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Identification of Androgen Response Elements in the Insulin-Like Growth Factor I Upstream Promoter

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Testosterone stimulates the expression of IGF-I in cells and tissues that include prostate, muscle and muscle satellite cells, and the uterus. Here, the molecular mechanisms of this effect of testosterone were explored. Testosterone increased IGF-I mRNA levels in HepG2 and LNCaP cells and stimulated the activity of reporter genes controlled by 1.6 kb of the upstream promoter of the human IGF-I gene. An androgen-responsive region that was located between −1320 and −1420 bases upstream of the first codon was identified by truncation studies. The androgen-responsive region was found to contain two sequences resembling known androgen receptor (AR)-binding sites from the Pem1 gene. Reporter genes incorporating these sequences were strongly stimulated by androgens. Each of the androgen-responsive elements (AREs) bound recombinant AR-DNA-binding domain in gel-shift experiments; binding was greatly enhanced by sequences flanking the apparent AR-binding half-sites. Testosterone induced recruitment of AR to sequences of genomic DNA containing these AREs. The two AREs were activated 5-fold more by AR than glucocorticoid receptor. Collectively, these findings indicate the presence of two AREs within the IGF-I upstream promoter that act in *cis* to activate IGF-I expression. These AREs seem likely to contribute to the up-regulation of the IGF-I gene in prostate tissues, HepG2 cells, and potentially other tissues. (*Endocrinology* 148: 2984–2993, 2007)

IGF-I IS A PLURIPOTENT trophic factor for many cells and tissues. The major source of IGF-I in plasma is the liver, which releases this hormone in response to GH. IGF-I is also released by nonhepatic tissues, including muscle, muscle satellite cells, and cultured prostate cancer cells. Locally released IGF-I is believed to result in autocrine and paracrine control of tissue function. Several types of evidence suggest that IGF-I levels are also affected by testosterone. Testosterone administration has been reported to increase IGF-I levels in men (1). Levels of IGF-I mRNA are also increased by testosterone in skeletal muscle (2–4), muscle satellite cells (5), uterine tissue (6), and prostate stromal cells and cancer cell lines (7, 8). The molecular mechanisms by which testosterone increases IGF-I expression in these cells and tissues are not known.

Testosterone acts by binding to and activating the androgen receptor (AR), a member of the steroid hormone receptor subfamily of nuclear receptors that also includes receptors for glucocorticoids (GR), mineralocorticoids (MR) and progesterone (PR) (9, 10). Steroid hormone receptors are ligand-activated transcription factors that contain an N-terminal transactivating domain, a highly conserved DNA-binding domain (DBD), and a C-terminal ligand-binding domain. Activation of gene expression by steroid hormone receptors generally involves binding of receptor homodimers to specific, *cis*-acting DNA sequences. The DBD of the AR, MR, PR, and GR is highly conserved and contains two zinc-binding modules. The first has the primary role in DNA binding, whereas the second has been suggested to have dual functions in DNA binding and in stabilization of receptor homodimers (11).

Steroid hormone receptors bind a consensus steroid hormone response element (SRE) (GGAACA nnn TGTTCT) consisting of two receptor-binding half-sites organized as inverted 6-bp repeats separated by three nucleotides (12). Although AR, GR, PR, and MR all bind such consensus sequences, naturally occurring binding sites for these receptors often diverge from the consensus sequence, providing one mechanism for specificity of receptor action at target genes (13–15). Androgen response elements (AREs) may consist of classical inverted repeats, but specificity for androgens has been associated with AREs that include direct repeats (16) or several contiguous AR-binding sites, each composed of sequences based on the consensus SRE (17). Similarly, features of glucocorticoid response elements that minimize activation by androgens include binding sites with overlapping sequences for GR half-sites (15). Specificity of binding of SREs to specific steroid hormone receptors may be further enhanced by interactions of sequences flanking the SRE with the second zinc finger of the DBD or the hinge region separating the DBD and ligand-binding domain (11, 18).

Mechanisms by which testosterone or its analogs increase IGF-I expression have not been examined. The IGF-I gene is under a complex system of transcriptional control in which two alternative promoters exist, one in exon 1 with the other

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Abbreviations: AR, Androgen receptor; ARE, androgen response element; CDS-FBS, charcoal-dextran-stripped FBS; DBD, DNA-binding domain; FBS, fetal bovine serum; GR, glucocorticoid receptor; GST, glutathione-S-transferase; MR, mineralocorticoid receptor; PR, progesterone receptor; SRE, steroid hormone response element.

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being in exon 2. These promoters are used in a tissue-specific manner (19). Both promoters seem to be used in liver and kidney, whereas only that in the first exon is employed in most other tissues. The exon 1 promoter has four transcriptional start sites, with the third being predominately used (19). Here, we tested the hypothesis that the androgen responsiveness of the IGF-I gene is attributable to AREs in the proximal promoter of this gene.

Materials and Methods

Plasmids

pREP4.hAR, expressing full-length human AR, and pCMV.Sport.β-Gal, expressing β-galactosidase under a CMV promoter, were as described (20). pcDNA5.rGR, expressing rat GR, and pSP72hAR-1, containing an insert of the full-length cDNA clone of the human AR, were gifts from Dr. Diane Robins (University of Michigan). A vector expressing rat AR with a C562G mutation in the DBD (rARC562G) leading to loss of DNA binding (21, 22) was provided by Dr. Jorma Palvimo (University of Helsinki). The IGF-I reporter genes pOLuc.1630, pOLuc1300, and pOLuc926 (23) were generously provided by Dr. Peter Rotwein (Oregon Health and Science University). These reporters expressed firefly luciferase under the control of 926-1630 bases upstream of the transcriptional start site of the human IGF-I promoter. pGEX 4T-1 hAR2, expressing glutathione-S-transferase (GST) fused to the N terminus of AR-DBD (24), was a generous gift from Dr. Robert J. Matusik (Vanderbilt University). The pBabe.puro retroviral backbone (25) with a *Not*1 site introduced into the polylinker was a generous gift of Dr. Robert Kraus (Mount Sinai School of Medicine). pRL-CMV expressing Renilla luciferase was from Promega (Madison, WI).

Cell culture

Cell lines were from the American Tissue Type Collection (Bethesda, MD). LNCaP cells were maintained at 37 C in humidified air supplemented with 5% carbon dioxide using RPMI 1640, 10% fetal bovine serum (FBS), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). HepG2 cells were maintained in humidified air containing 10% carbon dioxide using DMEM supplemented with 10% FBS and antibiotics.

Effect of testosterone on IGF-I mRNA levels

Incubations of cells and extraction of RNA. Cells were seeded into wells of six-well plates at 3×10^6 cells per well in medium supplemented with 10% charcoal-dextran-stripped FBS (CDS-FBS) and incubated overnight. Steroid hormones or ethanol were added, and cells were incubated overnight. For these experiments, and all others described below, solutions of testosterone were prepared freshly on the day of the experiment because preliminary studies indicated that this hormone began to lose activity in ethanol within 24–48 h. Total RNA was extracted from cultured cells using RNeasy columns (QIAGEN, Valencia, CA) after disruption of cells with QIAshredder columns (QIAGEN), freed of residual DNA by digestion on the column with RNase-free DNase (QIAGEN), and eluted with water.

Real-time PCR. RNA was quantified by absorbance at 260 nm. One microgram of total RNA was used to prepare cDNA libraries using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μl. Libraries were diluted 25-fold with water. Real-time PCR was performed with 12.5-μl reactions using TaqMan 2× PCR buffer (Applied Biosystems) and an Applied Biosystems 7500 thermocycler following the manufacturer's recommended procedures. Data were normalized relative to that for 18S RNA. TaqMan Assay on Demand probes (Applied Biosystems) were used for all assays. All determinations were performed in triplicate. Mean values for crossing points for the replicates were used in subsequent calculations. Levels of IGF-I mRNA were expressed relative to those in vehicle-treated cells using the $2^{-\Delta\Delta Ct}$ method (26).

HepG2 cells stably expressing AR

The retroviral backbone expressing human AR (pBabe hAR) was constructed by ligating the *Bgl*II-*Xba*I fragment of pSP72hAR-1, including the complete coding region for the human AR, into the *Bam*HI and *Hin*dIII sites of the pBabe puro vector (pBabe.puro.hAR). This construct was cotransfected together with vectors expressing gag-pol and VSV-G into 293T cells. The supernatants were harvested, and the cell debris was removed by centrifugation at $500 \times g$. The supernatant was used to infect HepG2 cells after addition of polybrene (4 μg/ml final concentration). After 24 h, puromycin (1000 ng/ml) was added to the culture medium and was present thereafter during culture and propagation of these cells. Pooled HepG2.AR cells were used for experiments after 1 wk of drug selection.

Luciferase assays

Cells were seeded at 5×10^4 cells per well into wells of 24-well plates containing media supplemented with 10% CDS-FBS. The next day, cells were transfected with a total of 300 ng DNA per well using Lipofectamine Plus (Invitrogen, Carlsbad, CA) with plasmids as indicated in the figure legends. Three hours after transfection, cells were covered with media supplemented with 10% CDS-FBS and either hormone dissolved in ethanol, or ethanol, as indicated in the figures. After overnight incubation, activities of firefly and Renilla luciferase were determined using the Dual Luciferase Assay system (Promega).

Construction of reporter genes

Reporter genes expressing firefly luciferase under the control of upstream regions of the IGF-I promoter were constructed by PCR amplification of segments of interest using primers shown in Table 1, using pOLuc-1630 as a template. PCR amplification was conducted using a high-fidelity *Taq* polymerase (Pfx; Invitrogen). PCR products were cloned into pCR2.1-TOPO TA (Invitrogen), and the sequences of the inserts were verified. Inserts were excised with *Kpn*I and *Xho*I and were subcloned into pGL3-Promoter (Promega) at these same sites. pGL3-Promoter expresses firefly luciferase under the control of a minimal promoter.

GST-hAR-DBD expression and purification

BL21:Des were transformed with pGEX 4T-1 hAR2, which encodes a GST-AR-DBD fusion protein under control of a Lac operon. A single colony was picked and grown at 37 C overnight in LB broth (5 ml) and then diluted 1:10 into 50 ml of this broth and grown 1 h at 37 C. Isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM. Cells were incubated for 2 h at 20 C, collected by centrifugation ($5000 \times g$ for10 min at 4 C), and frozen. Recombinant GST-AR-DBD was liberated using B-PER Bacterial Protein Extraction Reagent (Pierce,

TABLE 1. Primers used for amplification of regions of the human IGF-1 upstream promoter

Amplicon	Construct	Forward primer (5′–3′)	Reverse primer (5′–3′)
1	pGL3–13a	catagtgcaccattgacacaacat	tgagtctttctgtgtggttaatacattg
2	pGL3–210	ggtaccccaaagcctctcatgaca	ggtcaagtcccttcaggggctttcagc
3	pGL3–22	cacatgcccatcatatgactgtgaag	gaaagaggaataagatatggtcaagtc
4	pGL3–230	ggtaccccaaagcctctcatgacac	gaaagaggaataagatatggtcaagtc
5	pGL3–25a	catagtgcaccattgacacaacat	tgagtctttctgtgtggttaatacattg
6	pGL3–32	gggcacatagtagagctcacaaaatg	tgagtctttctgtgtggttaatacattg

The location of each amplified fragment is relative to the transcriptional start site.

Rockford, IL) following procedures recommended by the manufacturer. The supernatant obtained after centrifugation (15,000 × *g* for 10 min) was incubated (20 C for 30 min) with 1 ml 50% (wt/vol) glutathione-agarose beads with gentle mixing. The beads were washed three times with 10 ml ice-cold PBS. The fusion proteins were eluted by suspending the beads in 0.5 ml 50 mM Tris-HCl (pH 8.0) containing 15 mM reduced glutathione followed by incubation at 20 C for 4 min followed by centrifugation. Protein was stored at 4 C until use.

EMSAs

Oligonucleotides were NF-kB, 5′-AGTTGAGGGGACTTTC-3′ and 5′-GCCTGGGAAAGTCCCC-3′ (27); Oct-1, 5′-TGTCGAATGCAAATCACT-3′ and 5′-TTCTAGTGATTTGCATTC-3′ (28, 29); and SRE, 5′-ATGCATTGGGTACATCTTGTTCACATAGACA-3′ and 5′-TGTCAACAAGATGTACCCA-3′ (30). Sequences of all other probes used are shown in Fig. 7. Synthetic sense and antisense strands were annealed and then labeled with [γ^{32}P]dCTP by filling in the ends using the Klenow fragment of *Escherichia coli* DNA polymerase (New England Biolabs, Ipswich, MA) in the presence of dNTPs at 37 C for 30 min. Probes were purified using ProbeQuant G-50 Micro Columns (Amersham Biosciences, Piscataway, NJ) and eluted from the columns with water. Protein-DNA complexes were formed by incubation at 4 C for 30 min of recombinant GST-AR-DBD (16 μg) with 1 pmol ^{32}P-labeled probe and 2 μg poly-dI-dC in binding buffer (5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5). Some reactions were supplemented with unlabeled SRE (1, 2, 4, or 6 pmol). Complexes were resolved by nondenaturing electrophoresis for 10 min at 350 V with cooling on 5% polyacrylamide minigels containing 0.25× Tris-borate EDTA and visualized by phosphorimaging. Densitometry scanning was performed using Imagequant TL (GE Healthcare, Fairfield, CT). For each labeled probe, densities of bands in the presence of a competing SRE probe were normalized relative to densities for bands in the absence of competitor. The concentration of SRE causing a 50% reduction in binding of ARE1 or ARE2 was calculated by fitting the data to the curve for a one-site competition model using Prism 4.0b (GraphPad Software, San Diego, CA).

Chromatin immunoprecipitation

Hep G2 or LNCaP cells (2×10^7) were seeded into 150-mm dishes and incubated until confluent. Medium was replaced with fresh medium (RPMI or DMEM for LNCaP and HepG2, respectively) supplemented with 10% CDS-FBS and either 50 nM testosterone or ethanol. Cells were incubated overnight followed by chromatin immunoprecipitation using a commercially available kit (Upstate, Charlottesville, VA) according to the manufacturer's instructions. Immunoprecipitation was conducted with rabbit polyclonal anti-AR antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) or with normal rabbit serum. Immunoprecipitated DNA was eluted with high salt followed by detection of sequences containing ARE1 and ARE2 using primers employed to amplify sequences in pGL3–32 (Table 1).

Statistics

Data are expressed as mean values ± SEM. Comparisons between two means were performed using a Student's *t* test. Comparisons among multiple means were performed using one-way ANOVA with *post hoc* testing by the Newman-Keuls test. The significance of differences between values obtained for two fitted curves was determined using an *F* test. Where data were fitted to more than two curves, the significance of differences between pairs of mean values was determined by one-way ANOVA followed by the Newman-Keuls test. Statistical calculations were performed using Prism 4.0b.

Results

To confirm that testosterone increased IGF-I mRNA levels, effects of overnight incubation with testosterone on mRNA levels were determined for LNCaP and HepG2 cells. LNCaP cells constitutively express AR at levels sufficient to modulate gene expression. Although HepG2 cells are reported to express AR (31), in preliminary experiments, the level of expression was found to be below that needed to achieve testosterone-induced induction of reporters with known AREs such as MMTV-Luc. To permit us to assess the effects of testosterone on IGF-I mRNA levels in HepG2 cells, a retrovirally transduced version of these cells that stably express human AR was used (HepG2.AR cells). Levels of IGF-I mRNA were increased significantly by testosterone in both LNCaP (Fig. 1A) and HepG2.AR (Fig. 1B) cells. The possibility that testosterone increased transcription of the IGF-I gene was tested by determining whether testosterone altered the expression of a reporter gene expressing firefly luciferase under the control of 1.6 kb of the proximal promoter of the human IGF-I gene (pOLuc.1630). Testosterone significantly increased reporter gene activity in both cell types (Fig. 1, C and D). To establish whether the AR was necessary for testosterone-induced increases in reporter gene activity, HepG2 cells were cotransfected with pOLuc.1630 and either a vector expressing the human AR or one expressing β-galactosidase and then incubated overnight with testosterone or vehicle and assayed for luciferase expression. Testosterone had no effect on luciferase expression in cells cotransfected with β-galactosidase but caused a significant increase in expression in cells expressing AR (Fig. 1E).

These data indicate the presence of AR-dependent androgen-responsive sequences within the first 1.6 kb of the IGF-I upstream promoter. To localize the androgen-responsive sequences, this analysis was repeated using HepG2 cells and a series of reporter genes with 5′ truncations that removed between 300 and 900 bases from the 5′ end of the original 1.6-kb insert (Fig. 2). Androgen responsiveness was lost completely with removal of 300 bases from the 5′ terminus of the insert, indicating that the AREs are localized to between −1300 and −1600 bases upstream of the first codon of the IGF-I gene.

Inspection of the sequence of the androgen-responsive region of the IGF-I gene revealed two regions (ARE1 and ARE2, *bold* in Fig. 3) with similarities to the AREs of the Pem homeobox gene (30). To explore the possibility that these sequences represent authentic AREs, additional reporter genes were constructed by ligating sequences containing ARE1, ARE2, or both into pGL3-promoter, a reporter construct expressing firefly luciferase under the control of a minimal promoter (Fig. 4). HepG2 cells were then transfected with these reporter genes together with AR, and effects of incubation with testosterone on reporter gene activity were determined. Testosterone led to a more than 5-fold increase in expression of luciferase by reporter genes with inserts that included upstream sequences and ARE1 (pGL3–230 and pGL3–22 in Fig. 4). Testosterone responsiveness of a reporter gene with an insert containing only sequences upstream of ARE1 (pGL3–210) was reduced compared with that for pGL3–22 or pGL-230 (Fig. 4). Testosterone increased luciferase expression by more than 20-fold for the construct containing ARE2 and downstream sequences (pGL3–13A in Fig. 4), whereas little androgen responsiveness was observed with a reporter under control of sequences beginning just downstream of ARE2 (pGL3–25a in Fig. 4). The greatest induction was observed when cells were transfected with a reporter containing both ARE1 and ARE2 (pGL3–32 in Fig.

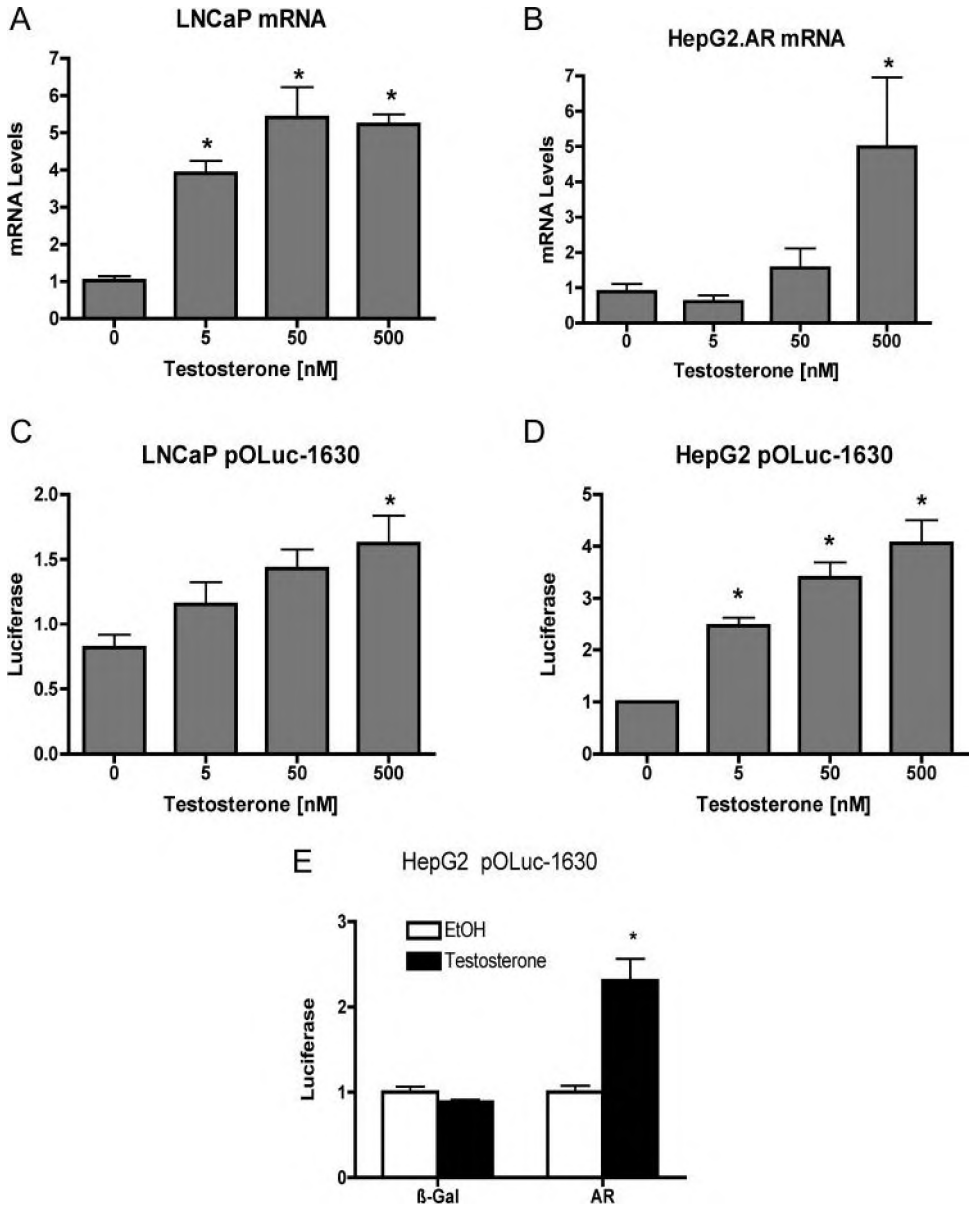

FIG. 1. The human IGF-I gene is androgen responsive. A, LNCaP cells were incubated overnight with testosterone at the concentrations indicated, or with vehicle, followed by quantitation of IGF-I mRNA. Data are from two experiments performed in duplicate. *, $P < 0.05$ *vs.* vehicle. B, HepG2.AR cells were incubated overnight with testosterone or vehicle. Levels of IGF-I mRNA were then quantified. Data are from two experiments, each performed in duplicate. C, LNCaP cells were cotransfected with pOLuc.1630 and pCMV-Renilla and then incubated overnight with testosterone or vehicle. Data are from three experiments with six replicates each. *, $P < 0.05$ *vs.* vehicle. D, HepG2 cells were cotransfected with pOLuc.1630, pCMV.Renilla, and pREP4.AR and then incubated overnight with testosterone or vehicle. Data are from four experiments, each with six replicates. E, HepG2 cells were cotransfected with pOLuc-1630, pCMV-Renilla, and vectors expressing either β-galactosidase or AR. Cells were incubated overnight with testosterone (50 nM) or vehicle. Data are from at least three experiments, each performed in triplicate. *, $P < 0.05$ *vs.* vehicle. EtOH, Ethanol.

4). These data are consistent with the presence of two androgen-responsive sequences located between −1420 and −1320 upstream of the first codon of the IGF-I gene.

The greater testosterone responsiveness of constructs containing both AREs, as opposed to either one alone, suggested the possibility of interactions between the two elements. To

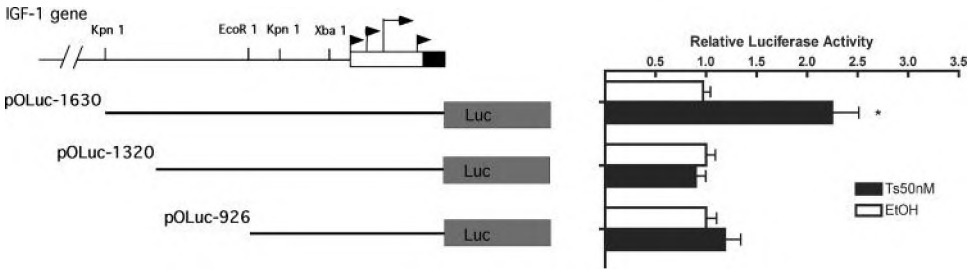

FIG. 2. Androgen responsiveness is ablated by 5′ truncation. *Left*, Map of the IGF-I gene and constructs used. *Arrows* indicate the approximate location of the four transcriptional start sites within exon 1, whereas the *black box* indicates the start of the coding sequence (19). *Right*, HepG2 cells were cotransfected with pOLuc-1630, or derivatives having 5′ truncations of the 1630 bp IGF-I promoter insert, together with pCMV-Renilla and a vector expressing AR. After incubation overnight with testosterone (Ts) or vehicle, luciferase activities were determined. Data are from at least three experiments, each having three replicates. *, $P < 0.05$ *vs.* ethanol (EtOH) (*t* test).

FIG. 3. Map of the androgen-responsive region of the human IGF-I upstream promoter. Sequences of the two proposed AREs are shown in *bold*. *Arrows* indicate regions of the IGF-I promoter included in the various reporter genes.

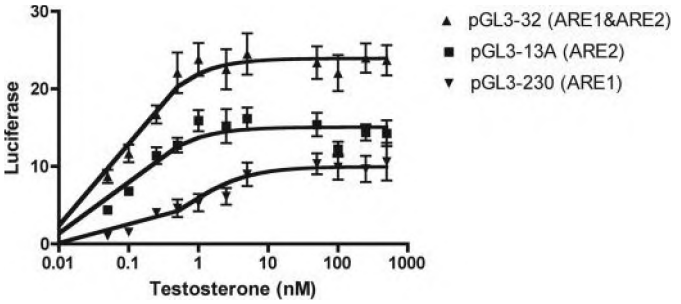

FIG. 5. Interactions between IGF-I AREs. HepG2 cells were cotransfected with the indicated reporter genes, pRL-CMV, and a vector expressing AR and then incubated with testosterone at the indicated concentrations or with ethanol (EtOH). The curves shown were fitted assuming a one-site binding model; values for R^2 were as follows: pGL3–13A, 0.93; pGL3–32, 0.98; pGL3–230, 0.95. Data are from three different experiments each with at least two replicates.

test for interactions more explicitly, cells were transfected with reporter genes under the control of ARE1, ARE2, or both and then incubated with increasing concentrations of testosterone (Fig. 5). Data were fitted to a curve assuming a one-site binding model. Values for EC_{50} were similar for constructs containing ARE2 compared with the construct containing both ARE1 and ARE2 (0.098 and 0.09 nM, respectively) and were significantly less than the EC_{50} for the construct containing ARE1 (0.68 nM, $P < 0.001$). Values for maximal reporter activity are each significantly different from values for the other two reporters and were greatest for the reporter containing both AREs (23.9) followed by a reporter containing ARE2 (15.0) and one containing ARE1 (10.0). The findings argue against synergist interactions on AR binding but are consistent with additive effects of the two AREs on transcriptional activation.

To determine whether these effects of androgens depended upon binding of the AR to specific DNA sequences within these two elements, the ability of testosterone to induce expression of reporter genes containing them was tested in cells transfected with a mutant AR with impaired DNA binding due to a C→G mutation in the fourth cysteine of the first zinc finger within the DBD (22) (AR-C562G). HepG2 cells were cotransfected with a vector expressing the mutant AR, and reporter genes containing ARE1 (pGL3–22), ARE2 (pGL3–13A), or both (pGL3–32). As a control, additional experiments were conducted in which cells were cotransfected with these reporters and a vector expressing wild-type AR. For each of the three reporter genes tested, induction of luciferase expression was virtually abolished when wild-type AR was replaced with the DBD mutant (Fig. 6A), consistent with a requirement for binding of AR to DNA sequences within ARE1 and ARE2 for transactivating activity.

To more directly test whether the AR bound to sequences with ARE1 and ARE2, binding of a GST-tagged version of recombinant AR-DBD to radiolabeled probes was examined using EMSAs. As expected, incubation of the AR-DBD with probes having the SRE consensus sequence yielded a complex that was diminished in amounts by the addition of excess unlabeled probe (Fig. 6B). Incubation with a probe including the sequence of either ARE1 or ARE2 yielded a band with a similar mobility to that observed for SRE (Fig. 6B). Intensity of the bands for ARE1 and ARE2 was diminished by unlabeled DNA having the sequence of the consensus SRE. No complex was apparent after incubation of the AR-DBD with probes containing irrelevant sequences consisting of the consensus binding site for either NF-κB or Oct-1 (Fig. 6C). These findings indicate specific binding of the IGF-I

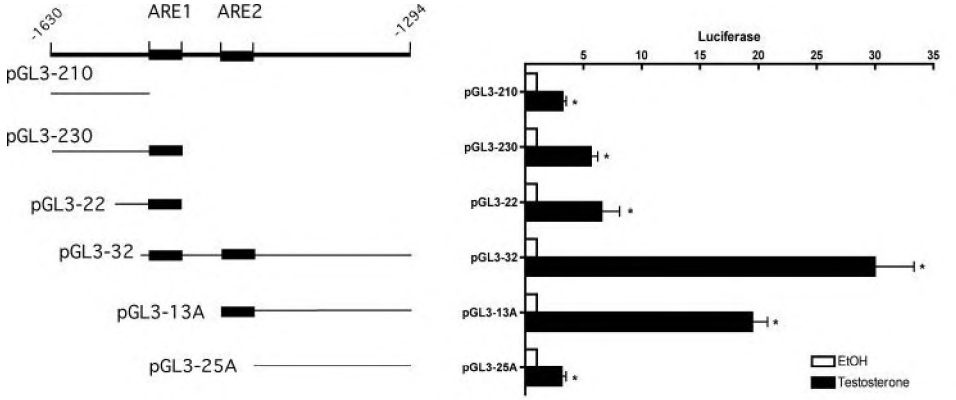

FIG. 4. Androgen responsiveness of the putative IGF-I AREs. *Left*, Map of reporter constructs tested. *Rectangles* indicate the approximate position of putative AREs in the human IGF-I promoter. *Right*, HepG2 cells were cotransfected with the indicated reporter constructs together with pCMV-Renilla and a vector expressing AR and then incubated overnight with testosterone (50 nM) or vehicle. Data are from at least three different experiments each performed in at least triplicate. *, $P < 0.05$ *vs.* ethanol (EtOH) (*t* test).

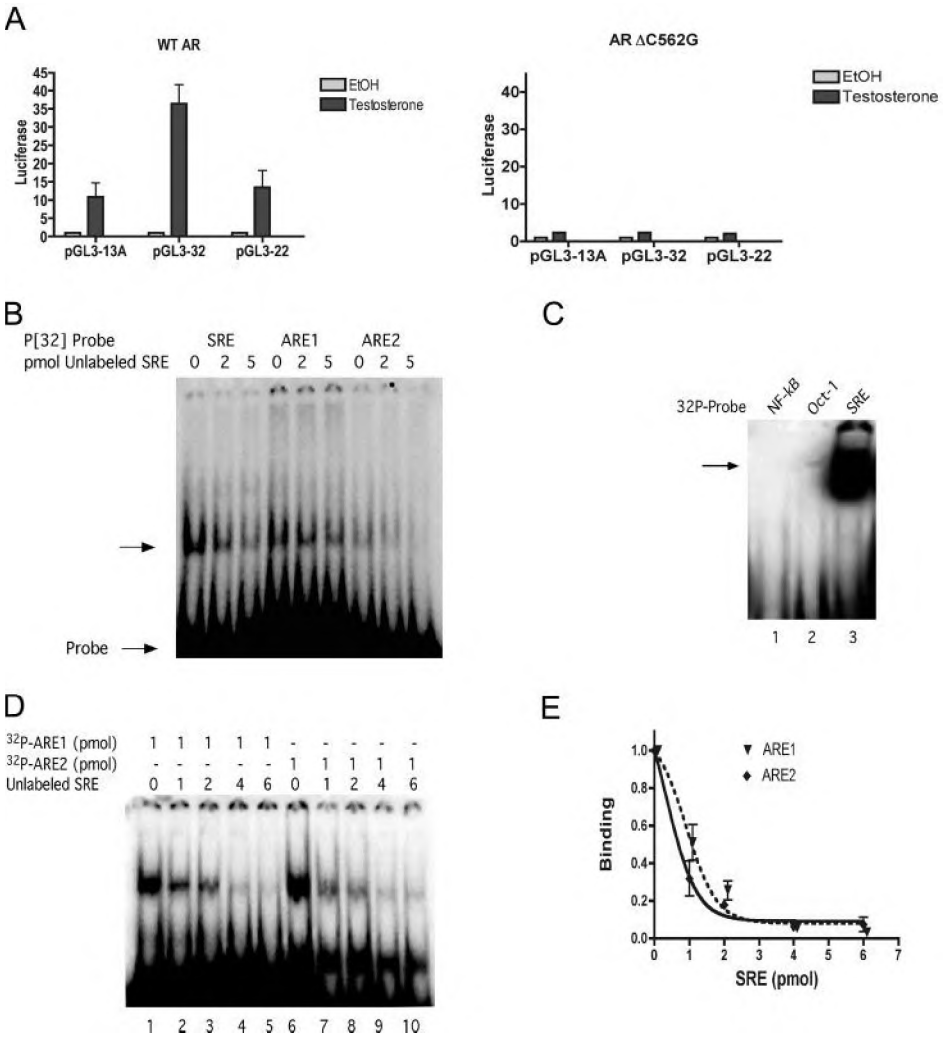

FIG. 6. The AR binds to ARE1 and ARE2 *in vitro*. A, HepG2 cells were cotransfected with a vector expressing mutant AR that is unable to bind DNA (pC562G) or wild-type AR together with the indicated reporter gene and pRL-CMV and then incubated overnight with testosterone (50 nM) or ethanol (EtOH). Data are from three separate experiments each performed at least two times. B, GST-AR-DBD was incubated with radiolabeled oligonucleotides as indicated in the absence or presence of excess unlabeled oligonucleotide with the SRE consensus sequence and then subjected to EMSA. The *arrow* indicates the band representing AR-oligonucleotide complexes. Probes 2 and 8 in Fig. 7B were used for ARE1 and ARE2, respectively. The gel shown is representative of two separate experiments. C, GST-AR-DBD was incubated with the indicated radiolabeled oligonucleotides and then subjected to EMSA. The *arrow* indicates the position of the AR-DBD-SRE complex. The gel shown is representative of findings in two separate experiments. D, GST-AR-DBD was incubated with radiolabeled oligonucleotides with or without unlabeled SRE as indicated and then resolved by nondenaturing electrophoresis. E, Intensities of bands in D were assessed by densitometry scanning. Binding in the presence of each amount of SRE was expressed as a fraction of binding without SRE. The curves shown were fitted using a one-site competition model. Values for R^2 were as follows: ARE1, 0.94; ARE2, 0.96. Data are from three separate experiments.

ARE1 and ARE2 to the AR-DBD, consistent with the interpretation that these DNA sequences represent *cis*-acting elements bound by the AR.

The relative binding affinities of ARE1, ARE2, and SRE were assessed by comparing the reduction in binding observed upon the addition of increasing amounts of excess, unlabeled SRE probe (Fig. 6, D and E). The concentration of SRE reducing binding by 50% was significantly greater for ARE1 than for ARE2 (8.2 nM for ARE1 *vs.* 2.1 nM for ARE2, $P < 0.016$), indicating that affinity of the AR was significantly greater for ARE1 than for ARE2.

In an effort to further localize sequences within each ARE that bound to AR, binding of to GST-AR-DBD of truncated probes based on sequences within ARE1 or ARE2 was examined (Fig. 7, A and B). A 5′ truncation of the original 36-bp ARE1 probe that removed the sequence GACTT (lanes 3 and 5, Fig. 7A) prevented binding. Addition of eight bases to the 3′ end of the probe did not appreciably increase binding (lane 6, Fig. 7A). In the case of the 37-bp ARE2 probe, a 3′ truncation removing nine bases greatly diminished binding, whereas a 5′ deletion removing this number of bases blocked binding completely (lanes 8 and 9, Fig. 7A).

These findings suggest that binding involves extended interactions between the AR and ARE1/ARE2. These findings could be explained by interactions of sequences flanking the DBD-binding motif with other domains of the AR (11) and/or by the presence of multiple half-sites within each ARE (15, 17). Accordingly, sequences within each ARE were inspected more closely for potential steroid hormone receptor-binding motifs (Fig. 8) similar to known AREs. Each ARE contained several half-sites (Fig. 7, A and B) similar to either a consensus SRE or to half-sites within other known AR-binding sites. Three such motifs were found in ARE1 (numbered 1–3 in Fig. 8), and an additional four motifs were identified within ARE2 (numbered 4–7 in Fig. 8). Assuming a usual spacer of 3 bp, each half-site appeared to be associated with a second half-site with a degenerate sequence lacking C/G in either position 3 or 5. Intriguingly, the potential binding sites were overlapped in both ARE1 and ARE2.

Within ARE1, the left half-site of the first binding site (labeled 1 in Fig. 8) closely resembled the consensus TGTTCT motif of the consensus SRE (Fig. 8), whereas the left half-site of the second site (labeled 2) was similar to the right half-site of the hAR ARE1. The third potential site (labeled 3) included a degenerate left half-site, with a right half-site similar to the

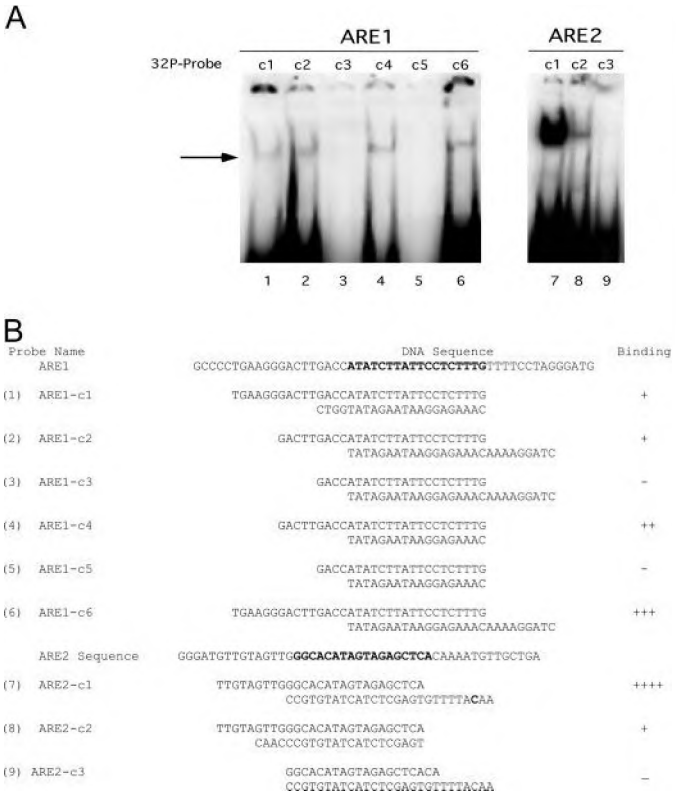

FIG. 7. Evidence that binding interactions are extended beyond the DBD. A, GST-AR-DBD was incubated with radiolabeled probes incorporating ARE1 (probes 1–6) or ARE2 (probes 7–9) and then subjected to EMSA. *Arrow* indicates the position of the AR-oligonucleotide complex. Gels are representative of results from two separate experiments. B, Sequences of the oligonucleotides used in studies in A; *bold* sequences are those similar to the AREs of the Pem gene (see Fig. 3).

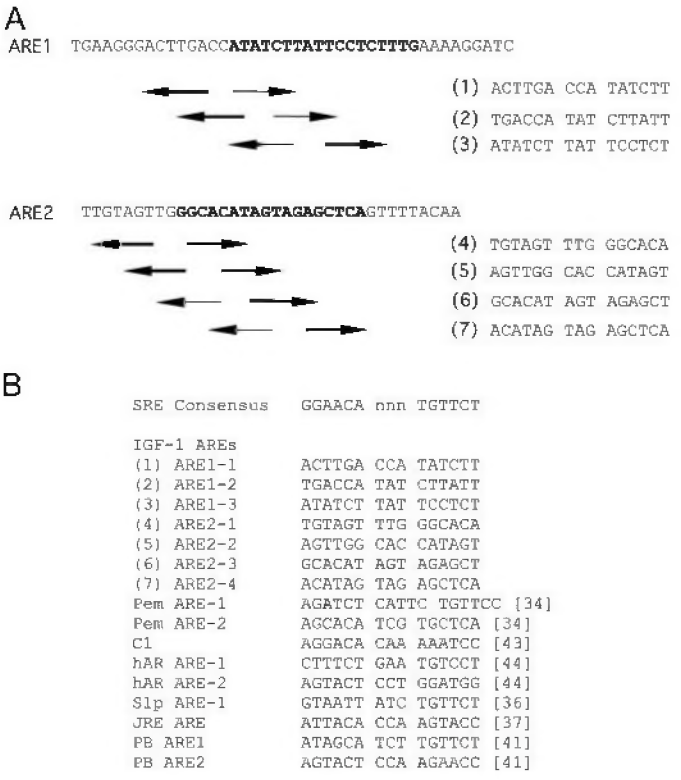

FIG. 8. A, Analysis of sequences within ARE1 and ARE2. *Thick arrows* indicate the location of half-sites resembling either a consensus SRE or half-sites in known AREs (B). *Thin arrows* indicate the location of degenerate half-sites. *Bold* characters indicate the location of sequences similar to AREs of the Pem gene. B, Sequences of IGF-I ARE1/2 binding sites and selected known AREs taken from the cited literature.

left half-site of the ARE in the C1 gene. The organization of this site as one half-site downstream of an incomplete half-site has also been observed in the first ARE from the Slp gene (32) and the JRE element (33) (Fig. 8B). Consideration of spacers shorter than three, or up to six, did not reveal combinations of half-sites more closely resembling classical AREs.

The first site in ARE2 (labeled 4) resembled an inversion of the consensus SRE but was degenerate in having a G in position 1 of the second half-site. A second motif (labeled 5) had a left half-site similar to the probasin ARE2 right-hand half-site. A third motif (labeled 6) had a degenerate left half-site with a right half-site similar to the hAR ARE1 right-handed half-site. The final motif (labeled 7) also contained a degenerate left half-site, with a right half-site similar to the C1 ARE, and the Pem ARE2 left-handed half-site. Of interest, if one assumed a spacer of six bases, the right half-site of this motif can be paired with the left half-site of the previous motif, yielding a sequence in which both half-sites contain G/C in positions 2 and 5 and in which the half-sites are arranged as a direct repeat (GGCACA tagtag AGCTCA).

If ARE1 and ARE2 contribute to testosterone-induced increases in IGF-I in intact cells, it would be predicted that testosterone would cause recruitment of the AR to the region of genomic DNA containing these AREs in intact cells. This prediction was tested in LNCaP and HepG2.AR cells using chromatin immunoprecipitation. This analysis revealed that a PCR product containing ARE1 and ARE2 was obtained when chromatin immunoprecipitation was performed with antibodies against AR (Fig. 9, A and B) but not when chromatin immunoprecipitation was done using normal rabbit serum. Moreover, amounts of the PCR product were increased by addition of testosterone, indicating that testos-

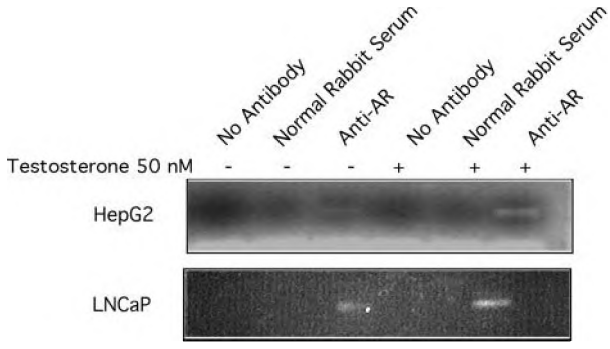

FIG. 9. The AR is recruited to IGF-I AREs in intact cells. Representative gels are shown. Each gel is representative of at least two experiments. Cells were incubated overnight with testosterone (50 nM) or vehicle followed by chromatin immunoprecipitation using either normal rabbit serum or anti-AR and PCR primers flanking the sequence containing ARE1/2.

terone induced the recruitment of the AR to chromatin containing ARE1 and ARE2.

AREs are also typically bound by GR resulting in transcriptional activation. Conversely, the sequence of AREs provides one means of discriminating AR from other steroid hormone receptors, such that transactivation of GR bound to AREs is often lower than that for AR. The ability of GR to cause transcriptional activation of reporter genes incorporating ARE1, ARE2, or both was therefore tested. Dexamethasone induced only weak induction of luciferase expression (less than 2-fold) in cells transfected with reporter genes incorporating ARE1 (p3–230 and p3–22 in Fig. 10B) compared with more than 5-fold increases observed in cells treated with testosterone (Fig. 10A). Stronger activation by glucocorticoids (5-fold) was observed for the reporters incorporating ARE2 or both AREs (p3–13a and p3–32, respectively), although activation remained more than 5-fold less than that observed for cells transfected with the same reporter genes and treated with testosterone (approximately 20- and 30-fold, respectively).

Discussion

The above findings provide evidence that, in hepatocytes and prostate epithelium, IGF-I is up-regulated by testosterone as a consequence of recruitment of the AR to two *cis*-acting DNA elements within the IGF-I promoter upon binding of testosterone to the AR. These elements are localized between −1320 and −1420 bases upstream of the first codon of the human IGF-I gene. Evidence supporting this conclusion includes findings that sequences from within the androgen-responsive region that contained one or both elements conferred strong activation by testosterone of reporter genes. Additionally, transcriptional activation via each of these androgen-responsive regions required a functional AR-DBD, and the AR was found to bind to DNA sequences within each of the elements. Moreover, in intact cells, testosterone caused recruitment of the AR to the DNA sequences containing them.

Our findings that testosterone up-regulates IGF-I expression in LNCaP and HepG2 cells are consistent with results from other studies that have examined the effects of androgens on expression of this gene. Up-regulation of IGF-I expression has been observed after exposure of prostate stromal cells or LNCaP cells to physiological concentrations (10–35 nM) (34) of testosterone (7, 8). Muscle satellite cells were also found to express higher levels of IGF-I mRNA after exposure to testosterone or to trenbolone, an anabolic steroid (5). These findings in cultured cells appear to have relevance for tissues. Testosterone administration led to more than 30-fold increases in uterine IGF-I mRNA levels (6). In elderly men with lower than normal concentrations of testosterone in blood, testosterone replacement therapy increased skeletal muscle expression of IGF-I mRNA and protein (2, 3), whereas in young men, ablation of testosterone production significantly reduced levels of IGF-I mRNA in skeletal muscle (4).

Although we observed increased IGF-I mRNA levels in HepG2 cells exposed to testosterone, and the liver is the primary source of IGF-I in blood, reports regarding the effects of testosterone on liver expression and secretion of IGF-I have reached conflicting conclusions. Studies of the effects of testosterone on hepatic IGF-I expression have been conducted in male peripubertal rats. When begun 1 wk after hypophysectomy, testosterone did not alter liver IGF-I mRNA levels or blood IGF-I levels (35). Similarly, no change in hepatic IGF-I expression was observed when testosterone was administered after castration with or without hypophysectomy (35). Testosterone did not alter blood IGF-I levels after castration and hypophysectomy and caused a small decline (14%) after castration only. Conversely, testosterone administration was found to increase IGF-I protein levels in blood in healthy young men (1).

The presence in the IGF-I gene of multiple AREs is similar to other genes where *cis*-acting DNA sequences are known to be involved in transcriptional activation by the AR (30, 36–38). These multiple AREs act together to control transcription in several different ways. In the probasin gene, cooperative interactions between AR bound to these different sites increases receptor-binding affinity for DNA at the two sites (24, 39). By contrast, in the Slp gene, only one of the three AREs is able to mediate transcriptional activation in isolation from the others, with activity of that consensus ARE being somewhat enhanced by one of the flanking, degenerate AREs while being repressed by the other (32). In the case of the AREs of the IGF-I gene, each ARE conferred testosterone-induced activation. The presence of both AREs did not seem to alter EC_{50} for activation by testosterone but did increase maximal activation achieved by testosterone. Transcriptional activation has been suggested to depend on multiple binding affinities that include those between the AR and DNA as well as between AR and other transcription factors (37) or between AR and coregulators. The EC_{50} would be expected to result in the sum effect of receptor affinity for DNA and receptor interactions with other transcription factors, coactivators, and corepressors; it would be predicted that coop-

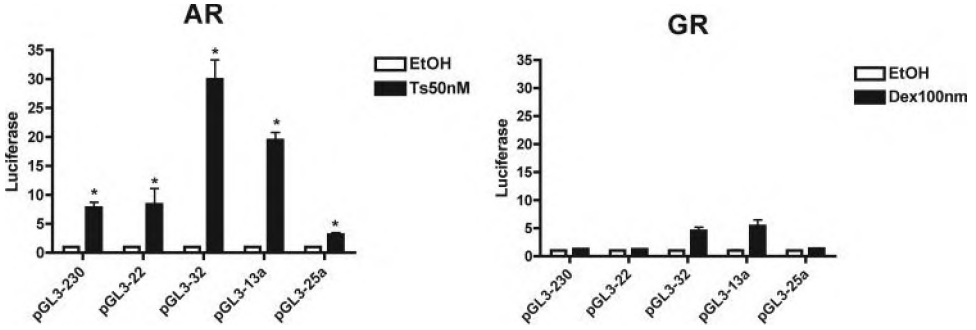

FIG. 10. Comparison of transactivation at ARE1 and ARE2 by AR and GR. HepG2 cells were cotransfected with the indicated reporter genes, pCMV-Renilla, and a vector expressing either AR or GR and then incubated overnight with hormone as indicated. Dex, Dexamethasone; EtOH, ethanol; Ts, testosterone. Data are from three separate experiments, each performed at least two times.

erative interactions between receptors would therefore reduce EC_{50}. Therefore, our findings argue against cooperative binding interactions between receptor and the two AREs and indicate instead additive effects of the two AREs on transcriptional activation.

Although a general relationship between AR binding affinities for DNA and transcriptional activation might be predicted, divergence from such a relationship could reasonably be expected to occur, and many disparities between these measures have been observed. For example, the binding affinities of the steroid hormone receptor DBDs to oligonucleotides based on the probasin ARE2 or C3(1) ARE were poor predictors of transcription activation of reporter genes based on these AREs in cultured cells. Similarly, ARE2 of the IGF-I bound AR with lower affinity but achieved greater transcriptional activation upon binding of AR.

The structure of the two AREs was complex and diverged from that of consensus SREs and most known AR-binding sites. Sequences within each ARE included what appeared to be overlapping half-sites resembling those in the consensus SRE, or in other known AREs, each linked to a second half-site markedly diverging from the consensus SRE sequence and unlike other known AREs described in the literature (24, 30, 40, 41). These overlapping motifs were compressed into approximately 18 bp of sequence, suggesting that only one AR dimer binds within each ARE at any one time. Our analysis does not exclude the possibility that a longer spacer is present between the two half-sites, and one motif consisting of a direct repeat with a spacer of six bases was observed in ARE2. Of note, ARE1 from the Pem gene includes a spacer of five bases, and the GR has been observed to bind motifs with up to nine base spacers (16), suggesting that the motif in ARE2 with an extended spacer may function in AR binding. It is both intriguing and unusual that binding of ARE1/ARE2 to the AR required extended interactions between the ARE and flanking sequences that were both 5′ and 3′ to the predicted AR-binding half-sites. One interpretation suggested by these findings is that because of the degeneracy of the second half-site in the primary AR-binding motifs, additional interactions were needed to stabilize AR-DNA complexes.

The presence of what appear to be overlapping half-sites within the AREs of the IGF-I gene is unique among AREs described thus far. There is one other example of an ARE having multiple AR-binding sites, which was identified in the human secretory component gene (17) and was found to consist of three contiguous (nonoverlapping) half-sites similar to the SRE consensus sequence, with a fourth half-site located just downstream. This ARE is highly selective for AR over GR, perhaps in part as a consequence of the presence of multiple half-sites. Conversely, a glucocorticoid response element composed of multiple overlapping half-sites has been described in the Ast gene (15) and has been found to be highly selective for transcriptional activation by the GR over AR or other receptors with overlapping DNA-binding specificities (15).

It is attractive to propose, therefore, that the organization of IGF-I AREs as several overlapping half-sites contributes to the selectivity of these AREs for AR over GR. However, other features of these AREs may also contribute to selectivity for transcriptional activation by AR over GR, including interactions of DNA sequences outside the half-sites with amino acids within the second zinc finger or hinge domain (18) and interactions with other transcription factors (42). It is notable that for the AREs of the IGF-I gene, sequences flanking each ARE interacted with the AR, suggesting that such interactions may contribute to discrimination by these AREs of AR and GR.

The IGF-I gene is an interesting example of a gene that is regulated in opposite directions by AR and GR. Whereas AR up-regulates IGF-I, GR represses it in cultured cells and reduces IGF-I protein levels in intact animals (43, 44). The relatively high degree of discrimination of ARE1 and ARE2 for AR *vs.* GR appears to be one way in which the cell ensures that these opposite effects of AR and GR on IGF-I expression occur. An additional mechanism has also been described. Glucocorticoids have been shown to up-regulate the expression of CAAT/enhancer binding protein δ, which then binds to downstream elements within the IGF-I promoter (43) and represses IGF-I transcription.

Thus, the human IGF-I gene contains two AREs localized between −1320 and −1420 bases upstream of the first codon, with each ARE organized as several overlapping half-sites and every half-site being paired with a degenerate half-site. Binding of these core motifs to the AR depends upon interactions between the AR and flanking sequences. This combination of degenerate core binding motif and interactions with flanking sequences confers selectivity for AR over GR that may be enhanced by additional downstream elements. The findings provide direct support for the view that androgens stimulate IGF-I expression in tissues and provide a molecular mechanism by which such expression occurs. Increased expression of IGF-I induced by androgens may contribute to many of the anabolic and growth-promoting effects of testosterone. Testosterone-induced IGF-I expression may also explain the beneficial, and apparently protective, effects of androgens in humans or animals exposed to glucocorticoids (45–48).

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