Function of Directly Repeated Half-sites as Response Elements for Steroid Hormone Receptors*

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The mouse mammary tumor virus promoter has been shown to be inducible by glucocorticoids and progesterone. Although steroid hormone receptors bind with high affinity to palindromic response elements, the hormone-responsive region of the mouse mammary tumor virus promoter contains a pair of directly repeated half-sites that are important for hormone inducibility. Recent experiments have also indicated that direct repeats can function as estrogen response elements. Here, we have investigated DNA binding by steroid receptors to direct repeats and provide evidence using gel retardation assays, methylation interference, and gene transfer experiments that direct repeats of TGGTCT or RGGTCA motifs function as response elements for glucocorticoid (GR) or estrogen receptors (ER), respectively, by binding receptor homodimers. Specific GR- or ER-DNA complexes were observed on direct repeats with different spacings between half-sites, indicating that binding of steroid receptors to direct repeats is more flexible than binding to palindromic elements. This flexibility was further emphasized by the observation that the GR could also bind to everted repeats of TGGTCT motifs separated by 9 base pairs. The isolated DNA binding domains of the GR and ER bound cooperatively to palindromes, but no evidence was observed for cooperative binding to direct repeats. Under similar conditions the DNA binding domains of retinoid receptors retinoid X receptor and retinoic acid receptor bound to direct repeats cooperatively as heterodimers. Similarly, ER derivative HEIS, which lacks a functional ligand binding domain, bound palindromic response elements but failed to bind direct repeats. These results indicate that the dimerization domain in the ligand binding domain is essential for binding of steroid receptors to direct repeats and that the dimerization domain in the D-box of the DNA binding domain is not functional under these conditions. Moreover, the results suggest that steroid receptor DNA binding domains may lack dimerization domains outside the D-box, which would function in binding to direct repeats, in contrast to receptors for retinoids and thyroid hormone. A comparison of the mechanisms of binding of steroid receptors and retinoid and thyroid hormone receptors to direct repeats is presented.

The nuclear receptors are a family of transcriptional enhancer factors that bind to specific DNA sequences in target promoters known as response elements (1–5). Specific members of the nuclear receptor family represent the primary intracellular targets for small lipid-soluble molecules, such as steroid and thyroid hormones, retinoids, and vitamin D₃, and act as ligand-inducible transcriptional regulators. These ligand-inducible receptors control a wide spectrum of developmental and physiological processes through modulating the transcription of target genes.

Sequence analyses of nuclear receptors have shown that they are composed of a series of conserved domains (1–6). The DNA binding domain (region C) is the most highly conserved and contains a 66–68-amino acid core composed of two zinc fingers that form a single structural domain (7–10). Three amino acids adjacent to the N-terminal zinc finger, known as the P-box, are critical for DNA sequence recognition (11–13). A subfamily of receptors, those for glucocorticoids (GR), progesterone, androgens, and mineralocorticoids, contain P-box amino acids Gly, Ser, and Val and recognize response elements composed of half-sites with the consensus AGAACA. In contrast, the discriminatory amino acids Glu, Gly, and Ala are found in the P-box of the estrogen receptor (ER), which binds to response elements with consensus RGGTCA half-sites. DNA binding domains (DBDs) of receptors for thyroid hormone, vitamin D₃, and retinoids contain a similar P-box (Glu, Gly, Gly) and recognize response elements containing the consensus RGG/TTCA.

The ligand binding domain (LBD) is the second most highly conserved among nuclear receptors. In addition to binding ligand, LBDs contain regions controlling transcriptional regulation, and receptor dimerization (14–20), which stabilizes receptor DNA binding. The action of LBD dimerization domains varies among receptors. In the absence of ligand, steroid receptors can form homodimers in solution through the dimerization domain in the LBD (17, 19, 21). In contrast receptors for thyroid hormone, vitamin D₃, and retinoids form heterodimers with the retinoid X receptors (RXRs) (21–25). Several studies have suggested that interaction with RXRs is stimulated by binding of ligand to the heterodimeric partner, whereas homodimerization of RXRs is stimulated by binding of their cognate ligand 9-cis-retinoic acid (5, 26, 27).

Hormone response elements for steroid receptors have been characterized as palindromic sequences, containing two half-sites separated by 3 base pairs. The estrogen response element (ERE) in the vitellogenin A2 gene of Xenopus laevis contains the perfectly palindromic element AGGTCA/NNTGACCT

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§ The abbreviations used are: ER, estrogen receptor; DBD, DNA binding domain; LBD, ligand binding domain; RXR, retinoid X receptor; ERE, estrogen response element; GRE, glucocorticoid response element; TR, thyroid hormone receptor; RAR, retinoic acid receptor; bp, base pair(s); MMTV, mouse mammary tumor virus; DTT, dithiothreitol; CAT, chloramphenicol acetyltransferase.
(28). A number of estrogen-responsive genes contain imperfectly palindromic EREs either in isolation or in multiple arrays (29–35). Similarly, the imperfectly palindromic glucocorticoid response element (GRE) tgtACANNTG TGTCT has been found in the rat tyrosine aminotransferase gene (36). Altering the spacing between half-sites disrupts the binding of steroid receptors to palindromic response elements (37, 38). In contrast to steroid receptors, RXR homo- and heterodimers of RXR receptors for thyroid hormone, vitamin D3, retinoids, or a number of orphan receptors preferentially recognize response elements composed of direct repeats of cognate half-sites (39–41). Different combinations of heterodimers discriminate between response elements based on the spacing between half-sites. Structure-function studies have shown that heterodimers bind with RXR in the 5′ position (42–45). The isolated DBDs of RXR and its heterodimeric partners can bind cooperatively to direct repeats, and discrimination between direct repeats with different spacings is controlled by the specific conformation of each DBD heterodimer (42–47).

Cooperative binding of steroid receptor DBDs to palindromic response elements is controlled by a dimerization domain in the second zinc finger of the DBD, known as the D-box (12, 48, 49). Substitution of the D-boxes of the GR or ER with the corresponding sequences of the thyroid hormone receptor (TR), or retinoic acid receptor (RAR) disrupts cooperative binding of DBDs to palindromic response elements, indicating that these regions of RAR and TR cannot homodimerize (48, 49). Heterodimerization between RXRs and RARs on response elements composed of half-sites separated by 5 bp (DR5) occurs through interaction of the D-box of RXRs with a region of the N-terminal zinc finger of RXR (46). In contrast, RXR/RAR heterodimers formed on response elements containing half-sites separated by 2 bp (DR2) require the extreme C terminus of the RXR DBD and a distinct N-terminal domain of the RAR DBD (47). Interestingly, the corresponding N-terminal domain of RXR, and the extreme C terminus of the RXR DBD, but not the D-box, have been implicated in binding of RXR homodimers to DR1 response elements (47). This indicates that multiple regions of RAR and RXR DBDs are capable of acting as dimerization interfaces.

Recent studies have suggested that direct repeats of RG-TGCA half-sites function as EREs (50–53). Moreover, the glucocorticoid-responsive region of the MMTV promoter contains a promoter proximal direct repeat of TGTTG T motifs required for optimal ligand inducibility (54, 55). We therefore investigated the mechanism of binding of the ER and the GR to direct repeats. Evidence is provided that the GR is capable of binding to the TGTTG T repeats of the MMTV promoter as a homodimer. Similarly, the ER can homodimerize on direct repeats. The roles of receptor dimerization domains in the binding of GR or ER homodimers to direct repeats and palindromes have been investigated using a series of truncated receptors. A comparison of the mechanisms of DNA binding by steroid receptors and receptors for thyroid hormone, vitamin D2, and retinoids is also presented.

**EXPERIMENTAL PROCEDURES**

**Materials**

Oligonucleotides were synthesized at the Sheldon Biotechnology Institute (McGill University). Steroid receptor expression vectors were a kind gift of Dr. Pierre Chambon (Illkirch, France). The plasmid pGRDBD was constructed by insertion of a KpnI-XhoI restriction fragment encoding human GR DBD amino acids 406–506, amplified by polymerase chain reaction using primers 5′-TGCGAAGGTACCCTACACGCTTCCTCAACAG and 5′-TGGCAACTGAACTGCTGATGGCCCTGCTGG into the bacterial expression vector pET32 (49) digested with KpnI and XhoI. Bacterial expression vectors for the ER DBD derivative H81, and RARa and RXRa DBDs have already been described (44, 49).

GRE reporter plasmids (see Fig. 3) were constructed by ligating pBLCAT8-28, previously digested with BamHI and treated with calf intestinal alkaline phosphatase, in the presence of a 50- or 200-fold excess of oligonucleotides GRE-D9 or GRE-D9M2 (see Fig. 1). Recombinants were screened for insert numbers by digestion with HindIII and BglII and insert number and orientation were verified by DNA sequencing. Partially purified human GR was the ammonium sulfate fraction from extracts of insect cells overexpressing the GR using the baculovirus expression system (Affinity Bioreagents Inc.). The rabbit anti-human GR antibody (AhuGR 150–175) was obtained from Affinity Bioreagents Inc. The anti-human ER monoclonal antibody F3 (56) was obtained from P. Chambon and D. Metzger (Illkirch, France).

**Methods**

**Cell Culture, Transient Transfections, and Cell Extracts**—HELa or COS-7 cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. COS-7 cells were transfected at 30–50% confluence by lipofection. Media was changed after 12–18 h, and cells were harvested after 44–48 h. For CAT assays, ov-1 cells were prepared as above for lipofection and lipofected with 0.5 μg each of H1G1 expression vector, GRE-CAT reporter recombinant (as indicated in the figure), and 10 μg of β-galactosidase expression vector p610AZ. Media was changed after 12–18 h and dexamethasone (25 μM) was added as indicated. Cells were harvested after 44–48 h.

For gel retardation assays, cells were harvested by combining those scraped from two or three 3-cm plates in 500 μl of ice-cold phosphate-buffered saline. Cells were centrifuged at 2500 rpm for 10 min at 4°C, the supernatant was carefully removed, and the pellet was resuspended in 30 μl of high salt extraction buffer (25 μM Tris (pH 7.9), 0.3 mM DTT, 0.1 EDTA, 400 mM NaCl, 10% (v/v) glycerol). Cells were lysed by 3 cycles of freezing at −70°C and thawing at room temperature, then centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were stored at −70°C.

For CAT assays, cells from individual plates were washed with 1.0 ml of phosphate-buffered saline, incubated for 15 min at room temperature in 250 μl of lysis buffer (Promega), harvested by scraping, and transferred to an Eppendorf tube and then centrifuged at 10,000 rpm for 5 min. Extracts were stored at −70°C.

Expression of ER, GR, RAR, or RXR DBDs in Bacteria—The ER, GR, RARα, or RXRa DBDs were expressed in 400-ml cultures of Escherichia coli BL21/pLyS5 containing pET32 derivatives at 37°C in L broth containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. Expression was then induced at an A600 of 0.6 by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 μM. Cells were harvested 1 h later. Bacterial pellets were resuspended in 10 μl of sonication buffer (25 mM Tris-HCl (pH 7.5), 0.2 mM NaCl, 1 mM DTT, 0.1 EDTA, 10% glycerol, and 2.5 μg/ml of protease inhibitors pepstatin, leupeptin, antipain, and pepstatin). Cells were lysed by sonication (3 × 40 s), and insoluble material was removed by centrifugation for 1 h and 40,000 rpm in a Beckman 50Ti rotor. ER or GR DBD derivatives expressed in pET32 were purified from a heparin-Sepharose column by salt step elution in 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.1 EDTA, 10% glycerol. ER DBD H81 elutes at 1 μM and the GR DBD at 300 μM NaCl. The DBDs were 50–80% pure as judged by Coomasie Brilliant Blue staining of SDS-polyacrylamide gels (data not shown).

**Gel Retardation Assays**—Bacterial extracts (0.1–0.3 μl), extracts of transiently transfected mammalian cells (2 μl), or 0.5-μl aliquots of partially purified GR expressed in baculovirus (Affinity Bioreagents Inc.) were added to incubations as indicated in the figures. Samples were incubated for 15 min on ice in 10 μl of final volume of 25 μM Tris-HCl (pH 8.0), 1 mM DTT, 50 mM KCl (plus 0.1% Triton X-100 for assays with the intact GR), 20% glycerol containing 1 μg of poly(dl-dC) and then for a further 20 min at 23°C after addition of 50,000–100,000 cpm (5–10 fmol) of 32P-end-labeled double-stranded oligonucleotide. Antibody shift experiments were performed by incubating together extracts of HEG0 with 0.5 or 1.0 μl of an ascites fluid of F3 monoclonal antibody (56) or 3 μl of rabbit anti-human GR antibody AhuGR 150–175 (Affinity Bioreagents Inc.), prior to addition of radioactive oligonucleotide. Competition experiments were performed by addition of 6.0 × 108 cpm of a preincubation of extract and poly(dl-dC) (1 μg/μl) to 4 μl of a mixture of radioactive probe (100,000 cpm; ~20 fmol) and unlabelled competitor as indicated in the figures. Concentrations of competitor probes were first determined by A260 and then verified on ethidium bromide-stained 5% polyacrylamide gels. Samples were loaded on 5% polyacrylamide gels (50:1 acrylamide:bis for all others) equilibrated in 25 mM Tris base, 31.3 mM boric acid, 1 mM EDTA (pH 8.0) and electrophoresed at 8 V/cm. Gels were dried prior to autoradiography.
Methylation Interference Experiments—Methylation interference was performed essentially by the method of Hendrickson and Schleif (57). Top and bottom strands of the ERE-D6 oligonucleotide were 32P-5'-end-labeled separately and hybridized to unlabeled complementary strands. Oligonucleotides were then partially methylated by incubation with 2 μl of dimethyl sulfate in 30 μl of 50 mM sodium cacodylate (pH 8.0) for 2 min at room temperature. DNA binding and electrophoresis were performed as for gel retardation assays, except that reactions were scaled up 5-fold. Retarded and unretarded bands were excised from 5% polyacrylamide gels and treated with 1 M piperidine for 60 min at 80 °C. Purified products were then electrophoresed on 15% urea-polyacrylamide gels. Relative intensities of bands on autoradiograms were determined using a Bio Image densitometric scanner (MilliGen/Biosearch).

CAT Assays—To correct for variations in transfection efficiency, 30 μl of cell extracts were used for β-galactosidase assays as described (58). CAT assays were performed as described (58) using extract volumes normalized for β-galactosidase activity. Reaction products were resolved by thin layer chromatography, and the degree of acetylation was determined using a PhosphorImager.

RESULTS

Binding of the Human Glucocorticoid Receptor to Response Elements Containing Direct Repeats and Everted Repeats of TGTTCT Motifs—Numerous studies have shown that steroid receptors bind to palindromic response elements as homodimers, with the subunits coupled by dimerization interfaces present in both the hormone binding and DNA binding domains of the receptors (1–4, 14, 16, 20). Interestingly, recent studies on the estrogen-responsive chicken ovalbumin gene (50), molecular genetic experiments in yeast (51, 52), and studies with extracts of the ER expressed in mammalian cells (53, 59) have suggested that directly repeated half-sites, as well as palindromes, are capable of acting as EREs. We therefore interested to know if direct repeats could also function as GREs. One of the best characterized glucocorticoid-responsive promoters is the long terminal repeat of MMTV, which contains a number of binding sites for the GR (54, 55). The binding site most proximal to the TATA box contains the sequence TGTTCTNGTTCT, which is a direct repeat of consensus GRE half-sites. Mutagenesis studies have shown that both half-sites participate in binding and transactivation by the GR (55).

We performed gel retardation experiments to test whether homodimers of the GR were capable of binding to this sequence. A number of oligonucleotides were used in this study (Fig. 1A), including the wild-type sequence from the MMTV promoter (GRE-D9), as well as mutant sequences in which either the upstream or downstream half-sites were disrupted (GRE-D9M1 and GRE-D9M2, respectively). A perfectly palindromic GRE (GRE-P3) was used as a positive control. The results show...
that a complex was formed on the GRE-P3 oligonucleotide upon incubation with extracts of COS-7 cells transiently transfected with a vector expressing the human GR, but not in extracts of cells transfected with a control vector (Fig. 1B, lanes 2 and 7; arrowhead). A similar complex was formed on the GRE-D9 oligonucleotide only in the presence of the GR (Fig. 1B, lanes 3 and 8). No such complex was formed on oligonucleotides GRE-D9M1 or GRE-D9M2 where either the upstream or downstream half-sites have been disrupted (Fig. 1B, lanes 4, 5, 9, and 10). Taken together, these results indicate that a complex is formed on an oligonucleotide containing TGTTCT half-sites separated by 9 bp in the presence of the human GR and that formation of this complex requires the integrity of both half-sites. Moreover, this complex comigrates with the one formed on palindromic response elements, suggesting that it corresponds to receptor homodimers.

Given the significant levels of nonspecific binding in COS-7 cell extracts, the presence of the GR in retarded complexes formed on GRE oligonucleotides was further tested using receptor purified from insect cells overexpressing the GR, along with polyclonal antibody raised against the human GR (Fig. 1C). A nonspecific complex was observed when oligonucleotides were incubated with antibody in the absence of GR-containing extract, but this complex did not correspond to the GR (Fig. 1C, lanes 1, 4, 7, 10, and 13; asterisk). The GR-specific complex formed on GRE-P3 is further retarded by incubation with a polyclonal anti-human GR antibody (Fig. 1C, lanes 2 and 3; arrowheads), confirming the presence of the GR. Similarly, a specific complex was formed on the GRE-D9 oligonucleotide, which was further shifted in the presence of antibody (Fig. 1C, lanes 5 and 6). Consistent with the results of Fig. 1B where no GR-specific binding was observed to oligonucleotides containing a single half-site, no specific complexes are formed on the GRE-D9M1 or GRE-D9M2 oligonucleotides, either in the absence or presence of antibody (Fig. 1C, lanes 11, 12, 14, and 15). Taken together, the results of Fig. 1 demonstrate that the GR can bind to direct repeats of TGTTCT motifs.

These results suggest that the GR DNA binding domain rotates in converting from a palindromic DNA binding mode to a direct repeat binding mode (see also Fig. 8). The flexibility of DNA binding by the GR is substantiated by the observation that specific retarded complexes formed on oligonucleotide GRE-E9 (see Fig. 1), which contains an everted repeat of TGTTCT motifs separated by 9 bp (Fig. 1C, lanes 8 and 9). Competition experiments were also performed with GRE oligonucleotides to analyze the relative efficiencies of palindromic GREs, direct repeats, and single half-sites in competing for GR binding. A typical result (Fig. 1D) showed that the GRE-D9 oligonucleotide competes 3.5-fold less efficiently for binding to the GR than GRE-P3 palindrome and 3.6-fold more efficiently than the GRE-D9M1 and GRE-D9M2 mutants, which contain only single half-sites. Taken together the above results indicate that the GR can bind cooperatively to direct repeats, suggesting that direct repeats may serve as response elements in vivo.

Function of Direct Repeats of TGTTCT Motifs as GREs in Vivo—Studies of steroid-inducible promoters have suggested that weak hormone response elements can combine synergistically to amplify the response to hormone (34, 36, 50, 54, 60). The function of direct repeats as GREs was studied in transiently transfected cells using a reporter plasmid containing one or three copies of the GRE-D9 element arranged head to tail upstream of a truncated herpes simplex virus thymidine kinase (tk) promoter, which does not respond to dexamethasone (Ref. 60 and data not shown). Similar recombinants containing one or three copies of GRE-D9M2 were used as negative controls (Fig. 2A). Given that the GRE-D9 direct repeats are derived from the MMTV promoter, the activity of 3GRE-D9tk-CAT was then compared with that of MMTV-CAT (Fig. 2B and C). No significant induction by dexamethasone was observed in CV-1 cells transiently transfected with a tk reporter construct containing one GRE-D9 element (Fig. 2B and C). However, in three different experiments the 3GRE-D9tk-CAT recombinant was induced an average of 29-fold by hormone (Fig. 2B and C). By comparison, MMTV-CAT was induced 32-fold by dexamethasone in CV-1 cells. In contrast, recombinants containing one or three GRE-D9M2 elements were not affected by hormone treatment (Fig. 2B and C, GRE-D9M2 and 3GRE-D9M2). Similar results were obtained in transfections of COS-7 cells (data not shown). These experiments indicate that the GRE-D9 element can function upstream of a heterologous promoter as part of an array of response elements, an action that is typical of a number of nonconsensus palindromic steroid response elements characterized to date.

Binding of the Human ER to Direct Repeats of RGTTCA Motifs—We have also analyzed by gel retardation assay the binding of the wild-type human and chicken ERs to direct repeats of cognate half-sites (Fig. 3A). A specific complex was formed on a palindromic ERE from the chicken vitellogenin gene (ERE-P3) with extracts of cells transiently transfected...
with either human or chicken ER expression vectors HEG0 (61, 62) or CE0 (6), respectively, but not in control extracts (Fig. 3, B and C, lanes 1 and 2). A complex of identical mobility was formed in the presence of the ER (Fig. 3, B and C, lanes 5 and 6) on an oligonucleotide containing two directly repeated RG- GTCA motifs separated by 6 bp (ERE-D6). A similar complex was formed at much lower levels on an oligonucleotide (ERE-D6M1) in which the downstream half-site has been mutated (Fig. 3, B and C, lanes 3 and 4). An identical reduction in binding of HEG0 was obtained when the upstream half-site (ERE-D6M2) was mutated (data not shown). Under these conditions, we have found that from 6- to 15-fold more retarded complex is formed when both half-sites participate in complex
form (Fig. 3, B and C, and data not shown). From 8- to 15-fold less retarded complex was formed on the ERE-D6 direct repeat than on the ERE-P3 palindrome (Fig. 3, B and C, and data not shown). Similar results were obtained with other gel retardation assays with the full-length human ER HEG0 and its truncated derivative HEG19 (see below).

The presence of the the human ER in specific retarded complexes observed on direct repeats was confirmed using the monoclonal F3 antibody that recognizes an epitope in the C-terminal F region of the receptor (Fig. 3D) and the ERE-D5 oligonucleotide. In the presence of antibody, complexes formed only in extracts of cells transfected with an HEG0 expression vector (Fig. 3D, lanes 1 and 6) were further retarded (Fig. 3D, lanes 2, 7, and B), indicating that, like the palindromic ERE, the ERE-D5 oligonucleotide binds the ER. These results are further supported by competition experiments which showed that ERE-D6 competes 8–12-fold more efficiently for ER binding than the ERE-D6 M1 mutant (Fig. 3E and data not known). Taken together, the results of Fig. 3 indicate that the ER can bind cooperatively to directly repeated half-sites.

The participation of both RGGTCA half-sites in ER binding to the ERE-D6 oligonucleotide was confirmed by methylation interference experiments which indicated that methylation of G residues in either half-site disrupted the formation of retarded complexes (Fig. 4). Oligonucleotides methylated at G residues present in either half-site were significantly under-represented in ER-specific retarded complexes (Fig. 4, asterisks). Note that methylation of the 5′ G residue of the GGGTCA half-site disrupted complex formation, implicating this residue in ER binding (Fig. 4, left panel). The consensus half-site for palindromic EREs is often given as GGTCGA. However, a survey of weaker imperfectly palindromic elements often shows a purine at the –1 position (29–35). In this respect EREs are similar to response elements for retinooids, thyroid hormone, and vitamin D3.

Flexibility of Binding of Steroid Receptors to Direct Repeats—
The above results indicate that the ER and the GR can bind to direct repeats separated by 6 and 9 bp, respectively. The directly repeated ERE half-sites in the chicken ovalbumin promoter are widely spaced (50). Taken together, this suggests that binding of steroid receptors to direct repeats may not display the same strict dependence on half-site spacing as binding to palindromic response elements. To further test this possibility, we have analyzed binding of the ER and GR to oligonucleotides containing direct repeats with different half-site spacings. We tested a series of oligonucleotides containing RGTTCA motifs separated by 2–5 bp (ERE-D2 to ERE-D5 only, the top strand is shown, B, gel retardation assays performed with HEG0 expressed in COS-7 cells. Extracts were incubated with oligonucleotides ERE-D2 to ERE-D6, as indicated. C, gel retardation assays performed with HGI expressed in COS-7 cells or with control COS-7 extracts with oligonucleotides GRE-D6, D9, and P3 as indicated. Specific complexes are indicated by arrowheads.

**Fig. 4.** Methylation interference analysis of ER binding to direct repeats. Methylation interference experiments (see "Experimental Procedures" for details) were performed with the ERE-D6 oligonucleotide and extracts of HeLa cells expressing HEG0. Cleavage products were resolved on a 15% urea/acylamide gel, T, F, and B refer to total, free, and bound oligonucleotide, respectively. G residues in the two half-sites of ERE-D6 are indicated. B, G residues whose methylation reduces binding by >50%, as determined by densitometric scanning of the autoradiogram, are indicated by asterisks.

**Fig. 5.** Flexibility of binding of steroid receptors to direct repeats. A, oligonucleotides used in this study. P and D refer to palindrome and direct repeat, respectively, and the number following refers to the inter-half-site spacing. For oligonucleotides ERE-D2 to ERE-D5 only, the top strand is shown. B, gel retardation assays performed with HEG0 expressed in COS-7 cells. Extracts were incubated with oligonucleotides ERE-D2 to ERE-D6, as indicated. C, gel retardation assays performed with HGI expressed in COS-7 cells or with control COS-7 extracts with oligonucleotides GRE-D6, D9, and P3 as indicated. Specific complexes are indicated by arrowheads.
the GRE-D9 sequence derived from the MMTV promoter (Fig. 5C, lanes 3 and 4), but also on the related oligonucleotide GRE-D6 with 6-bp spacing between half-sites (Fig. 5C, lanes 5 and 6). We have also seen binding of the GR to the GRE of the hepatitis B promoter (63), which is a direct repeat of TGCCT motifs separated by 6 bp (data not shown). While these are not exhaustive series of oligonucleotides, it is clear from the above results that a stringent inter-half-site spacing requirement (of 3 bp) for steroid receptor binding to palindromic EREs or GREs is not applicable to response elements formed from direct repeats.

Binding of Steroid Receptors to Direct Repeats Requires the Dimerization Function Present in the Ligand Binding Domain—Steroid receptors form homodimers through dimerization domains present in both the ligand binding and DNA binding domains of the receptors. We have tested the function of the dimerization domains of the ER in binding to direct repeats by using a series of truncated receptors. To analyze the possibility that the N-terminal A/B region of the estrogen receptor plays a role in binding to direct repeats, the binding of the truncated human ER derivative HEG19 (see Fig. 6A) was tested using extracts of transiently transfected COS-7 cells. Similar to results obtained with the full-length human ER, HEG0 (Fig. 3), HEG19 formed 15-fold more retarded complex on ERE-D6 than on the mutant ERE-D6M1 oligonucleotide (Fig. 6B, lanes 3–6), indicating that the A/B region is not required for binding to direct repeats. In contrast, no complex formation on direct repeats was observed with extracts of cells expressing the truncated human ER derivative HE15 (Fig. 6A), which lacks the C-terminal ligand binding domain of the receptor. Even under conditions where HE15 almost completely or completely saturates the palindromic ERE-P3 oligonucleotide (Fig. 6C, lane 1, and data not shown), no specific complexes were formed on ERE-D6 or ERE-D6M1 (Fig. 6C, lanes 3–6). This suggests that the dimerization function in the ER ligand binding domain is required for binding to direct repeats.

The requirement for the ligand binding domain in binding to direct repeats was further supported by studies with the ER and GR DNA binding domains expressed at high levels in E. coli. The ER DBD derivative HE81 (Fig. 7A) binds highly cooperatively as a dimer to palindromic response elements (Fig. 7B, lane 1, and Ref. 49). Previous studies have shown that this cooperativity requires a functional dimerization domain located in the D-box, which is situated in the C-terminal zinc finger of the DBD (49). No binding was observed to the ERE-D6 oligonucleotide in bacterial extracts not expressing HE81 (Fig. 7B, lane 2). A complex, corresponding to an HE81 monomer was formed on ERE-D6M1, which contains a single half-site (Fig. 7B, lane 4). A similar monomeric complex was formed on ERE-D6, which contains a direct repeat. No evidence for dimer formation was seen (Fig. 7B, lane 3, and data not shown). We have also tested binding of the ER DBD to the ERE-D5 element over a range of concentrations. Formation of a dimeric complex was observed on the ERE-P3 element (Fig. 7C, lane 1), even at the lowest concentration tested (data not shown). However, no evidence for dimer formation was seen on ERE-D5, even at concentrations which saturate ERE-P3 (Fig. 7C, lanes 1–5).

A similar result was obtained when binding of the GR DBD (Fig. 7A) to direct repeats was analyzed. While formation of a dimeric complex was readily observed on the palindromic oligonucleotide GRE-P3, but not on ERE-P3 (Fig. 7D, lanes 1 and 2), no dimer formation was observed on the half-site of GRE-D9M1 or the direct repeat of GRE-D9 (Fig. 7D, lanes 3 and 4). In addition, no dimeric complexes were formed on the GRE-D6 oligonucleotide (Fig. 7D, lane 5). The above experiments have been repeated several times, over a range of DBD concentrations, using crude bacterial extracts expressing the ER or GR DBDs, or with fractions of DBD purified to ~50–80% homogeneity, and no evidence for cooperative DNA binding was observed (data not shown).

In contrast to the results with steroid receptor DBDs, and consistent with previous findings (44), cooperative DNA binding was readily seen with combinations of RARα and RXRα DBDs expressed in E. coli (Fig. 7E). Formation of heterodimeric complexes was observed (Fig. 7E, lanes 3 and 6) under conditions where only low levels of monomeric complex are seen when RARα or RXRα are incubated alone. Taken together, the results of Figs. 6 and 7 indicate that the dimerization domain in the D-box of steroid receptor DBDs does not function in binding to direct repeats and suggest that steroid receptor DBDs may lack the dimerization interfaces located outside the D-box similar to those found in retinoid receptors. In addition, the results indicate that the dimerization function located in the ligand binding domain is essential for binding to direct repeats.
**DISCUSSION**

Direct Repeats as Steroid Response Elements—The experiments presented here indicate that both the GR and the ER are capable of binding to direct repeats of cognate half-sites as homodimers. These results extend the range of the DNA motifs constituting either a GRE or an ERE, as defined by the DNA sequences capable of binding steroid receptor homodimers. Our results have shown that the human GR is capable of forming complexes on direct repeats present in the MMTV promoter, as well as everted repeats of TGTTCT motifs. These complexes require the integrity of both half-sites and comigrate with complexes formed on a palindromic response element (Fig. 1). Similarly, complexes are formed by both the human and chicken ERs on direct repeats that comigrate with that formed on an ERE palindromic (Fig. 3).

The binding of either the ER or the GR to direct repeats is weaker than to palindromes. However, it should be pointed out that most GREs and EREs defined to date are not perfectly palindromic and are therefore bound more weakly by their corresponding receptors. The imperfect palindromic, AaGt-TCANNTGACCC, from the estrogen-responsive Xenopus vitellogenin B1 gene, competes ~8-fold less efficiently for ER binding than a perfectly palindromic ERE (64). Our results indicated that the direct repeat of ERE-D6 competes 6–10-fold less efficiently for ER binding than the palindromic sequence ERE-P3 and 8–12-fold more efficiently than an oligonucleotide containing only a single half-site (Fig. 3). This suggests that direct repeats of consensus half-sites could bind the ER roughly as efficiently as physiological imperfect palindromes. It is noteworthy that molecular genetic experiments in yeast have identified a number of DNA sequences that bind ER very weakly as potential EREs (52). In a similar study, a direct repeat of RGGTCA motifs separated by 3 bp mediated an estrogen response (51).

Direct repeats, like imperfectly palindromic elements, can be found in multiple arrays upstream of target promoters (50, 54, 55). Multiple imperfect palindromes of glucocorticoid- or estrogen-responsive promoters have been shown to combine to give a synergistic response to hormone (33, 34, 36). In the MMTV promoter, proximal response sequences, containing the sequences studied here, combine with more distal sites for an optimal response to glucocorticoid (55). Similarly, there is strong synergism between the four AGGTCA half-sites found far upstream of the chicken ovalbumin gene (50). In agreement with these results, our findings indicate that insertion of three, but not one, copies of the GRE-D9 element upstream of a heterologous promoter renders the promoter inducible by dexamethasone (Fig. 2).

Homodimerization and Binding of the ER and GR to Direct Repeats—The action of dimerization domains present in either the DBDs or LBDs of the ER and GR in binding to direct repeats was investigated using a series of truncated receptors and dimeric (D) complexes are indicated. Assays shown in lanes 1–5 were performed with 2 µl of 4-, 8-, 16-, and 32-fold dilutions of extracts, respectively. D, gel retardation assay performed with extracts of E. coli expressing the glucocorticoid receptor DNA binding domain derivative GR-DBD using ERE-P3, GRE-P3, GRE-D9, GRE-D9M1, or GRE-D6 oligonucleotides as indicated. Monomeric (M) and dimeric (D) complexes are indicated. E, gel retardation assay performed with extracts of E. coli expressing DNA binding domain derivatives of retinoid receptors RARα or RXRα (44) using the RARE-D5 oligonucleotide. Assays were performed with 0.25 µl (lanes 1 and 2) or 0.5 µl (lanes 4 and 5) of RARα- or RXRα-containing extracts alone or with 0.125 µl (lane 3) or 0.25 µl (lane 6) of each extract combined. Heterodimers of RARα and RXRα DNA binding domains are indicated by the arrowhead, and monomers and homodimers of RARα DNA binding domains are indicated by the asterisks.
ER derivative HE15, which contains regions A to D but lacks the ligand binding domain, failed to bind the direct repeats tested here, underlining the importance of the LBD in binding to nonpalindromic elements. In several experiments no evidence for cooperative binding of the GR DBD to direct repeats separated by 6, 8, or 9 bp was seen (Fig. 7D and data not shown). Similarly, no evidence for cooperative binding of the ER DBD to direct repeats separated by 2 to 6 bp was seen (Fig. 7B and C, and data not shown). These results indicate that the D-box of the ER and GR does not function in binding to direct repeats. Moreover, there is no evidence for the action of putative dimerization motifs located outside D-box of the ER or GR DBDs in binding to these elements. In contrast, the action of such motifs was observed under similar conditions in the cooperative binding of heterodimers of RXR and RAR to the RARE-D5 element (Fig. 7E). The truncated ER derivative HEG19 was found to bind to direct repeats, indicating that the ER A/B region is not essential for these interactions. It is noteworthy, however, that hormone-dependent interactions between the N- and C-terminal regions of the ER (65) and the androgen receptor (66) have been demonstrated recently. We cannot rule out the possibility that similar interactions may modulate binding to direct repeats.

Studies with RAR, TR, and RXR DBDs have suggested that cooperative binding to direct repeats only occurs on elements with specific inter-half-site spacings (42-47). Cooperativity between RAR and RXR DBDs is observed on elements with 2- or 5-bp spacings, with a different set of dimerization interfaces required in each case (46, 47). Binding of RAR/RXR heterodimers can occur to half-sites separated by 10 bp; however, specific interactions between the DBDs are apparently not required, and similar to the results presented here, DNA binding occurs exclusively through dimerization of the ligand binding domains (47). It remains possible that there exist a specific inter-half-site spacing(s) of direct repeats where cooperative binding by DBDs of steroid receptors can occur. However, accumulating evidence suggests that dimerization by DBDs is not necessary for direct repeats to function as response elements for steroid receptors and that, unlike binding to palindromic elements, binding of steroid receptors to direct repeats is very flexible. Each of the four RGGTCA half-sites located upstream of the chicken ovalbumin gene is separated by over 100 bp from its neighbor, and cooperative binding to two of the half-sites separated by 36 bp has been observed (50). Molecular genetic studies in yeast have suggested that direct repeats with 3-bp spacings can function as EREs (51, 52). The two GRE half-sites of the MMTV promoter, and hepatitis B virus promoter, are separated by 9 and 6 bp respectively. Finally, our DNA binding studies have suggested that steroid receptors will bind cooperatively to direct repeats with different spacings (Figs. 1 and 3-5).

Comparison of the Mechanisms of Binding to Palindromes and Direct Repeats by Nuclear Receptors—All of the above considerations are summarized in the models presented in Fig. 8. Binding of steroid receptors to palindromic elements is stabilized by dimerization domains located in the ligand binding domain and in the D-box of the DBD (Refs. 14, 48, and 49 and Fig. 8A, indicated by cross-hatches). Presumably, the dimerization domain in the D-box constrains the homodimer to recognize palindromes of a fixed spacing, i.e. 3 bp (12, 37, 38). Consistent with this interpretation, the TR, which lacks a functional D-box (12, 37), is capable of recognizing palindromes with a variety of inter-half-site spacings (67). Structure-function studies with both the ER and the GR have suggested that, unlike the dimerization by the ligand binding domain, DBD homodimers are unstable in solution (16, 37, 48, 49) and could therefore equilibrate between conformations that bind to palindromes or to direct repeats (Fig. 8A). This conformational change implies the presence of a flexible domain in region D necessary for equilibration between the two binding modes. This flexibility is reinforced by the observation that the GR can bind to everted TGTTCT motifs (Figs. 1 and 8A). A similar flexible domain must also exist in thyroid and retinoid receptors, which are capable of binding to both palindromes and direct repeats. We found no evidence for dimerization, and hence stabilization of isolated ER or GR DBDs bound to direct repeats, suggesting that the equilibrium of DNA binding domain conformations in homodimers is shifted toward a palindromic binding mode. The function of two sets of dimerization domains in binding to palindromes, as opposed to one set in binding to direct repeats, would account for the observations that steroid receptor homodimers bind to palindromes with higher affinity than to direct repeats.

The lack of apparent alternative dimerization domains in the DBDs of steroid receptors may account for the accumulating evidence of wide flexibility in their binding to direct repeats. Just as the ER and GR are constrained by D-box dimerization in recognition of palindromic elements, binding to direct repeats by heterodimers of RXR with retinoic acid, and thyroid hormone receptors is restricted by protein-protein interactions (Fig. 8B), which hold the DBDs in specific conformations. In contrast, binding of steroid receptor homodimers in the absence of DBD dimerization would be only constrained by a combination of the flexibility of the linker region between the DBD and the LBD and the flexibility of the DNA template. Thus binding to widely spaced half-sites, for example those upstream of the chicken ovalbumin gene (50), would be accompanied by a looping out of the intervening DNA (Fig. 8C). Interestingly in this regard, recent studies with the response element of the γ-F-crystallin promoter (68) showed that RXR/TR and RXR/RAR heterodimers can bind with high affinity to everted repeats separated by 6 or 8 bp. The everted half-sites of the γ-F-cry stallin response element are separated by an 8 bp (A + T)-rich linker, similar to the (A + T)-rich linker of the MMTV D9
element (see Fig. 1), and it was speculated that local DNA melting may contribute to flexibility of DNA binding (68).

In summary, the studies presented here extend our definition of the possible DNA sequences that can constitute steroid response elements, as defined by sequences recognized by receptor homodimers. They suggest that the DBDs of steroid receptors, similar to those of retinoid and thyroid hormone receptors, can adopt different conformations in recognizing response elements composed of either palindromes or direct repeats. The results also emphasize the importance of dimerization domains in controlling response element recognition by steroid receptors.

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