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Specificity of simple hormone response elements in androgen regulated genes

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Androgen (AR) and glucocorticoid (GR) receptors recognize a family of 15 base pair partial palindromic hormone response elements (HRE). We have studied receptor interactions with several HREs from androgen regulated genes to determine their potential to mediate a selective androgen response. Synthetic oligonucleotides corresponding to the elements were analysed for receptor binding and steroid dependent transcriptional enhancer activities. Each HRE contained the 3' half-site sequence (5'-TGTNCT-3') of the glucocorticoid response element (GRE) consensus sequence. HREs that countained the 5' half-site GRE consensus sequence (5'-A/GGNACA/G-3') had the strongest androgen response element (ARE) and GRE activities. In methylation interference assays, AR and GR interacted with identical base contact sites in the response elements. Two elements that deviated from the GRE consensus sequence by a single optimal base in the 5' half, had reduced ARE activity with no significant change in GRE activity and displayed lower binding of AR than GR in mobility shift assays using purified DNA binding domain peptides. Transfections with AR/GR and GR/AR chimeras containing the N-terminal domain of one receptor linked to the DNA-binding and C-terminal domains of the other suggested that N-terminal domain functions of GR also contributed to the greater GRE than ARE activities of the response elements.

Keywords: hormone response elements; androgen receptor; androgens; glucocorticoids; gene transcription; androgen response elements

Introduction

The androgen receptor (AR) belongs to the family of nuclear receptors that function as ligand-dependent transcription factors. These receptors enhance transcription of target genes by binding to specific nucleotide sequences termed hormone response elements (HREs). Steroid receptors have a modular structure defined by functional domains (Green & Chambon, 1987; Evans, 1988; Lubahn *et al.*, 1988a,b; Simental *et al.*, 1991; Carson-Jurica *et al.*, 1990). The centrally located DNA-binding domain mediates specific recognition of the HRE and is comprised of two zinc-binding motifs each formed by four cysteine residues. AR is one of a subfamily of steroid receptors of similar size and sequence similarity within their DNA binding domains that includes the glucocorticoid (GR), progesterone (PR) and mineralocorticoid (MR) receptors (Evans, 1988; Lubahn *et al.*, 1988a,b; Danielsen *et al.*, 1989; Carson-Jurica *et al.*, 1990). The glucocorticoid or progesterone response element (GRE/PRE) consensus sequence (5'-A/GGNACAnnnTGTNCT-3') is a partial palindrome of 15 base pairs arranged as two six base pair half-sites separated by a three base pair spacer (Beato, 1989; Nordeen *et al.*, 1990). GREs within the long terminal repeat of the mouse mammary tumor virus (MMTV-LTR) and tyrosine aminotransferase gene also mediated transcriptional responses to AR (Ham *et al.*, 1988; Denison *et al.*, 1989; Rundlett *et al.*, 1990) suggesting that AR shares a common HRE motif with GR and PR. GR and PR contact identical bases within the HRE; however, the contact points of AR have not been determined (Cairns *et al.*, 1991; Truss & Beato, 1993). GRE-like sequences identified in several androgen and glucocorticoid regulated genes, when isolated, function as androgen response elements (AREs) (Denison *et al.*, 1989; Claessens *et al.*, 1990; Rundlett *et al.*, 1990; Adler *et al.*, 1991; Tan *et al.*, 1992; Ho *et al.*, 1993; Rennie *et al.*, 1993). Using a DNA-binding site selection assay for random sequence oligonucleotides, Roche *et al.*, (1992) identified an AR binding sequence (5'-GGA/TACAnnnTGTTCT-3') that closely resembles the consensus GRE. Factors that could contribute to relative levels of response to androgens and glucocorticoids, where AR and GR recognize the same element, are ligand availability, receptor concentration and affinity for the response element. Moreover, other transcription control factors that interact with AR or GR likely influence differential gene regulation by enhancing or repressing receptor function.

In this report we compare several GRE-like sequences located in androgen regulated genes by analysis of AR and GR binding and transactivation. Two elements that deviated from the consensus 5' GRE by a single base had reduced ARE activity without loss of GRE activity.

Results

Analyses of response elements utilized synthetic oligonucleotides containing GRE-like sequences from five different androgen responsive genes (Figure 1) : C3-A, located 5' of the promoter and C3-B and C3-C in the first intron of the C3

	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7
Consensus GRE	A/G	G	N	A	C	A	N	N	N	T	G	T	N	C	T
MMTV 5'GRE	G	T	T	A	C	A	A	A	C	T	G	T	T	C	T
C3-A	T	G	A	A	A	C	C	A	G	T	G	T	T	C	T
C3-B	T	A	G	C	C	A	A	G	T	T	G	T	T	C	T
C3-C	A	G	T	A	C	G	T	G	A	T	G	T	T	C	T
C2	T	G	A	C	T	C	A	A	T	T	G	T	T	C	T
Probasin	A	T	A	G	C	A	T	G	A	T	G	T	T	C	T
GUS	A	G	T	A	C	T	T	G	T	T	G	T	T	C	T
GH	G	G	C	A	C	A	A	T	G	T	G	T	C	C	T

Figure 1 HRE sequences from androgen responsive genes. Oligonucleotides were synthesized corresponding to GRE-like sequences from the MMTV-LTR (MTV 5' GRE), the C3 subunit gene (C3-A, C3-B and C3-C) and C2 subunit gene (C2) of prostatein, rat probasin gene promoter (Probasin), mouse β-glucuronidase gene (GUS) and the human growth hormone gene (GH). The consensus GRE sequence and the bases most important for GRE activity (indicated by stars) are from: Beato (1989); Nordeen *et al.* (1990); Ham *et al.* (1988); Cairns *et al.*(1991); Truss & Beato, (1993). Bases identical to those in the consensus sequence are boxed

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subunit gene of prostatein (Tan *et al.*, 1992); C2, 5′ of the promoter of the C2 subunit gene of prostatein; Probasin, AR binding site 1 in the 5′ flanking region of the probasin gene (Rennie *et al.*, 1993, Kasper *et al.*, 1994); GUS, in the ninth intron of the mouse β-glucuronidase gene (Lund *et al.*, 1991); GH in the first intron of the human growth hormone gene (Moore *et al.*, 1985). These were compared with MMTV 5′ GRE, the 5′ most GRE of the MMTV LTR (Truss & Beato, 1993). All elements share the core GRE 3′ half site (TGTT/CCT), but differ in the 5′ half site. Each contains five or more of the bases considered optimal for GR binding and transcriptional enhancer activity (Figure 1) (Ham *et al.*, 1988; Beato, 1989; Nordeen *et al.*, 1990; Cairns *et al.*, 1991; Truss & Beato, 1993). Oligonucleotides were cloned upstream of the thymidine kinase promoter in the reporter vector, ptkCAT (Schule *et al.*, 1988; Ho *et al.*, 1993) for determination of ARE and GRE enhancer activities and were analysed in the mobility shift assay for AR and GR binding.

Transcriptional activation assays of HREs with AR and GR

CV1 cells were cotransfected with either a full length rat AR (pCMVrAR) (Tan *et al.*, 1992) or rat GR (pCMVrGR) Kupfer *et al.*, 1993) expression vector and the reporter vectors described above. With the MMTV 5′ GRE, optimal stimulation of CAT activity was observed with 0.1 nM R1881 and 10 nM dexamethasone, respectively (data not shown). These steroid concentrations were used for further analyses. MMTV 5′ GRE, C3-C, GUS, GH and Probasin, but not C3-A, C3-B or C2 oligonucleotides, mediated R1881-induced and dexamethasone-induced increases in CAT activity in CV1 cells cotransfected with rAR or rGR (Figure 2). AR and GR induced enhancer activities of C3-C and GH were similar, while induction by GR was significantly greater than AR with MMTV 5′GRE ($P<0.05$) and GUS ($P<0.01$). In the absence of pCMVrAR or pCMVrGR, no detectable stimulation of CAT enzyme activity was observed with the ptkCAT reporter vectors in the presence of R1881 or dexamethasone, respectively (data not shown).

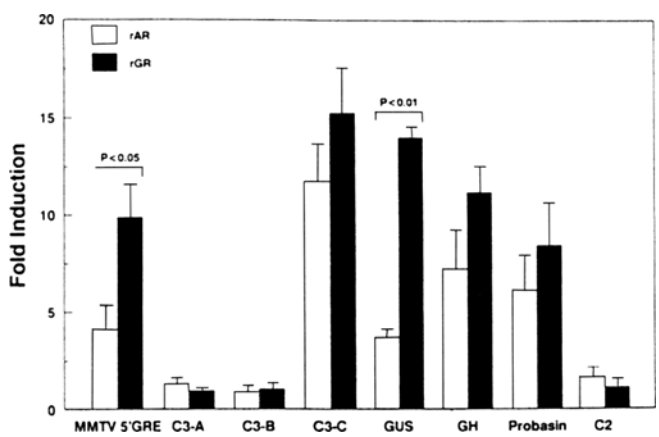

Figure 2 Androgen and glucocorticoid induced enhancer activities of HRE sequences. CV1 cells were transiently transfected with either 1 μg of pCMVrAR or pCMVrGR expression vector and ptkCAT reporter vector (5 μg) containing one of the synthetic oligonucleotides cloned upstream of the thymidine kinase promoter. Cells were incubated for 48 h in the presence or absence of 0.1 nM R1881 for AR or 10 nM dexamethasone for GR. Induction of CAT activity was maximal at these concentrations that reflect, in part, differences in receptor binding affinities of these hormones. CAT assays were performed as described in Materials and methods. Fold inductions are the means ± standard errors of five assays and were calculated from the percent acetylation of ^{14}C-chloramphenicol in extracts of CV1 cells incubated in the presence and absence of steroid. Differences in fold inductions between receptors for each sequence were determined by the paired *t*-test

HRE binding of AR and GR DNA binding domain peptides

To determine whether induction of CAT activity was related to differences in AR and GR binding to these elements, recombinant rat AR and rat GR DNA-binding domain (DBD) proteins (rAR-DBD, amino acids 496–665 and rGR-DBD, amino acids 407–556) were expressed in *E. coli* (Figure 3A). rAR-DBD and rGR-DBD contained epitopes recognized by receptor specific antibodies AR-52 (Quarmby *et al.*, 1990) and BUGR-2 (Gametchu & Harrison, 1984), respectively. Receptor proteins also contained N-terminal histidine residues which facilitated partial purification on a nickel-containing affinity resin (Figure 3B and C). Purification to near homogeneity was achieved by subsequent chromatography on a C3-C oligonucleotide affinity column, as shown by silver staining (Figure 3B) or immunoblotting (Figure 3C).

Receptor peptides, partially purified by nickel affinity resin, were incubated with ^{32}P end-labelled oligonucleotides and binding was analysed using the mobility shift assay. C3-C and GH formed strong complexes with rAR-DBD and rGR-DBD, while binding to MMTV 5′ GRE, GUS and Probasin was weaker (Figure 4). Secondary shifts in mobility observed with addition of AR-52 or BUGR-2 antibodies confirmed the presence of receptor protein-DNA complexes. Receptor:HRE

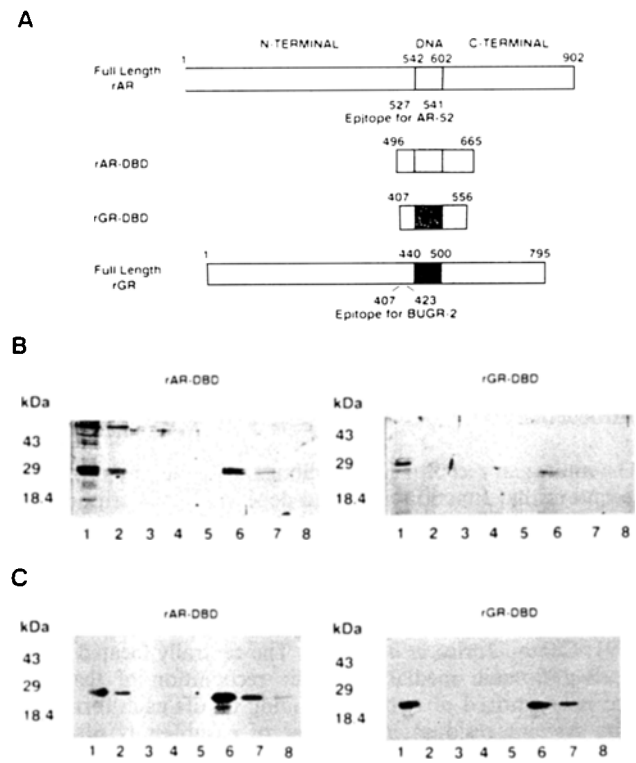

Figure 3 Expression and Purification of Recombinant rAR and rGR DNA-binding Domain Proteins in *E. coli*. (A) Rat cDNAs encoding amino acids 496–665 of rAR or amino acids 407–556 of rGR were cloned into the T7 RNA polymerase dependent expression vector, pET-16b, for expression in *E. coli*. The DNA-binding domain proteins include epitopes recognized by the AR antibody, AR-52, and the GR antibody, BUGR-2. Recombinant DNA-binding domain proteins were expressed in *E. coli* and purified by sequential affinity chromatography. Purified proteins were resolved on 15% SDS polyacrylamide gels and visualized by silver-staining (B) or immunoblotting using AR-52 or BUGR-2 (C). Lane 1, peak fractions from Ni^{2+}-affinity resin (His-Bind); lane 2, flow-through from C3-C DNA affinity resin; lane 3, low salt wash of DNA affinity resin; lanes 4–8, fractions from high salt elution of DNA affinity resin. Molecular weights correspond to the positions of prestained standards. Predicted molecular weights of rAR-DBD and rGR-DBD are approximately 25 kDa and 23 kDa, respectively

complexes were enhanced in the presence of antibody, presumably due to stabilization of the complexes (Tan *et al.*, 1992). rGR-DBD formed both monomer and dimer complexes similar to those observed by Alroy & Freedman (1992), while monomer rAR-DBD complexes were either weak or undetectable. C3-A, C3-B and C2 binding of rAR-DBD and rGR-DBD could be detected only in the presence of antibody (Figure 4). The sparsity of monomer GR complexes with the weaker elements (consensus right half site only) suggested that GR bound the stronger palindromic elements as a dimer and the monomer complexes (Figure 4) resulted from dissociation of dimers.

To compare the binding of rGR-DBD and rAR-DBD to C3-C, GH (Figure 5A) MMTV5′ GRE and GUS (Figure 5B) saturation analysis was performed using more highly purified receptor preparations. Binding curves for rGR-DBD and rAR-DBD were similar with C3-C and GH, however with MMTV5′ GRE and GUS the binding of rAR-DBD was somewhat lower than GR-DBD. The similar binding of AR and GR-DBD to C3-C in our studies differed from the quantitative footprinting of C3 intron-1 (Rundlett & Miesfeld, 1995) that showed a twofold higher affinity of GR than AR for the HRE.

Sites of AR and GR interactions with HREs determined by methylation interference

Contact sites of rAR-DBD and rGR-DBD with MMTV 5′ GRE and C3-C were compared by methylation interference. C3-C was analysed because of its strong ARE activity (Tan *et al.*, 1992), and MMTV 5′ GRE because it was used previously to compare contact points of GR and PR (Cairns *et al.*, 1992; Truss & Beato, 1993). Methylation of G residues at four positions each indicated by an asterisk (Figure 6), in the top and bottom strands of MMTV 5′ GRE and C3-C interfered with rAR-DBD and rGR-DBD binding indicating they are essential sites for receptor interaction. However, the G at −2 in the top strand of C3-C (an A in MMTV 5′ GRE) did not interfere with binding when methylated and therefore is likely not required for AR or GR recognition. Methylation of Gs within the spacer regions also had no effect on AR binding in agreement with observations that the base composition of the spacer region is of relatively little importance to response element function (Ham *et al.*, 1988; Nordeen *et al.*, 1990; Roche *et al.*, 1992).

Functional assays of HREs with chimeric receptors

The amino-terminal domains of AR (Simental *et al.*, 1991) and GR (Evans, 1988; Beato, 1989; Jurica *et al.*, 1990; Gronemeyher, 1991; Rennie *et al.*, 1993) contain transcriptional activation functions but share little primary amino acid sequence similarity. Thus differences in AR and GR

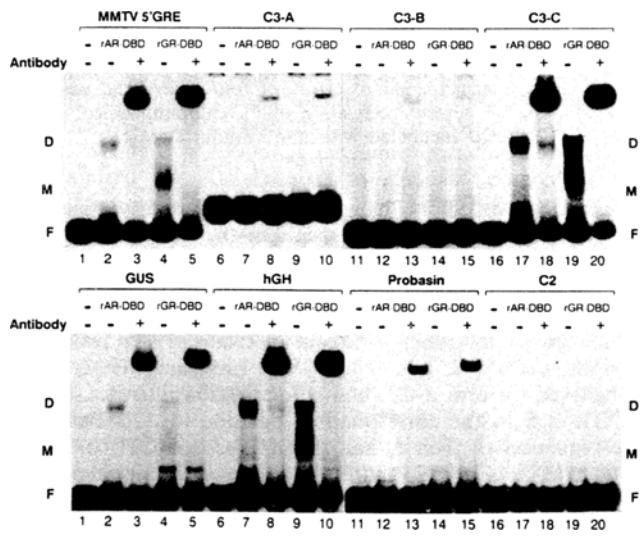

Figure 4 Binding of recombinant rAR and rGR DNA-binding domain peptides to GRE-like sequences. Mobility shift DNA binding assays were performed with recombinant rAR and rGR DNA-binding domain peptides partially purified by His-Bind affinity chromatography (Ni^{2+}-affinity resin) and ^{32}P end-labeled HRE probes. Probes were prepared as 50 base pair restriction fragments from inserts of ptkCAT reporter vectors containing the indicated GRE-like sequences. Proteins were incubated with ^{32}P-labeled probes (10 000 c.p.m.) in the presence or absence of receptor specific antisera and protein-DNA complexes were resolved on 6% nondenaturing polyacrylamide gels as described in Materials and methods. Lanes 1,6,11 and 16, no receptor DBD protein; lanes 2,7,12,17, contained 0.25 μg rAR-DBD; lanes 3,8,13,18,0.25 μg rAR-DBD and 1.0 μg of AR-52 IgG fraction; lanes 4,9,14,19,0.5 μg rGR-DBD; lanes 5,10,15,20,0.5 μg rGR-DBD and 1:2 dilution of BuGR-2 antiserum. F indicates unbound (free) probe; M and D indicate bound monomeric and dimeric complexes, respectively

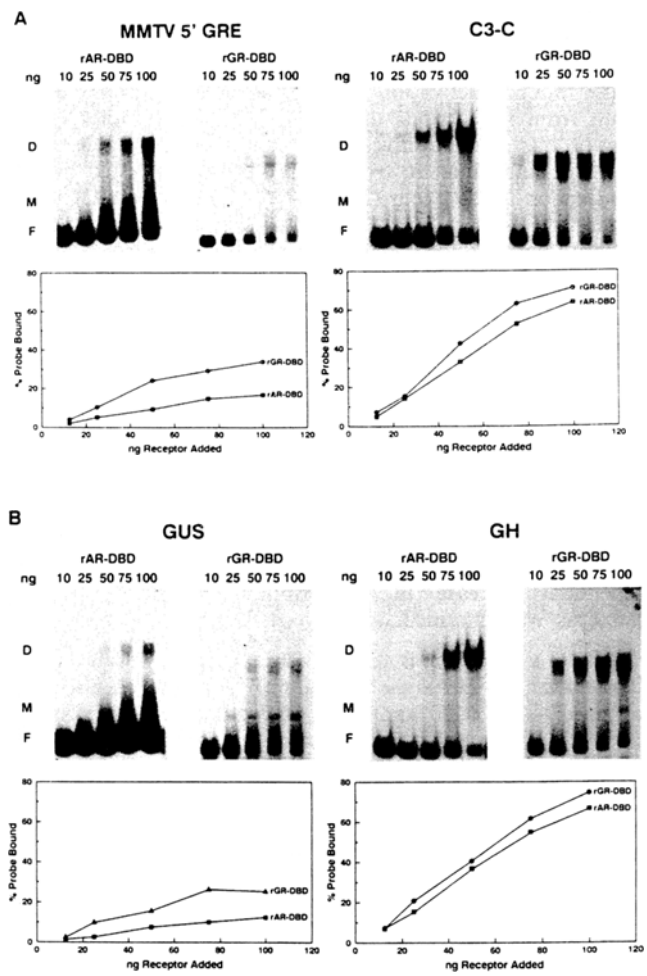

Figure 5 Saturation binding analysis of purified rAR-DBD and rGR-DBD to HRE sequences. Mobility shift assays of purified rAR-DBD and rGR-DBD binding to MMTV 5′ GRE and C3-C (A), and GUS and GH (B) sequences were performed as described in Figure 4. Receptor DBD peptides were purified by sequential affinity chromatography on His-Bind and C3-C DNA affinity resins as shown in Figure 3. The total amount (ng) of receptor protein in each binding reaction is indicated above the lanes. Equal amounts of ^{32}P-HRE were used. F, Free DNA probe; M, DNA bound by a monomer; D, DNA bound by a dimer. Individual bands were excised from the gel and counted in a scintillation counter. The graphs of these counts show the proportion of ^{32}P end-labeled DNA probe bound by a dimer as a percentage of total probe added to the reaction. Autoradiograms are not directly comparable with the graphs since the exposure times were not identical

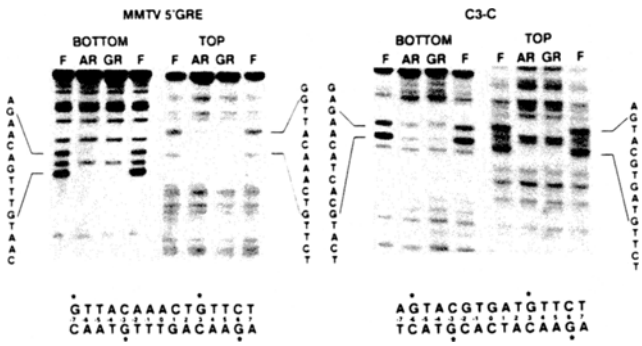

Figure 6 Methylation interference assay to determine DNA contact sites of rAR-DBD and rGR-DBD. Partially purified rAR-DBD and rGR-DBD were incubated with MMTV 5′ GRE or C3-C containing DNA fragments labeled at the 5′ or 3′ ends with ^{32}P and methylated by treatment with dimethylsulfate. Free (F) and receptor-bound DNA (AR, GR) were separated by mobility shift assay. DNA was eluted from the gel, cleaved at methylated residues with piperidine and loaded on an 8% denaturing polyacrylamide gel. Lines and *designate nucleotides in which methylation interfered with receptor binding

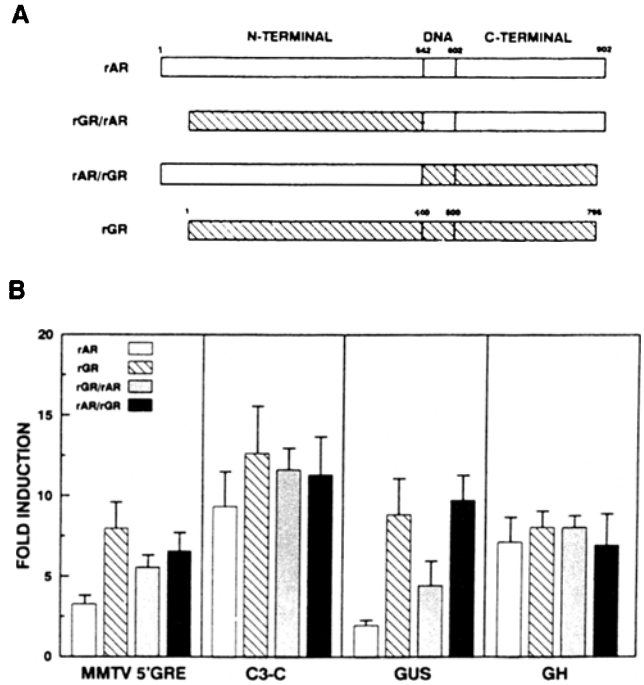

Figure 7 Transcriptional activation by rAR and rGR chimeric proteins through HRE sequences. (A) Chimeric receptor expression vectors pCMVrGR/rAR and pCMVrAR/rGR were constructed by combining the N-terminal domain of rGR (amino acid codons 1–446) with the DNA- and steroid-binding domains of rAR (amino acid codons 549–902) or the N-terminal domain of rAR (amino acid codons 1–548) with the DNA- and steroid-binding domains of rGR (amino acid codons 447–795), respectively. (B) CV1 cells were transiently transfected with 1 μg of either pCMVrAR, pCMVrGR, pCMVrGR/rAR or pCMVrAR/rGR expression vector and ptkCAT reporter vector (5 μg) containing one of the GRE-like sequences indicated. Cells were incubated for 48 h in the presence or absence of 10 nM R1881 for AR or 10 nM dexamethasone for GR. CAT assays were performed as described in Materials and methods. Fold inductions are the means ± standard errors of four assays and were calculated from the percent acetylation of ^{14}C-chloramphenicol in extracts of CV1 cells incubated with and without steroid

transcriptional activation through a common HRE might result from interactions of these domains with other transcription factors. To investigate this possibility, full-length wild-type and chimeric receptor proteins were tested by co-transfection in CV1 cells (Figure 7). One chimera, rAR/rGR, contained the rat AR N-terminal domain (amino acids 1–548) linked to the rat GR DNA and C-terminal domains (amino acids 447–795). The other chimera, rGR/rAR, contained the rGR N-terminal domain (amino acids 1–446) linked to the rAR DNA and C-terminal domain (amino acids 549–902). Both chimeras functioned as ligand-inducible transcription factors and increased CAT activity in the presence of the appropriate ligand, dexamethasone or R1881 (Figure 7). With C3-C and GH, elements that were equally strong with wild-type AR and GR, induction of CAT activity by the two chimeric receptors was similar. GUS mediated induction of CAT activity by the chimera AR/GR was like wild-type GR and greater than that induced by GR/AR or wild-type AR. However, with MMTV 5′ GRE, the steroid dependent increase in transcription stimulated by the chimeras was intermediate between that of AR and GR.

Discussion

Simple HRE sequences from androgen regulated genes and the strongest HRE of the MMTV-LTR were compared with respect to ARE and GRE activities. The stronger AREs, C3-C, GH and Probasin had similar GRE activities, while GUS and the MMTV 5′ GRE were stronger GREs than AREs. The elements C3-C, C3-B and C2 were equally weak as AREs or GREs. Within this group as a whole, binding of receptor DNA binding domains to response elements correlated with ligand-receptor dependent transactivation (C3-A, C3-B and C2 *vs* C3-C, GH, GUS and Probasin). The lower ARE than GRE activities of MMTV 5′ GRE and GUS related to their weaker binding of AR than GR suggesting that receptor binding contributed to the differential activities of these HREs. Our results agree with the report of DeVos *et al.* (1993) and Rundlett & Miesfeld (1995) on AR and GR footprinting of the C3 gene and together with earlier studies on rGR (Cairns *et al.*, 1991; Truss & Beato, 1993) demonstrate that AR and GR DNA binding domains interact with the same G residues and bind to both half sites of the MMTV 5′ GRE and C3-C.

It is noteworthy from the crystal structure of the GR-DBD/GRE complex that all of the amino acids shown to make direct base-pair or phosphate backbone contacts are identical in GR and AR (Luisi *et al.*, 1991; Freedman, 1992; Truss & Beato, 1993) (Figure 8). Sequence identity exists also between AR and GR amino acids in the region of the first zinc finger that imparts specificity of HRE recognition (Danielsen *et al.*, 1989). There is a difference between GR and AR in the D-box (Figure 8) (Lubahn *et al.*, 1988a,b; Danielsen *et al.*, 1989; Umesona & Evans, 1989; Dahlman-Wright *et al.*, 1991; Luisi *et al.*, 1991; Freedman, 1992) which is believed to form a GR homodimerization interface. G 478 in GR is S at the corresponding position in AR. Based on the sequences of their 5′ half-sites (C3-C and GH compared with MMTV 5′ GRE, and GUS) it would appear that T at −6 or −2 reduces AR relative to GR binding (Figure 1). The G at −6 in C3-C is a base contact site and both position −6 and −2 are close to phosphate backbone interaction sites of GR, amino acids Y452 and Y474 (Umesono & Evans, 1989; Freedman, 1992; Truss & Beato, 1993). Three amino acids adjacent to Y474 in GR, QHN (471–473) are KQK (590–592) in AR (Figure 8).

Examination of the different oligonucleotide sequences shown in Figure 1 suggests that optimal bases for GRE activity in the 5′ half site are also important for ARE activity. This is in agreement with GRE analysis by mutagenesis (Nordeen *et al.*, 1990) and the consensus ARE identified by Roche *et al.* (1992). In the studies of Nordeen *et al.* (1990) changing single bases optimal for GRE activity (Figure 1) in the left half of the MMTV 5′ GRE to those in the corresponding positions of C3-A (positions −2, −3, or −7), C3-B (positions −4 or −7), or C2 (positions −2, −3, or −4) greatly reduced GRE activity. These sequences lacked

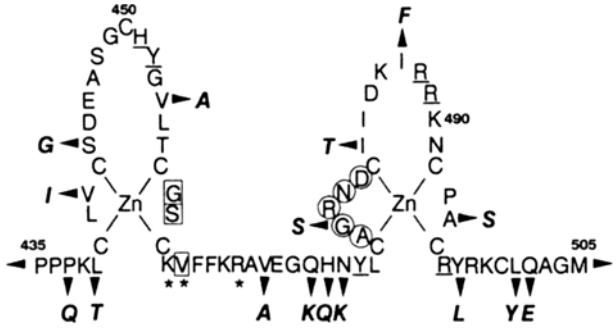

Figure 8 Amino acid differences between rAR and rGR DNA-binding domains. The amino acid sequence of the rGR DNA-binding domain is shown with the positions of zinc atoms. Numbering is based on full length rGR (Freedman, 1992). Amino acids in rAR different from rGR are indicated by arrowheads and bold, italicized letters. Amino acids in rGR that confer specificity of recognition (Danielsen *et al.*, 1989) are boxed. Those within the dimerization interface or D box are in circles (Dahlman-Wright *et al.*, 1991; Freedman, 1992). rGR residues making direct base contacts are indicated by asterisk; those making specific phosphate backbone contacts are underlined (Luisi *et al.*, 1991; Freedman, 1992; Truss & Beato, 1993)

both GRE and ARE activities in the present study. Comparison of MMTV 5′ GRE and GUS with other sequences in the left half of the palindrome (Figure 1) illustrated substitutions of pyrimidines for purines at positions −1 and −6 that reduce ARE relative to GRE activity.

Although AR and GR-DBD contact the same G residues in methylation interference assays, monomer GR-DBD formed HRE complexes better than did monomer AR-DBD suggesting a difference in the way these two receptor DNA binding domains interact with HREs. In agreement with this result Rundlett & Miesfeld (1995) reported recently that GR-DBD had a greater ability to form monomer:HRE complexes than did AR-DBD. Similarly full-length GR binds the HRE as a monomer or dimer, although predominately as dimer (Drouin *et al.*, 1992) whereas full-length AR binds only as a dimer (Wong *et al.*, 1993). Thus, dimer formation appears to be required for AR binding to response element DNA. Strong homodimer but not monomer-HRE binding was also observed with AR-DBD expressed in the baculovirus system (C.I. Wong & E.M. Wilson, unpublished). With GR, two types of interactions with the HRE have been suggested: (1) primary binding of monomers to the stronger 3′ half-site of the HRE followed by cooperative enhancement of dimerization and binding of a second monomer to the 5′ half-site (Green & Chambon, 1987); and (2) direct binding of homodimers (Tsai *et al.*, 1988; Dahlman-Wright *et al.*, 1990; Gronemeyer, 1991; Drouin *et al.*, 1992; Truss & Beato, 1993). The apparent lower capacity of AR than GR binding to MMTV 5′ GRE and GUS may be related in part to the inability of AR to interact as a monomer. Monomer GR binding to DNA might enhance the reformation of dimers and contribute to the stability of GR-DBD:HRE complexes.

Because steroid receptors have distinct functional domains (Green & Chambon, 1987; Evans, 1988; Lubahn *et al.*, 1988a,b; Carson-Jurica *et al.*, 1990; Simental *et al.*, 1991), it was possible to create chimeric receptors to test the relative effects of N- and C-terminal domains on transactivation. With the weaker AR binding elements, MMTV 5′ GRE and GUS, activities of both chimeras were somewhat greater than that of wild-type AR. The greater effect of AR/GR than wild-type AR with GUS correlated with the DNA binding of wild-type GR versus AR DNA binding domains in mobility shift assays. Rundlett *et al.* (1990) obtained higher CAT activity induction by rGR than rAR with a reporter vector containing the MMTV-LTR and another containing a GRE from the tyrosine aminotransferase gene linked to a thymidine kinase promoter. They also found that a rAR/rGR chimera, consisting of the rAR amino terminal and DNA-binding domains linked to the rGR steroid binding domain, was less active than wild-type rGR with the two reporter vectors. GR has been reported to function cooperatively with a number of other transcription factors (Shule *et al.*, 1988; Strahle *et al.*, 1988; Truss & Beato, 1993), however similar results with AR have not been reported. Thus it is possible the difference we observed could be explained in part by cooperativity with other factors.

The HREs, C3-C and GUS have in common that they are located in introns of known androgen regulated genes. C3-C is a strong simple response element and functions in cotransfections as both ARE and GRE even within the context of a 0.5 kbp intron sequence (Tan *et al.*, 1992). C3-C may be a site for AR and GR regulation of the C3 subunit gene (Rennie, 1989; Tan *et al.*, 1992) and the androgen dependence of C3 gene expression a result of its requirement for other androgen regulated factors (Tan *et al.*, 1992; Ho *et al.*, 1993). The GUS response element is located in intron 9 of the murine β-glucuronidase (GUS^a) gene, which is androgen regulated in kidney (Lund *et al.*, 1991). In the GUS^{OR} mouse, β-glucuronidase gene expression is unresponsive to androgen stimulation and the 9th intron HRE is deleted, suggesting it has an essential role in the androgen response. Although this HRE, as an isolated sequence, is a stronger GRE than ARE, GUS^a transcription is not maintained by glucocorticoids alone in mouse kidney perhaps because of 11-β-hydroxysteroid dehydrogenase inactivation of corticosterone in kidney (Funder, 1993) and/or a requirement for other androgen dependent regulatory factors (Lund *et al.*, 1991).

GH is a response element in intron 1 of the human growth hormone gene (Moore *et al.*, 1985) and may have a role in its transcriptional regulation. Androgens enhance the growth hormone secretory response to growth hormone releasing hormone by a direct action on the pituitary (Wehrenberg *et al.*, 1985; Akira *et al.*, 1988; Hertz *et al.*, 1989). Dexamethasone also increases growth hormone mRNA in the pituitary adenoma cell line, GH3 (Strobl *et al.*, 1989). Thus, actions of both AR and GR may be mediated by the 1st intron HRE. HREs acting alone or within the context of complex elements provide a diversity of mechanisms for AR and GR transactivation ranging from hormone specific to combinations of activities that are likely important in a wide range of physiological functions.

In studies of simple HREs thus far, (Ham *et al.*, 1988; Denison *et al.*, 1989; Rundlett *et al.*, 1990), a 15 bp palindromic element with stronger ARE than GRE activity was not reported. However, a 17 bp sequence with ARE>GRE activity was identified recently by binding site selection from a pool of degenerate double-stranded oligonucleotides (Zhou *et al.*, 1994). Whether or not this sequence is present in native DNA, it could provide important insights into the structural determinants of specific AREs. ARE specificity has been observed in larger, complex elements (>100 bp) such as those 5′ of the sex limited protein (SLP) gene (Adler *et al.*, 1992) in the promotor region of the probasin gene (Rennie *et al.*, 1993; Kasper *et al.*, 1994) and in intron 1 of the 20 KDa protein gene (Ho *et al.*, 1993). These complex AREs bind both AR and GR but mediate stronger transcriptional responses to AR. ARE specificity is cell type dependent suggesting that interactions with non-receptor host cell factors have a role in the selective response.

Materials and methods

Plasmid constructs

Complementary strands of oligonucleotides corresponding to HREs in the C3 subunit gene of rat prostatein (C3-A, C3-B and C3-C, Tan *et al.*, 1992); the C2 subunit gene of pros-

tatein (C2), the ninth intron of the mouse β-glucuronidase gene (GUS, Lund *et al.*, 1991), the 5' most GRE of the MMTV-LTR (MMTV 5' GRE, Truss & Beato, 1993), the probasin gene promoter (ARBS-1, Rennie *et al.*, 1993) and the first intron of the human growth hormone gene (GH, Moore *et al.*, 1985) were synthesized using the phosphoramide method with an Applied Biosystems Model 380B DNA synthesizer. Complementary strands of C2, GUS, probasin and GH oligonucleotides, when annealed, contained *Sal*I compatible single stranded ends (5'-TCGA-3'). Annealed complementary strands of oligonucleotides C3-B, C3-C and MMTV 5' GRE contained blunt ends. Annealed complementary strands of C3-A contained *Bam*HI compatible single stranded ends (5'-GATC-3'). C3-B, C3-C, and MMTV 5' GRE oligonucleotides were cloned into the blunt ended *Sal*I site, C2, GUS probasin and GH into the *Sal*I site, and C3-A into the *Bam*HI site of the reporter vector, ptkCAT (Tan *et al.*, 1992). Each reporter vector contained a single response element oriented 5'-3' in the sense strand. Constructs were verified by double-stranded sequencing using Sequenase (US Biochemical Corp.).

pCMVrAR is a pCMV1 expression vector containing full-length rat AR cDNA (Tan *et al.*, 1988, 1992; Ho *et al.*, 1993). pCMVrGR was constructed by cloning the full-length rat GR (Kupfer *et al.*, 1993) into pCMV5. The chimeric receptor expression vector pCMVrAR/rGR was constructed by removing the DNA fragment coding for the rAR N-terminal domain (amino acid codons 1-548) from pCMVrAR by digestion with *Bgl*II and *Hind*III and the DNA fragment encoding the rGR DNA-binding and C-terminal domains (amino acid codons 447-795) from pCMVrGR by digestion with *Bam*HI and partial digestion with *Hind*III. These fragments were subcloned by ligation into pCMV5 digested with *Bam*HI. pCMVrGR/rAR was constructed by removing the DNA fragment coding for the rGR N-terminal domain (amino acid codons 1-446) from pCMVrGR by digestion with *Bam*HI and partial digestion with *Hind*III and by removing the DNA fragment encoding the rAR DNA-binding and C-terminal domains (amino acid codons 549-902) from pCMVrAR by digestion with *Hind*III and *Bam*HI. Fragments were subcloned by ligation into pCMV5 digested with *Bam*HI.

Cell culture, transfection and CAT assay

African green monkey kidney cells, CV1, were maintained at 37°C under 5% CO_2 in DMEM-H medium supplemented with 5% bovine calf serum (BCS). One day before transfecting, cells were plated in 6 cm culture dishes at 0.4×10^6 cells per dish and incubated in the same medium for 20 h when they were 70-80% confluent. Cells were transfected with 1 μg of receptor expression vector DNA and 5 μg of reporter plasmid DNA using the $CaPO_4$ method as described previously (Tan *et al.*, 1992). Cells were washed twice and placed in DMEM-H (without phenol red) containing 0.2% BCS and either with or without the synthetic androgen, R1881 (methyltrienolone) or dexamethasone, at the indicated concentrations. After incubation for 20 h, medium was replaced with or without hormone at the same concentration. Cells were incubated for another 24 h, harvested in PBS (0.15 M NaCl, 0.01 M Na_2 HPO_4, pH 7.2) and assayed for CAT activity as described by Gorman *et al.*, (1982) except that the cells were broken by freeze-thaw five times. Thin layer plates were exposed on Kodak X-Omat AR film for 24-72 h. For quantitation, the radioactive spots were cut out and counted in a liquid scintillation counter. Background was defined as the amount of radioactivity in the absence of ligand. Stimulation of CAT activity is expressed as fold increase over background and was based on at least five independent experiments.

Overexpression of recombinant androgen and glucocorticoid receptor

ADNA fragment containing sequences encoding the rat GR-DBD (amino acid codons 407-556) fused in-frame with the first 10 amino acids of T7 gene 10 was excised from the expression vector pT7X556 (Freedman *et al.*, 1988) and subcloned into the T7 RNA polymerase-dependent expression vector, pET16b (Novagen), at the same restriction sites. A 510 bp DNA fragment containing sequences encoding rat AR amino acids 496-665 encompassing the DNA binding domain and hinge region was generated by PCR amplification using oligonucleotides that introduce *Bam*HI sites at either end of the amplified fragment. The PCR product was digested with *Bam*HI and inserted into the expression vector pET-16b. Receptors were overexpressed in *E. coli.* strain BL21(DE3)pLysS and purified by absorption to His-Bind resin as described previously (Ho *et al.*, 1993). Further purification was achieved by subjecting the fractions from the His-Bind resin to DNA affinity chromatography according to the procedure of Kadonaga & Tjian (1986) using a Sepharose CL-4B column containing oligomers of the C3-3 double stranded oligonucleotide. Receptor preparations (5 ml) were incubated with poly (dI-dC) (10 μg) for 10 min at 4°C. The protein-DNA solutions were mixed with 1 ml DNA affinity resin equilibrated with $TEGDZ_{50}$ buffer (50 mM Tris pH 7.5, 1 mM EDTA, 10% glycerol, 50 mM NaCl, 4 mM $CaCl_2$, 40 mM $MgCl_2$, 5 mM DTT) and the suspension was incubated for 1 h at 4°C with constant mixing. The protein-resin suspension was packed into a Bio-Rad Econo-Column and the flow through was collected. The resin was washed four times with 2 ml of $TEGDZ_{50}$ and the receptor protein was eluted from the column with 5 ml of $TEGDZ_{500}$ (TEGDZ containing 500 mM NaCl) collecting 1 ml fractions. Silver staining and immunoblotting using AR-52 or BuGR-2 antibodies of 15% SDS polyacrylamide gels indicated that the peak fraction contained nearly homogeneous rAR-DBD or rGR-DBD. Protein concentration in the peak fractions was quantitated by Bradford assay (Bio-Rad).

Mobility shift assay

DNA-receptor binding was analysed by the mobility shift assay using rAR-DBD or rGR-DBD polypeptides and was performed as described previously (Tan *et al.*, 1992). Briefly, partially purified or purified receptor proteins were incubated with 500 ng or 20 ng of poly (dI-dC), respectively, at 4°C for 15 min in a binding buffer containing 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 25 mM KCl, 20 μg BSA and 1 mM DTT. Labeled DNA probes were added and the reactions were incubated for 15 min at room temperature. Specific binding of receptor to DNA was demonstrated using the protein A purified IgG fraction of anti-AR antiserum (AR-52, Quarmby *et al.*, 1990) or anti-GR antiserum (BUGR-2, Gametchu & Harrison, 1984). Antiserum was added after the addition of the labeled DNA and the reaction was incubated an additional 15 min at room temperature. Protein-DNA complexes were resolved by electrophoresis on 6% nondenaturing polyacrylamide gels. DNA probes used were 50 base pair fragments from ptkCAT vectors containing the synthetic HRE oligonucleotide inserts. Fragments were removed by restriction enzyme digestion to generate 5'-protruding ends and purified by electroelution from polyacrylamide gels. The 5'-protruding ends were filled in with [β^{32}P-]dCTP (Amersham Corp.) using Klenow fragment of DNA polymerase I (Gibco/BRL life Technologies).

Methylation interference assay

Interference with the binding of rAR-DBD and rGR-DBD by methylation of DNA was measured essentially as

previously described (Baldwin, 1988). DNA fragments labeled at one end with α^{32}P-dCTP were treated with dimethylsulfate for 5 min. The methylated fragments were included in preparative gel mobility shift assays with partially purified rAR-DBD and rGR-DBD. Following electrophoresis, radioactive bands representing the free and receptor bound DNA were localized by autoradiography of the wet gel. DNA was electroeluted from the gel into dialysis bags containing 300 µl Tris-acetate buffer. After phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation, the DNA was cleaved at guanine residues with piperidine and electrophoresed on an 8% polyacrylamide-8 M urea gel.

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References

Adler, A.J., Scheller, A., Hoffman, Y. & Robins, D.M. (1991). *Mol. Endocrinol.*, **5**, 1587–1596.

Adler, A.J., Danielsen, M. & Robins, D.M. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 11660–11663.

Akira, S., Wakabayashi, I., Sugihara, H., Minami, S., Takahashi, F. & Motoyama, A. (1988). *Neuroendocrinology*, **47**, 116–124.

Alroy, I. & Freedman, L.P. (1992). *Nucleic Acids Research*, **20**, 1045–1052.

Baldwin, A.S. (1988). *Current protocols in molecular biology*. Ausubel, F.M., Brent, R.M., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, (eds.). Greene Publishing Associates & Wiley-Interscience: New York. pp. 12.3.1–12.3.6.

Beato, M. (1989). *Cell*, **56**, 35–344.

Carson-Jurica, M.A., Schrader, W.T. & O'Malley, B.W. (1990). *Endocr. Rev.*, **11**, 201–220.

Claessens, F., Rushmere, N.K., Davies, P., Celis, L., Peeters, B.L. & Rombauts, W.A. (1990). *Mol. Cell. Endocrinol.*, **74**, 203–212.

Cairns, C., Gustafsson, J-A. & Carstedt-Duke, J. (1991). *Mol. Endocrinol.*, **5**, 598–604.

Dahlman-Wright, K., Siltala-Roos, H., Carlstedt-Duke, J. & Gustafsson, J-A. (1990). *J. Biol. Chem.*, **265**, 14030–14035.

Dahlman-Wright, K., Wright, A., Gustafsson, J-A. & Carlstedt-Duke, J. (1991). *J. Biol. Chem.*, **266**, 3107–3122.

Danielsn, M., Hinck, L. & Ringold. G.M. (1989). *Cell*, **57**, 1131–1138.

Denison, S.H., Sands, A. & Tindall, D.J. (1989). *Endocrinology*, **124**, 1091–1093.

DeVos, P., Claessens, F., Peeters, B., Rombauts, W., Heyns, W. & Verhoeven, G. (1993). *Mol. Cell. Endocrinol.*, **90**, 11–16.

Drouin, J., Sun, Y.L., Tremblay, J., Lavender, P., Schmidt, T.T., de Lean, A. & Nemer, M. (1992). *Mol. Endocrinol.*, **6**, 1299–1309.

Evans, R.M. (1988). *Science*, **240**, 889–895.

Freedman, L.P., Luisi, B.F., Korszun, Z.R., Basavappa, R., Sigler, P.B. & Yamamoto, K.R. (1988). *Nature*, **334**, 543–546.

Freedman, L.P. (1992). *Endocrine Reviews*, **13**, 129–145.

Funder, J.W. (1993). *Science*, **259**, 1132–1133.

Gametchu, B. & Harrison, R.W. (1984). *Endocrinology*, **114**, 274–279.

Gorman, C.M., Moffat, L.F. & Howard, B.H. (1982). *Mol. Cell. Biol.*, **2**, 1044–1051.

Green, S. & Chambon, P. (1987). *Nature*, **325**, 75–78.

Gronemeyer, H. (1991). *Ann. Rev. Genet.*, **25**, 89–123.

Ham, J., Thomson, A.A., Needham, M., Webb, P. & Parker, M. (1988). *Nucleic Acids Res.*, **16**, 5263–5276.

Hertz, P.P., Silbermann, M., Even, L. & Hochberg, Z. (1989). *Endocrinology*, **125**, 581–585.

Ho, K-C., Marschke, K.B., Tan, J-A., Power, S.G.A., Wilson, E.M. & French, F.S. (1993). *J. Biol. Chem.*, **268**, 27226–27235.

Kadonaga, J.T. & Tjian, R. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 5889–5893.

Kasper, S., Rennie, P.S., Bruchovsky, N., Sheppard, P.C., Cheng, H., Lin, L., Shiu, R.P.C., Sudek, R. & Matusik, R.J. (1994). *J. Biol. Chem.*, **269**, 31763–31769.

Kupfer, S.R., Marschke, K.B., Wilson, E.M. & French, F.S. (1993). *J. Biol. Chem.*, **68**, 17519–17527.

Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S. & Wilson, E.M. (1988a). *Science*, **240**, 327–330.

Lubahn, D.B., Joseph, D.R., Sar, M., Tan, J-A., Higgs, H.N., Larson, R.E and French F.S. (1988b). *Mol. Endocrinol.*, **2**, 1265–1275.

Luisi, B.F., Xu, W.X., Otwinoski, Z., Freedman, L.P., Yamamoto, K.R. & Sigler, P.B. (1991). *Nature*, **352**, 497–505.

Lund, S.D., Gallagher, P.M., Wang, B., Porter, S.C. & Ganchow, R.E. (1991). *Mol. Cell. Biol.*, **11**, 5426–5434.

Moore, D.D., Marks, A.R., Buckley, D.I., Kapler, G., Payvar, F. & Goodman, H.M. (1985). *Proc. Natl. Acad. Sci. USA*, **82**, 699–702.

Nordeen, S.K., Suh, B.J., Kuhnek, B. & Hutchison, C.A. (1990). *Mol. Endocrinol.*, **4**, 1866–1873.

Quarmby, V.E., Kemppainen, J.A., Sar, M., Lubahn, D.B., French, F.S. & Wilson, E.M. (1990). *Mol. Endocrinol.*, **4**, 1399–1407.

Rennie, P.S. (1989). *Mol. Endocrinol.* **3**, 703–708.

Rennie, P.S., Bruchovsky, N., Leco, K.J., Sheppard, P.C., McQueen, S.A., Cheng, H., Snoek, R., Hamel, A., Bock, M.E., MacDonald, B.S., Nickel, B.E., Chang, C., Liao, S., Cattini, P.A. & Matusik, R.J. (1993). *Mol. Endocrinol.*, **7**, 23–36.

Roche, P.J., Hoare, S.A. & Parker, M.G. (1992). *Mol. Endocrinol.*, **6**, 2229–2235.

Rundlett, S.E., Wu, X-P. & Miesfeld, R.L. (1990). *Mol. Endocrinol.*, **4**, 708–714.

Rundlett, S.E. & Miesfeld, R.L. (1995). *Mol. Cell. Endocrinol.*, **109**, 1–10.

Shule, R., Muller, M., Kaltschmidt, C. & Renkawitz, R. (1988). *Science*, **242**, 1418–1420.

Schule, R., Muller, M., Otsuka-Murakami, H. & Renkawitz, R. (1988). *Nature*, **332**, 87–90.

Simental, J.A., Sar, M., Lane, M.V., French, F.S. & Wilson, E.M. (1991). *J. Biol. Chem.*, **266**, 510–518.

Strahle, U., Schmid, W. & Schutz, G. (1988). *EMBO J.*, **7**, 3389–3395.

Strobl, J.S., van Eys, G.J.J.M. & Thompson, E.B. (1989). *Mol. Cell. Endocrinol.*, **66**, 71–82.

Tan, J-A., Joseph, D.R., Quarmby, V.E., Lubahn, D.B., Sav, M., French, F.E. & Wilson, E.M. (1988). *Mol. Endocrinol.*, **2**, 1276–1285.

Tan, J-A., Marschke, K.B., Ho, K-C., Perry, S.T., Wilson, E.M. & French, F.S. (1992). *J. Biol. Chem.*, **267**, 4456–4466.

Tan, J-A. (1992). *Ph.D. Thesis, Dept. of Biology, University of North Carolina at Chapel Hill.*

Truss, M. & Beato, M. (1993). *Endocrine Reviews*, **14**, 459–479.

Tsai, S.Y., Carlstedt-Duke, J., Weigel, N.L., Dahlman, K., Gustafsson, J-A., Tsai, M-J. & O'Malley, B.W. (1988). *Cell*, **5**, 361–369.

Umaesono, K. & Evans, R.M. (1989). *Cell*, **57**, 1139–1146.

Wehrenberg, W.B., Baird, A., Ying, S-Y. & Ling, N. (1985). *Biol. Reprod.*, **32**, 369–375.

Wong, C-I., Zhou, Z-X., Sar, M. & Wilson, E.M. (1993). *J. Biol. Chem.*, **268**, 19004–19012.

Zhou, Z., Corden, J.L. & Brown, T.R. (1994). *Program of the 76th Annual Meeting of the Endocrine Society*, **Abstract 530**.