A Complex Response Element in Intron 1 of the Androgen-regulated 20-kDa Protein Gene Displays Cell Type-dependent Androgen Receptor Specificity*

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The androgen-regulated 20-kDa protein gene consists of four exons that code for a major secretory protein of rat ventral prostate. Analysis of its potential cis-acting transcriptional regulatory elements revealed that a large intron 1 region (In-1c) had stronger androgen response element (ARE) activity than did the 5'-flanking DNA. In cotransfected CV1 cells, In-1 and its most active subfragment In-1c functioned as AREs but not glucocorticoid response elements (GRE). Nevertheless, several ARE/GRE-like partial palindromic sequences are present in In-1c, and it bound both androgen receptors and glucocorticoid receptors in mobility shift assays. A cluster of three ARE/GRE-like sequences contained within a 39-base pair sequence of In-1c had both ARE and GRE activities when analyzed as an isolated oligonucleotide, suggesting that other elements within In-1c determined its ARE specificity. In addition to ARE/GRE-like sequences, In-1c contains putative response elements for the transcription factors AP1, CREB, AP2, OCT-1, C/EBP, and a number of inverted and direct repeats. The ARE specificity of In-1c observed in CV1 cells was diminished in PC3 and HeLa cells transiently cotransfected with an androgen receptor or glucocorticoid receptor expression vector together with an In-1c reporter vector; however, the ARE activity of In-1c was greater than its GRE activity in these cell lines. Interestingly, a 131-base pair subfragment of In-1c retained ARE specificity in all three cell lines.

Regulatory effects of steroid hormone receptors on gene transcription are initiated by their interactions with nucleotide sequences referred to as hormone response elements (1-3). These elements are recognized by the receptor DNA binding domain, a cysteine-rich region containing two zinc fingers (4). The subfamily of nuclear receptors, which includes androgen (AR), glucocorticoid (GR), progesterone, and mineralocorticoid receptors, contains a high degree of sequence similarity within the DNA binding domains of the receptors (5). This sequence homology is reflected in their common interactions with a similar class of response elements consisting of 15 bp partial palindromic sequences (3, 6-12). Studies to identify androgen-regulated prostate C3 subunit gene identified a potent androgen response element (ARE) A GTACGCU TGTCTT (9, 70, 13) within the first intron which also functions as a glucocorticoid response element (GRE) in transient cotransfection assays (14). Several weaker elements were identified within the C3 first intron and 5'-flanking regions, and these too displayed both ARE and GRE activities (10, 14). This set of simple response elements provides a plausible basis for the overlapping androgen and glucocorticoid effects on gene expression which have been demonstrated in rat ventral prostate. However, the predominant androgen dependence of gene expression in rat prostate suggests that there exist more specific AREs that respond selectively to androgen.

Thus far an AR-specific single 15-bp palindromic sequence has not been identified, but receptor specificity may be determined by more complex response elements. Recent studies on the AR, GR, progesterone receptor subfamily have shown that transactivation can be mediated by complex elements that include multiple simple steroid response elements (15-bp partial palindromes or half-sites) together with recognition sequences for other transcription factors. A response element of this type could provide a framework for modulation of receptor transactivation by other factors through both protein-protein and protein-DNA interactions (3, 12, 15-21).

Herein we report on a complex element that responds selectively to the androgen receptor. The complex androgen response element is located in the first intron of the androgen-regulated 20-kDa protein gene that codes for one of the major secretory proteins of rat ventral prostate and lacrimal gland (22-24). This intron element conferred androgen but not glucocorticoid receptor responsiveness to a heterologous promoter in transient cotransfection experiments utilizing monkey kidney CV1 cells. In the human prostate cancer cell line PC3 and in HeLa cells, both AR and GR transactivation was

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank†/EMBL Data Bank with accession number(s) L12454.

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1 The abbreviations used are: AR, androgen receptor; GR, glucocorticoid receptor; bp, base pair(s); ARE, androgen response element; GRE, glucocorticoid response element; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; CAT, chloramphenicol acetyltransferase; DBD, DNA binding domain; C/EBP, CCAAT and enhancer core binding protein; CREB, cyclic-adenosine monophosphate response element binding protein.
FIG. 1. Nucleotide sequence of the rat 20-kDa protein gene. Panel A, schematic diagram of the 20-kDa protein gene and its sequencing strategy. The sequence GATAAAA is the putative TATA box. Cross-hatched bars are exons. Open bars are 5'-flanking, introns 1, 2, 3, and 3'-flanking regions. Residue numbers are indicated relative to the predicted mRNA start site. The restriction enzyme sites shown were used in sequencing: R, EcoRI; Hi, HinfI; A, AluI; H, HincII. The sequence between nucleotides 3001 and 5399 was analyzed in...
Double-stranded DNA was synthesized by polymerase chain reaction (PCR), purified by agarose gel electrophoresis, and cloned into pGEM3zf (+) for sequencing. Single-stranded DNA was synthesized by asymmetric PCR, purified by centrifugation in Centricon-30 or -100 (Amicon Division, W. R. Grace & Co.), and sequenced using Sequenase.

**Primer Extension Analysis**—The 5′ start site of mRNA transcription was determined by primer extension analysis as described previously (27). Total RNA was isolated from Sprague-Dawley rat (250-350 g) ventral prostate using the acid guanidinium thiocyanate-phenol-chloroform method (28) and enriched for poly(A) RNA by oligo(dT)-cellulose chromatography (20). The oligo(dT) fragment was ligated to EcoRI genomic DNA fragments hybridizing to cDNA probes. DNA fragments were labeled with [a-'32P]dCTP and cloned into pGEM3zf (+) at the EcoRI site. Subfragments were obtained by restriction of the EcoRI fragment containing a potential GRE sequence (29). All constructs were verified by double-stranded sequencing using oligonucleotide primers. Clone pFL-1 contains the flanking region fragment FL-1, nucleotides -895 to -38. FL-1 was prepared using PCR primers 5′-GTAATTAAAGATAACTAAG-3′ (nucleotides -895 to -876) and 5′-CCTCCTACTTGGACCGAGCCTCC-3′ (nucleotides -38 to -60) on the 1,426-bp EcoRI fragment. Fragment FL-1 was cloned into the T4 DNA polymerase-blunted BamHI site of pCAT. The flanking region containing nucleotides -901 to +8 was generated by cleaving the 1,426-bp EcoRI fragment with MboII and cloned into the BamHI site of pCAT by blunt end ligation. Clone pIn-1 containing the intron 1 fragment In-1 was generated by restriction of the 2,558-bp EcoRI fragment with BamHI and cloning into the BamHI site of pCAT by blunt end ligation.

Clone pIn-1a contains the intron 1 subfragment In-1a (nucleotides 1419-1779). The fragment was generated by restriction digestion of the fragment In-1 with HindII then AulI. In-1a was cloned into the BamHI site of ptkCAT by blunt end ligation. Clones pIn-1b and pIn-1c were constructed similarly but contain the intron 1 subfragments In-1b, nucleotides 2013-2290, and In-1c, nucleotides 2549-2907, respectively. However, pIn-1c was first cloned in reverse orientation, cut with BamHI, and reinserted in the correct orientation to preserve the BamHI sites. Clone pd1 contains the larger subfragment (D1) of In-1c (nucleotides 2980-2907) cleaved by Ddel and cloned into the Sall site of ptkCAT by blunt end ligation. Clone pd2 contains the smaller subfragment (D2) of In-1c, nucleotides 2549-2860, obtained by Ddel cleavage. Clone pIn-1c contains a 39-bp oligonucleotide of fragment In-1c, nucleotides 2684-2722. The oligonucleotide was prepared with Sall sites at both ends and cloned into Sall site of ptkCAT. pCMV-AR is a pCMV1 vector containing full-length rat AR cDNA (10, 30). pCMV-GR was constructed by cloning the full-length rat GR (31) into pCMV1.

**Cell Culture, Transfection, and CAT Assay**—African green monkey kidney cells, CV1, were maintained at 37 °C under 5% CO2 in Dulbecco's modified Eagle’s medium-H supplemented with 5% fetal bovine serum. Human prostate cancer cells, PC3, were maintained at

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**FIG. 2. Primer extension analysis of the transcription start site of the 20-kDa protein gene. Lanes 1-4**, sequencing ladders of M13mp19 using the 17-mer sequencing primer -20 in the order of G, A, C, T. **Lanes 5-7**, the products of extension using 1.0, 0.1, and 0.01 μg of rat ventral prostate poly(A) RNA as template. The arrow indicates the position of the longest DNA product which corresponds to a G residue of M13mp19, 372 nucleotides from the 5′ start point of the 17-mer sequencing primer -20. This located the mRNA transcription start site at an A residue of the genomic DNA sequence shown in Fig. 1.

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**Materials and Methods**

**Genomic Clones and DNA Sequencing**—EcoRI and HaeIII partially digested rat genomic DNA libraries constructed in λ Charon 4A were provided by T. Sargent, R. Wallace, and J. Bonner (California Institute of Technology). Libraries were screened for the 20-kDa gene using two EcoRI cDNA fragments (nucleotides 237-352 and 353-809) digested rat genomic DNA libraries constructed in pGEM3zf (+) at the SmaI site. Sequencing in M13mp19 was performed using T4 DNA polymerase and cloned into M13mp19 or pGEM3zf (+) at the EcoRI site. Subfragments were obtained by restriction of the EcoRI fragment containing a potential GRE sequence (29). All constructs were verified by double-stranded sequencing using oligonucleotide primers. Clone pFL-1 contains the flanking region fragment FL-1, nucleotides -895 to -38. FL-1 was prepared using PCR primers 5′-GTAATTAAAGATAACTAAG-3′ (nucleotides -895 to -876) and 5′-CCTCCTACTTGGACCGAGCCTCC-3′ (nucleotides -38 to -60) on the 1,426-bp EcoRI fragment. Fragment FL-1 was cloned into the T4 DNA polymerase-blunted BamHI site of pCAT. The flanking region containing nucleotides -901 to +8 was generated by cleaving the 1,426-bp EcoRI fragment with MboII and cloned into the BamHI site of pCAT by blunt end ligation. Clone pIn-1 containing the intron 1 fragment In-1 was generated by restriction of the 2,558-bp EcoRI fragment with BamHI and cloning into the BamHI site of pCAT by blunt end ligation.

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**Fig. 3.** Androgen-dependent enhancer activities of the 5′-flanking region and intron 1 fragment In-1 in CV1 cells. Schematic diagram of the 20-kDa protein gene and the two fragments, FL-1 and In-1, cloned into the reporter vector ptkCAT for assay of androgen-dependent enhancer activity (see “Materials and Methods” for construction). CV1 cells were cotransfected with the androgen receptor expression vector pCMVrAR (1 µg) and (5 µg) of pFL-tkCAT containing the flanking region FL-1 or pIn-1-tkCAT containing the intron 1 fragment In-1. Lane 1, recombinant reporter vector in the presence of 50 nM R1881. Lane 2, recombinant reporter vector plus pCMVrAR without R1881. Lane 3, the same as lane 2 but with 50 nM R1881. CAT activities were measured in CV1 cell extracts as described under “Materials and Methods.”

**Fig. 4.** Androgen-dependent enhancer activities of intron 1 subfragments in CV1 cells. The diagram at the top indicates relative locations of In-1 subfragments In-1a, In-1b, In-1c, and arrows point to CAT assays with these fragments shown below. CV1 cells were cotransfected with 5 µg of recombinant reporter vector (tkCAT) containing In-1a, In-1b, or In-1c, and 1 µg of the androgen receptor expression vector, pCMVrAR. Lane 1, recombinant reporter vector (5 µg) in the presence of 50 nM R1881. Lane 2, recombinant reporter vector plus pCMVrAR (1 µg) without R1881. Lane 3, the same as lane 2 but with 50 nM R1881. Data shown are representative of at least five experiments. CAT activities were measured in CV1 cell extracts as described under “Materials and Methods.”

37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium and F-12 medium supplemented with 5% fetal bovine serum. Human cervical carcinoma cells, HeLa, were maintained in Eagle’s minimal essential medium supplemented with 5% fetal bovine serum. One day before transfecting, cells were plated in 10-cm culture dishes at 1.2 × 10⁶ cells/dish and grown in the same medium for 20 h when they were 70–80% confluent. (See the legend to Table III for modifications of the protocol in experiments comparing regulation in CV1, PC3, and HeLa cells.) Cells were transfected with 1 µg of receptor expression vector DNA, pCMVrAR or pCMVrGR and 5 µg of reporter plasmid DNA using the CaPO₂ method as described previously (10). Cells were washed twice, placed in Dulbecco’s modified Eagle’s medium-H without phenol red and supplemented with 0.2% fetal bovine serum in the presence or absence of the indicated concentration of synthetic androgen, R1881 or synthetic glucocorticoid, dexamethasone. After incubation for 20 h, the medium was replaced with or without hormone at the same concentration. Cells were incubated for another 24 h, harvested in phosphate-buffered saline (0.15 M NaCl, 0.01 M Na₂HPO₄, pH 7.2), and assayed for CAT activity as described by Gorman et al. (32) except that the cells were broken by freeze-thaw three times. Thin layer plates were exposed on Kodak X-Omat AR film for 24–72 h. For quantitation, the radioactive spots were cut out from the plates and counted in a liquid scintillation counter. Stimulation of CAT activity was expressed as fold increase over background and was based on at least five independent experiments.

**Mobility Shift: DNA Binding Assay—DNA receptor binding was analyzed by gel mobility shift using AR or GR DNA binding domain polypeptides and was performed as described previously (10). Specific binding of receptor to DNA was demonstrated using the protein purification IgG fraction of anti-AR antisemur (AR-52) or unpurified anti-GR antisemur (BuGR-2). 20-kDa protein gene fragments used in the mobility shift assay were inserts of ptkCAT vectors removed...
by restriction enzyme digestion to generate 5'-protruding ends and purified by electrophoresis from polyacrylamide gels. The 5'-protruding ends were filled in with [α-32P]dCTP (Amersham Corp.) using Klenow fragment of DNA polymerase I (Life Technologies, Inc.).

Overexpression of Recombinant Androgen and Glucocorticoid Receptor—The rat GR DNA binding domain (DBD) (amino acid codons 407–556) was excised from the expression vector pT7X556 by NdeI and Clal digestion and subcloned into the T7 RNA polymerase-dependent expression vector pET16b (Novagen) at the same restriction sites. In addition to non-GR residues present in T7X556, the resulting construct, pET16b-rGR-DBD, contained 19 non-GR amino acids at the amino terminus contributed by pET16b including 10 histidine residues which permit rapid purification using a nickel-containing affinity resin.

A 510-bp DNA fragment corresponding to rat AR amino acid codons 495–665 encompassing the DNA binding domain and hinge region of AR was generated by PCR amplification using oligonucleotides that introduce BamHI sites at either end of the amplified fragment. The PCR product was digested with BamHI and cloned into the expression vector pET16b. The resulting expression vector, pET16b-rAR-DBD, contained 19 non-AR amino acids at the amino terminus including 10 histidine residues and 19 non-AR amino acids at the carboxyl terminus including a stop codon.

BL21(DE3)pLysS, a strain carrying a stable integrant of T7 RNA polymerase under the control of the lac UV5 promoter, was transformed with pET16b-rGR-DBD or pET16b-rAR-DBD, grown at 30°C in LB medium containing carbenicillin and chloramphenicol, and induced at midlogarithmic growth (A600 = 0.6–0.8) by the addition of isopropyl-1-thio-β-D-galactopyranoside at 1 mM. After 2 h of induction, cells were collected by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10% glycerol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 1 μg/ml aprotinin), and 5 mM imidazole, 0.04 ml/ml of culture) and sonicated on ice for three 10-s intervals. After 15 min on ice, sodium deoxycholate was added to 0.05% and the suspension stirred for 30 min in an ice bath. The suspensions were centrifuged at 40,000 × g for 30 min at 4°C. Supernatants were filtered through a 0.45-μm filter and mixed with a nickel-containing affinity resin (His-Bind™, Novagen), 1 ml of resin, and 10 ml of supernatant, for 1 h at 4°C by inversion. Resin suspensions were packed into columns, washed sequentially with 10 ml of lysis buffer, 10 ml of lysis buffer containing 25 mM imidazole, and 10 ml of lysis buffer containing 50 mM imidazole. Receptor proteins rGR-DBD or rAR-DBD were eluted with 5 ml of lysis buffer containing 200 mM imidazole. Protein fractions were dialyzed against TEGDZn (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM TIOH, 50 μM ZnSO4, and 50 mM NaCl) for 4 h at 4°C, aliquoted, frozen in liquid N2, and stored at −80°C. Eluted protein fractions were approximately 20 and 40% rGR-DBD and rAR-DBD, respectively, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis Coomassie Blue staining and Western blotting.

RESULTS

Structure and Nucleotide Sequence of 20-kDa Protein Gene—Two genomic clones of the 20-kDa protein gene were selected, clone R6 from the EcoRI partially digested library, and clone H8 from the HaeIII partially digested library (also cloned in Charon 4A at EcoRI site with EcoRI linker). When these genomic fragments were restricted with EcoRI, clone R6 yielded 7 bands and clone H8 11 bands on agarose gel electrophoresis.

In Southern blot analysis, two fragments of clone R6 (2,564 and 1,423 bp, respectively) hybridized to the 5' portion of 20-kDa protein cDNA (EcoRI fragment, nucleotides 237–352 in Ref. 22). The 3' portion of the 20-kDa protein cDNA (nucleotides 353–809) hybridized with two fragments of clone H8 (1,255 bp and approximately 4 kilobases, respectively) (data not shown). Clone H8 also contained a 258-bp EcoRI fragment that did not hybridize with the cDNA probe. It was demonstrated by PCR that this fragment is in the second exon-intron region of 20-kDa gene (see Fig. 1A). The sequencing strategy and nucleotide sequence are shown in Fig. 1, A and B. Primer extension analysis (Fig. 2) together with the cDNA sequence reported earlier (22, 23) indicated that this genomic DNA sequence contains the entire 20-kDa gene sequence including 901 bp 5’ of the transcription start site with a TATA box at −22, a 293-bp first exon, 2,709-bp first intron, 96-bp second exon, 762-bp second intron, 114-bp third exon, 838-bp third intron, 325-bp fourth exon, and 267-bp nontranscribed 3' region.

Androgen Response Element Activities of the 5'-Flanking Region and Intron 1 Fragments—Androgen-dependent enhancer activities of the 20-kDa protein gene 5'-flanking region and intron 1 fragment were compared in CV1 cells. The 5'-flanking and intron 1 fragments were cloned into pCAT and ptkCAT, respectively, both at the BamHI site, and cotransfected with the rat androgen receptor expression, pCMVrAR, in the presence or absence of the synthetic androgen R1881 (50 nM). No increase in CAT activity was detected with the 5' fragment, nucleotides −901 to +8 (created by MboII cleavage) in pCAT (data not shown). When a fragment of this region, FL-1 (nucleotides −895 to −38) without the putative TATA box, was tested in ptkCAT, CAT activity was stimulated only about 2-fold by R1881 (50 nM) (Fig. 3). A subfragment of FL-1, nucleotides −503 to −181, was also tested in ptkCAT, and CAT activity was stimulated only 2-fold by
The intron 1 fragment, In-1, was cleaved with the restriction enzyme HincII to yield two fragments which were digested with AluI. Three fragments containing sequences resembling GREs were selected, and their androgen-dependent enhancer activities were tested (Fig. 4). Fragments In-1a, nucleotides 1420–1779, and In-1b, nucleotides 2083–2290, yielded low levels of activity (2-fold or less). However, the fragment In-1c, nucleotides 2549–2907, resulted in a 9-fold stimulation of CAT activity in initial experiments (Fig. 4). For this reason, further studies were focused on fragment In-1c.

**Androgen and Glucocorticoid Response Element Activities of Intron 1 Fragment, In-1, and Subfragment, In-1c, in CV1 Cells**—The ability of the intron 1 fragment, In-1, and its subfragment, In-1c, to enhance GR as well as AR transactivation was tested by cotransfection analysis in CV1 cells (Fig. 5). With the large intron 1 fragment, In-1, CAT activity increased with AR and R1881 in concentrations up to $10^{-8}$ M (Fig. 5). However, there was no increase in CAT activity with GR and dexamethasone up to $10^{-8}$ M. With In-1c there was strong androgen-stimulated CAT activity but again no response to GR and dexamethasone even at $10^{-7}$ M (Fig. 5). This result was in contrast to the activities of simple response elements which are capable of directing transactivation with all members of the androgen, glucocorticoid, and progesterone subfamily of nuclear receptors (3, 6–11). The sequence specific to AR was further investigated by DdeI restriction digestion of fragment In-1c into two fragments, D1 and D2 (Fig. 6). In addition, we synthesized a 39-bp oligonucleotide (N39) corresponding to the portion of In-1c containing a cluster of three ARE/GRE-like half-site sequences. Fig. 6 shows that the two In-1c subfragments, D1 and D2, retained androgen receptor specificity in the CAT assay (lanes 1 and 2) and did not mediate GR transactivation (lanes 3 and 4). In contrast, N39 containing three GRE-like sequences mediated increases in CAT activity in response to both R1881-AR and dexamethasone-GR (Fig. 6). The larger fragment (D1) contains 11 ARE/GRE-like sequences including the cluster of three in N39 (Fig. 6 and Tables I and II) and potential transcription factor recognition sites for AP2, C/EBP, and OCT-1. Responsiveness of N39 to both AR and GR indicates that other factors interacting with sequences outside the 39-bp region determine androgen receptor specificity in CV1 cells. The smaller fragment (D2) contains potential transcription factor recognition sites for AP1 and CREB but lacks a strong candidate ARE/GRE.
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Table III

Effect of cell type on response element specificity of In-1c and its subfragments D1 and D2

|        | CV1                     | PC3       | HeLa   |
|--------|-------------------------|-----------|--------|
|        | AR                      | GR        | AR     | GR     |
| In-1c  | 19.8 ± 1.7              | 0.7 ± 0.1 | 10.5 ± 2.1 | 2.9 ± 0.8 |
| D1     | 3.9 ± 0.7               | 0.5 ± 0.1 | 3.8 ± 0.9 | 1.9 ± 0.5 |
| D2     | 15.4 ± 2.5              | 0.5 ± 0.1 | 7.5 ± 0.1 | 0.7 ± 0.1 |

![Fig. 7.](image)

Fig. 7. Androgen receptor binding to In-1c. Each binding reaction contained 32P end-labeled In-1c (10,000 cpm) and 1 μg of poly(dI-dC). Recombinant rAR-DBD partially purified from Escherichia coli was incubated with labeled probe in the presence or absence of unlabeled In-1c and loaded onto a 4% nondenaturing polyacrylamide gel. Lane 1, free labeled probe; lanes 2-7, 1.0 μg of AR-DBD; lanes 3-7, 0.28 μg of antibody AR-52; lanes 4 and 5 each contained the indicated molar excesses of unlabeled In-1c; lanes 6 and 7 contained molar excesses of unlabeled non-specific DNA (N.S. DNA).

a 15-bp partial palindromic that functions as a strong ARE or GRE (oligo(C) in the C3 gene, Ref. 10). When the ARE/GRE was inserted into pIn-1ctkCAT 5′ of In-1c and cotransfected into CV1 cells with either AR or GR expression vectors, responses to dexamethasone and androgen were similar to those observed with oligo(C) alone, indicating that In-1c did not suppress the GRE activity of this ARE (data not shown). However, strong ARE activity of oligo(C) may have overcome a suppressor of weaker hormone response elements.

Influence of Cell Type on Intron Response Element Specificity—Possible effects of cell specific factors on androgen receptor specificity of In-1c and its subfragments D1 and D2 were investigated by transient cotransfection again in CV1 cells and in two additional cell lines, PC3, a human prostate cancer cell line, and HeLa cells. None of these cell lines contained detectable levels of AR. CV1 and PC3 cells also lack GR; however, HeLa cells contain sufficient endogenous GR such that transfected mouse mammary tumor virus-CAT responded to dexamethasone without cotransfection of a GR expression vector (33). Fragment In-1c (pIn-1ctkCAT) was not as selective for androgen in PC3 and HeLa cells and mediated responses to both AR and GR; however, R1881 stimulated a greater increase in CAT activity than did dexamethasone in both cell lines (Table III). The response of subfragment D1 (pDltkCAT) to R1881-AR was less than that of In-1c in all three cell lines. D1 was only slightly responsive to dexamethasone-GR in PC3 cells and unresponsive in CV1 and HeLa cells. On the other hand, the smaller subfragment, D2 (pD2tkCAT) retained its selectivity for androgen receptor in all three cell types, CV1, PC3 and HeLa cells. Dexamethasone-GR did not increase CAT activity with D2 in any of these cell lines and the response to R1881-AR was greater than with D1.

Androgen (AR) and Glucocorticoid (GR) Receptor Binding to Intron 1 Fragment, In-1c, and Its Subfragments, D1 AND D2—Binding of purified recombinant AR DNA binding domain polypeptide (rAR-DBD) to fragment In-1c was examined using the mobility shift DNA binding assay. When rAR-DBD was incubated with 32P end-labeled In-1c (Fig. 7), a protein-DNA complex was detected which was shifted farther with anti-AR antibody (AR-52) confirming the binding of AR to this fragment. AR binding to labeled In-1c could be inhibited with unlabeled In-1c up to 1,000-fold molar excess (lanes 4 and 5). Nonspecific DNA was a less effective competitor of AR binding to In-1c (lanes 6 and 7). Recombinant GR DNA binding domain polypeptide (rGR-DBD) shifted radiolabeled In-1c as did rAR-DBD (Fig. 8A, lanes 1-5), and the anti-GR antibody (BuGR) caused a farther shift confirming the binding of GR to this element. Excess unlabeled In-1c also inhibited binding of rGR-DBD to labeled In-1c (data not shown). These results demonstrated that fragment In-1c contains recognition sequences for AR and GR. However, by comparison with the strong binding to the C3 gene first intron ARE/GRE, C3-C (10) binding of AR and GR to In-1c was weaker (Fig. 8B, lanes 11-14). We also compared rAR-DBD and rGR-DBD binding to the D1 and D2 subfragments of In-1c and the N39 sequence located at the 5′ end of D1 (Fig. 8, A and B). D1, D2, and N39 all bound both AR and GR as indicated by the shifted complexes in the presence of receptor antibody. However, using amounts of protein and antibody that yielded similar AR and GR binding to C3-C, binding of GR to In-1c and its subfragments was less than that of AR, especially with D2 and N39 (Fig. 8, A and B).

Discussion

The 20-kDa protein gene was cloned from a rat genomic DNA library and found by sequence analysis to contain four exons, a large intron 1 and smaller introns 2 and 3. Within intron 1 is a region containing multiple palindromic GRE-like sequences, suggesting that it might function as a
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FIG. 8. Androgen and glucocorticoid receptor binding to In-1c and its sequences D1, D2, and N39. Each binding reaction contained 32P-labeled probe (10,000 cpm) and 0.5 μg of poly(dIC-dC) in a 20-μl total volume. Recombinant rAR-DBD or rGR-DBD partially purified from E. coli was incubated with labeled probe and loaded onto 4% (panel A) or 5% (panel B) nondenaturing polyacrylamide gels. Panel A: lanes 1–5 contained 32P-labeled In-1c; lanes 6–10 contained 32P-labeled D1; lanes 1 and 8, free labeled probe; lanes 2, 3, 7, and 8, 1.0 μg of total protein of rAR-DBD; lanes 4, 5, 9, and 10, 2.0 μg of total protein of rGR-DBD; lanes 5 and 10, 2 μl of a 1:5 dilution of BuGR antiserum. Panel B: lanes 1–5 contained 32P-labeled D2; lanes 6–10 contained 32P-labeled N39; lanes 11–14 contained 32P-labeled C3-C; lanes 1 and 6, free labeled probe; lanes 2, 3, 7, 8, 11, and 12, 1.0 μg of total protein of rAR-DBD; lanes 4, 5, 9, 10, 13, and 14, 2.0 μg of total protein of rGR-DBD; lanes 5, 8, and 12, 1.2 μg of protein of antibody AR-52; lanes 5 and 10, 2 μl of a 1:5 dilution of BuGR antiserum. Note that for assays with In-1c and its subfragments, AR-DBD and GR-DBD and their corresponding antibodies were used in the same amounts in A and B as those used for C3-C.

Complex androgen response element. Intron 1 fragment In-1c was a stronger androgen-dependent enhancer in CV1 cells than two other first intron regions, In-1a and In-1b, each selected by the presence of GRE-like sequences. GRE-like sequences were noted also in the 5' flanking region; however, this region was less responsive than either the large first intron fragment In-1 or its subfragment In-1c. Low levels of androgen-stimulated CAT activity were observed with two reporter constructs each containing fragments of the 5' flanking region in ptkCAT. However, the 5' flanking DNA (−901 to +8) with its own promoter (cloned in pCAT) was not an effective androgen-dependent enhancer of transcription. We observed a similar lack of androgen regulation of the C3 subunit gene promoter in CV1 cells (10).

Several features of the first intron element In-1c are noteworthy. Although In-1c bound both AR and GR in the DNA mobility shift assay, only AR induced a transcriptional response in CV1 cells. A cluster of GRE-like sequences, N39, consisting of a 15-bp partial palindrome and two right half-sites, when isolated from In-1c functioned as a weak ARE or GRE, although it bound AR better than GR. Thus the selective ARE activity of In-1c was likely determined by AR interactions with other nucleotide sequences or with one or more nonreceptor regulatory proteins interacting outside the GRE-like cluster. In CV1 cells, ARE specificity of the D1 and D2 subfragments of In-1c was retained even though they also bound AR and GR. However, transfection of reporter vectors containing In-1c, D1, or D2 into PC3 and HeLa cells revealed differences in their transcriptional responses. In PC3 and HeLa cells, 1c and D2 were less responsive to AR than in CV1 cells. In-1c also mediated a weak response to GR in these cells, whereas D2 remained AR-specific. D1 was a weaker ARE than In-1c or D2 in all three cell lines. It exhibited GRE activity only in PC3 cells and at a lower level than ARE activity. The selective AR responsiveness of D2 in the three different cell lines suggests that it is intrinsically AR-specific and is consistent with its stronger binding of AR than GR. However, the AR specificity of In-1c was confined to CV1 cells. Thus, the In-1c subfragments, D1 and D2, combined to make In-1c a stronger ARE than GRE in PC3 and HeLa cells. The relatively weak GRE activity of In-1c could result from a permissive factor for GR transactivation which is not present in CV1 cells (34). The factor might be a specific GR coactivator or an inhibitor of a selective GR suppressor.

The absence of In-1c GRE activity in CV1 cells and its presence in HeLa cells are reminiscent of studies on a composite GRE of the proliferin gene that contains an AP1 recognition sequence and responded with repression or stimulation of reporter gene expression depending on the relative amounts of c-Jun and c-Fos in the cell (16, 35-39). In CV1 cells, repression of GR transactivation was linked to the predominance of Fos/Jun dimers, whereas in the HeLa cells, GR-dependent enhancement of reporter gene expression was related to the presence of AP1 as Jun/Jun homodimers (16). In-1c mimics this pattern of cellular response in that it is a GRE in HeLa cells but not in CV1 cells. However, subfragment D2, which contains both AP1 and CREB recognition sites, did not mediate a response to GR in either HeLa or CV1 cells, suggesting that factors other than or in addition to Jun and Fos may influence the receptor specificity of this complex element.

AR binding to In-1c and its subfragments D1 and D2 was weaker than to the 15-bp partial palindrome ARE within intron 1 of the C3 subunit gene (10). Low receptor binding activity is a feature of the complex ARE controlling the mouse sex-limited protein gene (17) and of complex response elements described for GR (15, 16). GRE-like sequences within In-1c, although not tested individually, would be considered weak GREs according to the nucleotide sequence criteria of Nordeen et al. (40). In general, it has been found that within this family of simple response elements the 15-bp partial palindromes have both ARE and GRE activities (7, 14). Based on a consensus sequence (10, 14, 40), the strongest simple elements of In-1c are in the 39-bp sequence at the 5' end of the D1 subfragment, and they too were relatively weak as an isolated unit when analyzed in CAT assays. The presence of strong individual response elements within a complex element may allow for modulation by different factor interactions (17),...
thus enabling the element In-1c to mediate AR-specific transcription in CV1 cells and yet transact responses to both AR and GR in PC3 and HeLa cells. Interestingly, D2 was a stronger ARE than D1 yet lacks a sequence conforming to that of a consensus ARE/GRE (7, 12, 40). The closest to an ARE/GRE-like 15-bp partial palindrome is GTCTACTTTTGTAAAC (nucleotides 2614–2600, 5' to 3' on the antisense strand). It remains to be determined if this is an effective AR binding site.

Windericks et al. (23) demonstrated that androgen regulation of 20-kDa protein gene expression requires protein synthesis. The testosterone-stimulated increase in 20-kDa protein mRNA was blocked by cycloheximide, indicating that rapidly turning over factors might interact with AR and GR to control transactivation. The multiplicity of potential controlling elements in In-1c provides a structural framework for cooperativity among ARE/GRE-like sequences as well as interactions with transcription control sequences (Tables I and II). Functional cooperativity was demonstrated among GREs and recognition sequences for several transcription-regulating proteins including NF1, SP1, CCAAT box, OTF, and CACCC box-binding proteins (41–43). NF1 appears to be the subject of intensive investigation in future years. It remains to be determined if this is an effective AR binding site.

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REFERENCES

1. Yamamoto, K. R. (1985) Annu. Rev. Genet. 19, 209-252
2. Evans, R. M. (1988) Science 240, 889-896
3. Beato, M. (1989) Cell 56, 335-344
4. Franzen, L. P. (1992) Endocr. Rev. 13, 129-144
5. Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J.-A., Higgs, H. N., Larson, R. E, French, F. S., and Wilson, E. M. (1988) Mol. Endocrinol. 2, 1265-1275
6. Cato, A. C. B., Henderson, D., and Ponta, H. (1987) EMBO J. 6, 363-368
7. Han, J., Thomson, A., Needham, M., Webb, P., and Farkas, M. (1988) Science 240, 889-895
8. Denison, S. H., Sands, A., and Tindall, D. J. (1989) Endocrinology 124a, 1091-1093
9. De Vos, P., Claassens, P., Windericks, J., Van Dijck, P., Celis, L., Peeters, B., Rombaums, W., Heyns, W., and Vemaenin, E. (1991) J. Biol. Chem. 266, 3429-3443
10. Tan, J.-A., Marschke, K. B., Ho, K.-C., Perry, S. T., Wilson, E. M., and French, F. S. (1992) J. Biol. Chem. 267, 4460-4466
11. Rieger, P. H. J., Vlieistra, R. J., van der Korput, J. A. G. M., Brinkmann, A. O., and Treppe, J. (1991) Mol. Endocrinol. 5, 1921-1930
12. Rennie, P. S., Bruchovsky, N., Lenci, K. J., Sheppard, P. C., McQueen, S. A., Cheng, H., Snoek, R., Hansel, A., Bock, M. E., MacDonald, B. S., Nickel, B. E., Chang, C., Liao, S., Cattini, P. A., and Matsuji, R. J. (1990) Mol. Endocrinol. 4, 148-157
13. Claassens, F., Celis, L., Peeters, B., Heyns, W., Verboeven, G., and Rombaums, W. (1989) Biochem. Biophys. Res. Commun. 164, 835-840
14. Marschke, K. B., Tan, J.-A., Wilson, E. M., and French, F. S. (1993) in Perspectives in Endocrinology (DeBelliis, A., and Marschke, K. B., ed) Vol. 9, pp. 41-50, Raven Press, New York
15. Imai, E., Stromstedt, P. E., Quinn, P., G., Carlstedt-Duke, J., Gustafsson, J. A., and Graner, D. K. (1990) Mol. Cell. Biol. 10, 4712-4719
16. Diamond, M. I., Mittler, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266-1277
17. Adler, A. J., Scheller, A., Hoffman, Y., and Robin, M. D. (1991) Mol. Endocrinol. 5, 1057-1066
18. Adler, A. J., Danelsen, M., and Robbins, D. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11680-11685
19. Lund, D. S., Gallagh, P. M., Yang, B., Porter, S. C., and Ganschow, R. E. (1991) Mol. Cell Endocrinol. 11, 5427-5434
20. Langer, A. J., Esipchin, C., Hall, R., El-Maghrabi, M. R., Vaross, A. M., Mi Zacsek, K. J., Graner, D. K., and Pilak, S. J. (1992) J. Biol. Chem. 267, 10673-10680
21. Kostons, T., Williams, P. M., DiLenore, D., and Ringold, G. M. (1991) J. Biol. Chem. 267, 11311-11319
22. Ho, K.-C., Shouk, R., Quarany, V. E., Viaskovich, D. H., Rennie, P. S., Marschke, K. B., French, F. S., Bruchovsky, N. (1989) Biochemistry 28, 6373-6377
23. Windericks, J., Henschoote, K., De Cleyn, N., Van Dijck, P., Peeters, B., Rombaums, W., Verboeven, G., and Heyns, W. (1990) Mol. Endocrinol. 4, 657-667
24. Verasen, R., Windermers, J., debo, A. Peeters, B., and Heyns, W. (1992) Endocrinology 131, 2496-2502
25. Maniata, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 525-530, and 197-198, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
27. Ho, K.-C., Quarany, V. E., French, F. S., and Wilson, E. M. (1992) J. Biol. Chem. 267, 12660-12666
28. Chmeczenski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159
29. Schule, R., Muller, M., Otsuka-Murakami, H., and Renkawitz, R. (1988) Nature 333, 87-90
