinsic property of the AR. Amero et al. [34] and Laudet et al. [35,36] have demonstrated that the AR is the most divergent member of this subgroup of nuclear receptors, which includes the GR, the PR, the MR and the AR. Within the 75 amino acids that form the DNA-binding domain, 17 residues differ between the AR and the GR, whereas 10 of these residues are conserved between the MR, GR and PR. This divergence between the AR and the GR could explain their differential binding to sequences derived from GRE A.

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