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Discrimination of DNA response elements for thyroid hormone and estrogen is dependent on dimerization of receptor DNA binding domains

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ABSTRACT **We and others have previously shown that a two-amino acid substitution in the base of the first zinc finger of the glucocorticoid receptor DNA binding domain (DBD) is sufficient to alter the receptor's target DNA from a glucocorticoid response element (GRE) to an estrogen response element (ERE). Activation of a thyroid hormone response element (TRE) has been shown to require an additional five-amino acid change in the second zinc finger of the thyroid hormone receptor (TR). Using closely related TRE and ERE sequences, we report that a receptor containing the TR DBD activates the ERE poorly, and receptors containing essential amino acids of the estrogen receptor (ER) DBD activate the TRE poorly. The ER DBD (expressed in *Escherichia coli*) selectively bound to a ^{32}P-labeled ERE (^{32}P-ERE) as a dimer and a ^{32}P-TRE as a monomer, whereas the TR DBD bound ^{32}P-TRE as a dimer and ^{32}P-ERE as a monomer. When hybrid receptor DBDs were examined, we found that the five amino acids in the second zinc finger of the TR necessary for TRE activation were also essential for dimer formation on a TRE. Dimer formation of ER on an ERE was localized to the second half of the second zinc finger. These results suggest that the ability of ER and TR to functionally discriminate between an ERE and a TRE is a result of dimerization of their DBDs.**

Steroid hormone receptors are a class of transcriptional activators that produce their biological effects through discrete gene enhancers termed hormone response elements (HREs) (1, 2). Glucocorticoid receptors (GRs) and estrogen receptors (ERs) are composed of modular domains, each with ascribable functions related to ligand binding, DNA binding, and transcription activation (2–5). Binding of the receptor to a specific HRE occurs through a DNA binding domain (DBD), which consists of two fingers, each formed by the coordination of a single zinc atom with four cysteine residues (6, 7). This structural configuration is consistent with evidence that receptors interact with their response elements in the form of homodimers (8–10) or heterodimers (11, 12).

To elucidate the mechanism by which a receptor recognizes its own response element, we and others recently constructed cDNAs carrying systematic substitutions of amino acid residues within the zinc fingers of either the GR (13, 14) or the ER (15). These studies identified seven amino acids that are critical for determining sequence-specific recognition by the DBD. Selective activation from a glucocorticoid response element (GRE) vs. an estrogen response element (ERE) is dependent on two amino acids in the base of the first finger; activation from a thyroid hormone response element (TRE) requires an additional five amino acids in the second finger.

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Here we have focused on the ability of hybrid receptors to distinguish between an ERE and a TRE. Experiments indicated that while chimeric receptors containing the entire DBD of the thyroid hormone receptor (TR) are able to activate transcription from a TRE, they are unable to do so from an ERE. This was intriguing because the difference between some EREs and TREs is the spacing of the palindromic half sites (16). Using an *Escherichia coli* expression system, we synthesized the DBDs of hybrid receptors and examined their ability to bind HREs *in vitro* by an electrophoretic mobility-shift assay (17). Our results suggest that the spacing of the TRE and ERE half sites is critical in the formation of DBD dimers. The portion of the TR DBD required for dimerization appears to be the first five amino acids in the second finger previously identified as essential for TRE activation (14). Dimerization of ER DBDs was also found to occur through the second finger; however, the critical residues are C-terminal to those identified for the TR.

MATERIALS AND METHODS

Construction of Reporter Plasmids and Chimeric Receptors in Expression Vectors. The first finger constructs, pGE9 and pGT1, have been described (13). The interfinger constructs, pGT2 and pGT3, were derived from pGT1 and pSV2Xrec and pGTR was derived from pGT3.

For protein expression of the DBDs, the T7 *E. coli* expression system was used (18). A blunted *Sty* I/*Nar* I (amino acids 386–581) fragment from pSV2Xrec, a modified version of the wild-type GR (13), was ligated into the *Bam*H1 site of the pET3a expression vector. This yielded Xrec, a 237-amino acid fusion protein that contains 10 amino acids of T7 gene 10 on the N terminus and 31 amino acids of T7 gene 10 on the C terminus with a calculated molecular weight of 28,649. Amino acids 420–496 of pGT1, pGT2, pGT3, pGTR, and pGER were used to replace the *Kpn* I/*Xho* I fragment of Xrec. All constructs were confirmed by DNA sequencing and also examined by Western blot analysis using the monoclonal antibody BuGR1 (a generous gift of Robert Harrison, Vanderbilt University School of Medicine, Nashville, TN) to confirm that receptor protein was being expressed.

Reporter plasmids were constructed from pMCAT5 (19), which lacks the endogenous GRE. pMECAT and pMTCAT were made by inserting either an ERE-containing oligonucleotide (GATCAAGCTTAGATCAGGTCACTGTGACCTAGATCTAAGCTT) or a TRE-containing oligonu-

Abbreviations: HRE, hormone response element; GR, glucocorticoid receptor; ER, estrogen receptor; DBD, DNA binding domain; GRE, glucocorticoid response element; ERE, estrogen response element; TRE, thyroid hormone response element; TR, thyroid hormone receptor.

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cleotide (GATCAAGCTTAGATCAGGTCATGACCTA-GATCTAAGCTT) into a *Bgl* II site.

Tissue Culture and Transfections. COS-7 cells grown to near confluence were transfected by the lipofection procedure as described (13) using 0.5 μg of receptor DNA and 1.5 μg of the reporter plasmid. Cells were harvested after 3 days and treated with vehicle (ethanol) or 1 μM dexamethasone 24 hr before harvesting. Chloramphenicol acetyltransferase assays were performed as described (13).

Protein Expression of Chimeric DBD. pET expression vectors with or without receptor sequence were transfected into BL21DE3 *E. coli* carrying the LysS plasmid (obtained from F. W. Studier; ref. 18). One-liter cultures in L broth were grown to an OD_{600} of 0.3. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 1 mM and cultures were grown an additional 5–6 hr. Extracts were prepared as described (7).

Electrophoretic Mobility-Shift Assay. Three double-stranded oligomers, two 35 base pairs and one 34 base pairs long, were synthesized and used in the mobility-shift assay. These were called ERE (GATCGCTAATAGGTCACAGT-GACCTGATGCCGTCC), TRE (GATCGCTAATCAGGT-CATGACCTGGATGCCGTCC), and GRE (GATCGCTAA-TAGAACAAAATGTTCTGATGCCGTCC). When used as probes, the oligonucleotides were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) using T4 polynucleotide kinase (20). In a 25-μl binding assay mixture, each sample contained buffer [10 mM Tris·HCl, pH 7.5/0.5 mM EDTA/10% (vol/vol) glycerol/50 mM sodium chloride/0.2% Nonidet P-40], 100 ng of poly(dI-dC), 10–20 pg of labeled DNA probe (1–1.5 $\times$ 10^9 dpm per μg of DNA), and (added last) 1–2 μl of a 1:50–1:100 dilution of extract (100–500 ng of protein). Samples were incubated at room temperature for 20 min and then applied to an 8% native gel [8% polyacrylamide/0.5$\times$ TBE (1$\times$ TBE = 89 mM Tris, pH 8.3/89 mM boric acid/8 mM EDTA)] equilibrated at 4°C and run $\approx$3 hr at 10 V/cm.

Methylation Interference. The method used for this assay is described elsewhere (21). End-labeled ERE and TRE fragments were prepared (7.5 $\times$ 10^7 dpm per μg of DNA) and exposed to dimethyl sulfate as described (22). To obtain adequate radioactivity in the recovery, all components in the assay were increased 50-fold over our typical assay sample. The appropriate bands from the mobility-shift assay were localized by autoradiography, excised from the gel, and recovered by electrophoresis onto DEAE paper. After extraction from the paper, the DNA was cleaved with piperidine (22), dried under vacuum, and resuspended in formamide; 50,000 cpm of each sample was run on a 10% sequencing gel.

RESULTS

Transcriptional Activity of GRs Containing Chimeric DBDs. We (13) and others (14, 15) have demonstrated that substituting two amino acids in the base of the first zinc finger of the GR (glycine and serine) with those found in the ER (glutamic acid and glycine) alters the specificity of transcriptional activation of this hybrid protein from that of a GR to an ER. These substitutions, however, are not sufficient to activate transcription from a TRE. Umesono and Evans (14) revealed that, in addition to the substitution in the first finger, the five amino acids between the two cysteines at the base of the TR second finger (termed the D box) are critical for activation of a TRE. We also find that a receptor with the two-amino acid substitution in the first finger (pGE9; Fig. 1) or a receptor with the entire ER DBD (ref. 13; data not shown) activates an ERE, whereas both pGE9 (Fig. 1*B*) and the human ER (14) activate a TRE poorly. Substituting a TR sequence for the entire first finger (pGT1), as well as the interfinger region (pGT2), yields a receptor that fails to substantially activate a TRE. However, when the D box is included (pGT3), TRE activation ensues (Fig. 1*B*). Furthermore, insertion of both TR zinc fingers (pGTR) greatly reduces activation from an ERE. These results suggest that the second finger plays a critical role in discrimination between the closely related HREs for estrogen and thyroid hormones. The difference between the ERE (AGGTCA-CAGTGACCT) and TRE (AGGTCATGACCT) used in these studies is the three inserted bases (CAG) in the ERE. This change in spacing of the inverted repeats would be expected to alter the spatial relationship between proteins binding to the two halves of the palindrome.

Binding Specificity of Chimeric DBDs. To explore the basis for TRE/ERE specificity among the chimeric receptors, we produced a portion of the receptor protein using a T7 *E. coli* expression system. The initial constructs contained a fragment of the mouse GR (amino acids 386–575) spanning the DBD in which the zinc fingers were swapped for those of the ER or the TR DBD, as well as the two-amino acid change of pGE9 (GE9 DBD). Crude *E. coli* extracts containing the ER DBD were incubated with the ^{32}P-labeled ERE (^{32}P-ERE) and then analyzed by electrophoretic mobility-shift assay (Fig. 2*A*). Several bands representing protein–probe complexes were observed, but only one prominent band was found in extracts expressing the receptor (lane 1 vs. lane 12). In competition experiments, this band could best be displaced by the identical unlabeled ERE, partially by the TRE, and very poorly, if at all, by the GRE. The data from multiple experiments depict the relative binding affinities of the three HREs (Fig. 2*B*).

In experiments examining extracts containing TR DBD incubated with ^{32}P-TRE, we observed two receptor-specific bands (Fig. 2*C*). The upper band corresponds to that seen when extracts containing the ER DBD were used and probably represents receptor binding to the DNA as a dimer. Consistent with other published data (8), the faster-migrating band probably represents receptor binding to DNA as a monomer. Indeed, as we demonstrate below by methylation interference, a monomer of the ER DBD migrates at the same position when bound to a TRE. This lower band is unlikely to be a proteolyzed form of the protein since Western blotting

A

GR	CLVC SDEASGCHYGVLT CGSC KVFFKRAVEGQ HN YL CAGRNDCIIDKIRRKN CPAC
TR	CVVC GDKATGYHYRCIT CEGC KGFFRRTIQKNLHPSYS CKYEGKCVIDKVTRNQ CQEC
ER	CAVC NDYASGYHYGVWS CEGC KAFFKRSIQGH NDYM CPATNQCTIDKNRRKS CQAC

B

		CAT ACTIVITY (% max) pMECAT	pMTCAT
pGE9	•••• ••••••••••••• •EG• ••••••••••• •• •• •••••••••••••••• ••••	100	8
pGT1	•V•• G•K•T•Y••RCI• •EG• •G••••••••• •• •• •••••••••••••••• ••••	80	20
pGT2	•V•• G•K•T•Y••RCI• •EG• •G••R•TIQKNL•PS•S •••••••••••••••• ••••	27	24
pGT3	•V•• G•K•T•Y••RCI• •EG• •G••R•TIQKNL•PS•S •KYEGK•V•••••••• ••••	33	94
pGTR	•V•• G•K•T•Y••RCI• •EG• •G••R•TIQKNL•PS•S •KYEGK•V•••VT•NQ•Q••	8	100

FIG. 1. DBDs and reporter gene activity of transfected receptors. (*A*) Amino acid sequence of DBDs of the GR, TR, and ER. (*B*) Amino acid sequence of chimeric receptor DBDs of the indicated plasmids. Dots indicate amino acids of GR. COS-7 cells were cotransfected with chimeric plasmid and either pMECAT or pMTCAT. Data were normalized to results from pGE9 for pMECAT and to pGTR for pMTCAT. Numbers are mean values for that construct obtained from five independent transfections (each in triplicate).

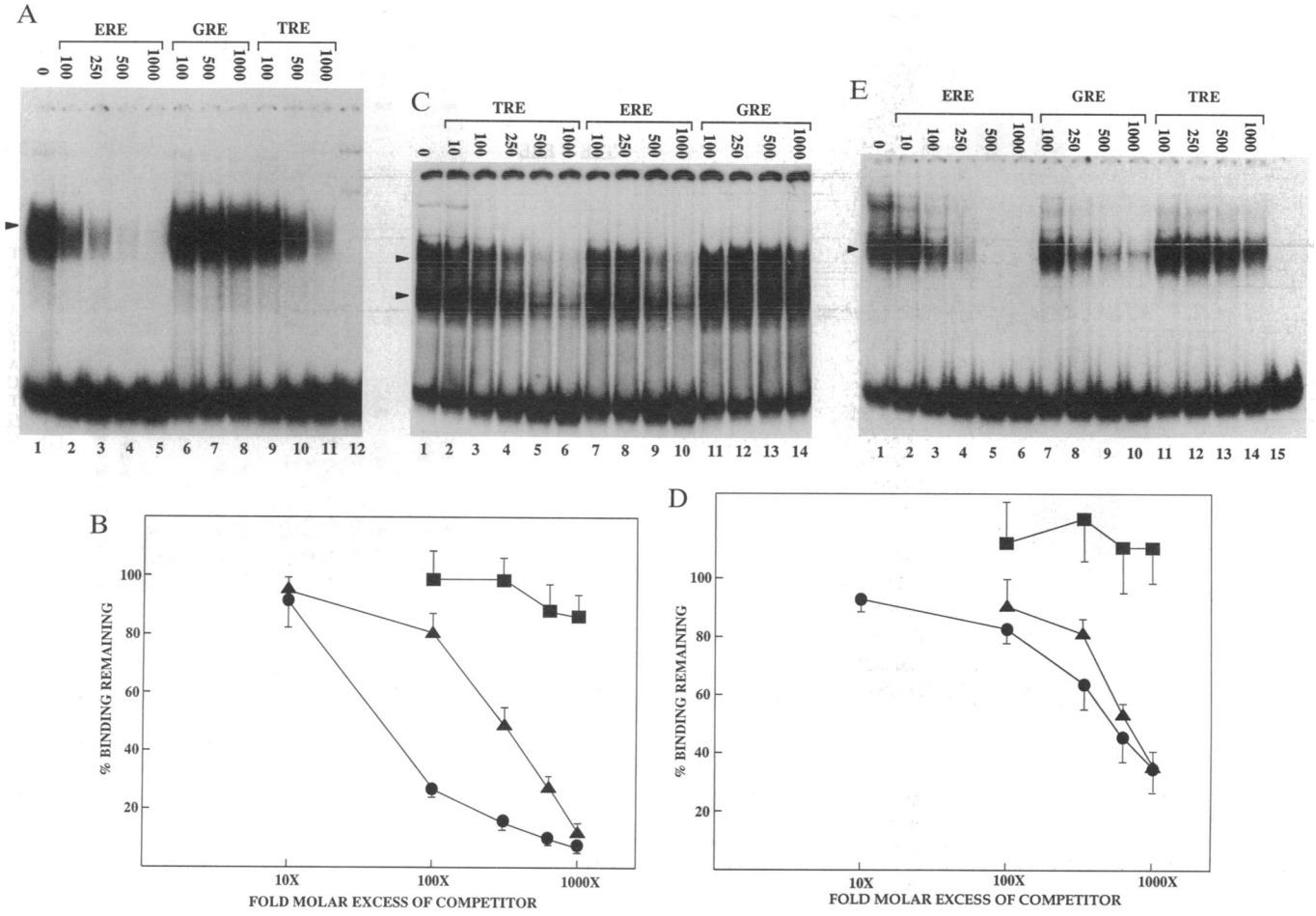

FIG. 2. Mobility-shift assay of binding specificity of ER, TR, and GE9 DBDs. (*A*) ER DBD extracts were incubated with a ^{32}P-ERE oligonucleotide in the presence or absence of various quantities of competitor DNA. Arrowhead indicates the receptor-specific band. Numbers above lanes indicate concentration of added competitor (ERE, GRE, or TRE) expressed as -fold molar excess. Lane 12, extract from *E. coli* carrying a pET expression plasmid with no receptor insert. (*B*) Bands in *A* were excised and assayed for radioactivity. Data points represent means ± SE from at least five determinations. Radioactivity assayed within the indicated band in the absence of competitior was assigned a value of 100%. Competition with unlabeled ERE (circles), TRE (triangles), and GRE (rectangles). (*C*) TR DBD extracts were incubated with a ^{32}P-TRE oligonucleotide plus or minus competitor DNA as in *A*. Arrowheads indicate receptor-specific bands. (*D*) Bands were assayed and data were calculated as in *B*. Competition with unlabeled TRE (circles), ERE (triangles), and GRE (rectangles). (*E*) GE9 DBD extracts were incubated with a ^{32}P-ERE oligonucleotide plus or minus competitor DNA as in *A*. Arrowhead indicates receptor-specific band. Lane 15, no extract.

of the extracts used in these experiments detects predominantly intact protein (data not shown). Competition experiments demonstrate that TRE and ERE are the best competitors for TR DBD binding, while GRE does not compete (Fig. 2*D*).

Extracts containing GE9 DBD yielded two major bands when incubated with ^{32}P-ERE (Fig. 2*E*). The upper band was not receptor specific as we observed this band in control extracts (see Figs. 2*A* and 3*B*). The lower receptor-specific band had an identical mobility to that seen with ER DBD. The competition profile, however, was unlike that observed for ER and TR DBD. The most effective competitor was ERE followed by GRE and finally TRE. When extracts containing GE9 DBD were incubated with ^{32}P-TRE, no receptor-specific bands were observed (data not shown).

The ability of the TRE and ERE to bind *in vitro*, respectively, to ER and TR DBDs may reflect the similarity in sequence of the HREs. The finding that TRE is a relatively poor competitor for GE9 DBD binding to an ERE supports the functional data indicating that the second finger imparts the ability to distinguish between a TRE and an ERE.

DNA Binding of ER and TR Zinc Fingers to Radiolabeled Estrogen and Thyroid HREs. We examined the binding of ER and TR DBD to ^{32}P-TRE and ^{32}P-ERE (Fig. 3). The bands observed in control extracts (Fig. 3, lanes 7b and 8b) are not receptor specific and presumably represent binding of *E. coli* proteins. When extracts containing ER DBD were incubated with a ^{32}P-TRE, we observed a lower molecular weight complex than we did with ^{32}P-ERE (lane 4a vs. lane 1a). This lower band was more easily blocked by competition with unlabeled ERE than with unlabeled TRE (lane 5a vs. lane 6a), indicating that the protein associated with the TRE is in fact the ER DBD. These data are consistent with a monomer of ER DBD binding to half palindromic binding sites on the TRE. When TR DBD is incubated with ^{32}P-ERE, the only receptor-specific band observed is the lower band (lanes 4b–6b). This band is also seen with ^{32}P-TRE (lanes 1b–3b). Like ER DBD binding to a TRE, TR DBD association with an ERE appears to be in the form of monomers. Thus, while the data in Fig. 2 demonstrate that ERE can compete for TR DBD binding to a TRE, and TRE can compete for ER DBD binding to an ERE, this cross competition is occurring via monomer binding.

Methylation Interference of the ER DBD and Radiolabeled HREs. To provide direct evidence that the difference in migration patterns in the band-shift assay is the result of receptor DBDs associating with the HRE as dimers or monomers, we performed a methylation interference assay

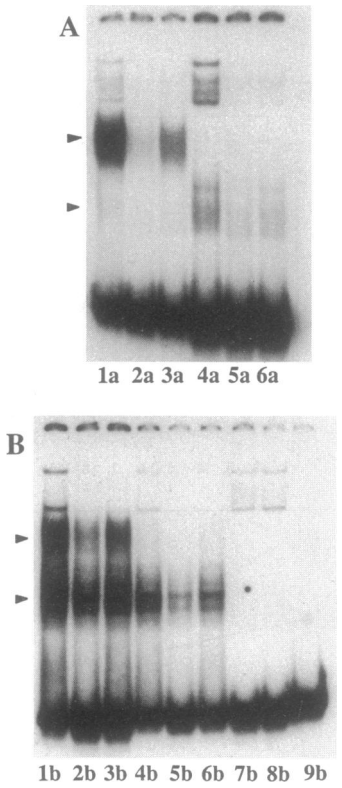

FIG. 3. Mobility-shift assay of ER and TR DBD binding to ^{32}P-TRE and ^{32}P-ERE. (*A*) ER DBD binding to ^{32}P-ERE and ^{32}P-TRE. Lanes: 1a–3a, ER DBD-containing extracts incubated with ^{32}P-ERE; 4a–6a, extracts incubated with ^{32}P-TRE; 1a and 4a, no competitior; 2a and 5a, 250-fold molar excess unlabeled ERE; 3a and 6a, 250-fold molar excess unlabeled TRE. (*B*) TR DBD binding to ^{32}P-TRE and ^{32}P-ERE. Lanes: 1b–3b, TR DBD-containing extracts incubated with ^{32}P-TRE; 4b–6b, extracts incubated with ^{32}P-ERE; 1b and 4b, no competitor; 2b and 5b, 250-fold molar excess unlabeled TRE; 3b and 6b, 250-fold molar excess unlabeled ERE; 7b, extract with no receptor fragment incubated with ^{32}P-ERE; 8b, extract with no receptor fragment incubated with ^{32}P-TRE; 9b, ^{32}P-TRE probe with no extract. Arrowheads indicate receptor-specific bands.

(21). We reasoned that if the ER DBD was binding the TRE as a monomer, then 50% of the DNA molecules recovered from the shifted band in the ^{32}P-TRE-labeled sample would have a receptor DBD bound to one half of the palindrome and 50% would have a receptor bound to the other half. This would result in a footprint (interference region) with a 50% decrease in radioactivity of the critical guanosine residues when compared to the unbound probe. If the ER DBD binds to the TRE palindrome as a dimer, there would be a 100% decrease in radioactivity, indicating that both halves of the palindrome of all DNA molecules were bound. When the ER DBD was complexed with ^{32}P-TRE, only partial diminution of radioactivity in the critical guanosine residues resulted (indicative of monomer formation). When complexed with a ^{32}P-ERE, however, the nearly complete absence of radioactivity in the same guanosine residues indicated dimer formation (Fig. 4). Quantitative data are presented in Table 1. This experiment provides positive evidence that a receptor DBD can associate with a HRE in either a monomeric or a dimeric configuration, which can be detected in a mobility-shift assay as a difference in migration.

Localization of the Dimerization Domains Within TR and ER DBDs. To ascertain the portion of the zinc fingers necessary to enable dimer binding to the HRE, three chimeric DBDs (GT1, GT2, GT3) were tested for DNA binding *in vitro*. PGT1, which contains TR sequence of the entire first finger activates a TRE poorly (Fig. 1); GT1 DBD binds to a labeled

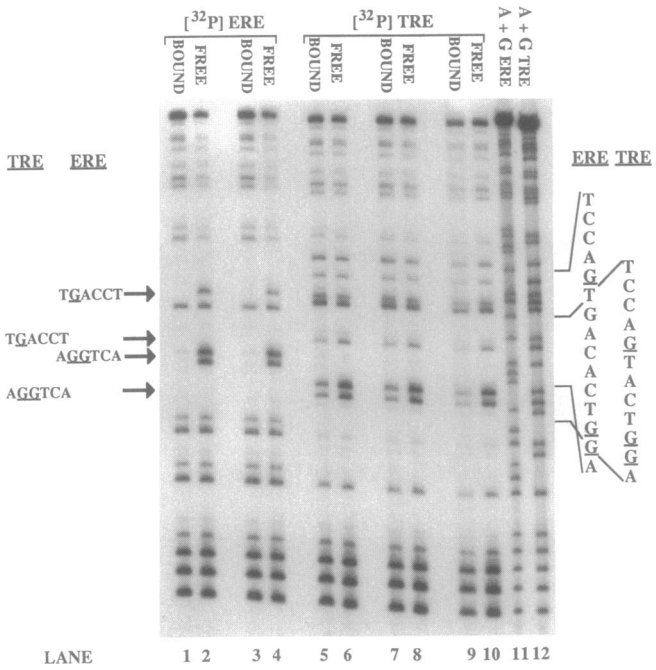

FIG. 4. Methylation interference of ER DBD complexed with ^{32}P-ERE and ^{32}P-TRE. Extracts containing ER DBD were incubated with either end-labeled and methylated TRE or ERE and were analyzed as described. Radioactivity recovered from receptor-specific shifted bands (bound) or from unshifted probe (free) is shown in duplicate (^{32}P-ERE) and triplicate (^{32}P-TRE). Lanes 11 and 12, adenine and guanosine ladder of ^{32}P-ERE and ^{32}P-TRE, respectively. Locations of HREs are shown in brackets next to lanes 11 and 12. Critical methylated guanosine residues are indicated on the left.

TRE *in vitro* only as the faster-migrating monomeric species (Fig. 5, lane 2). pGT2 (and GT2 DBD) contains TR sequence up to but not including the second zinc finger and yields results similar to pGT1; that is, pGT2 activates a TRE poorly and GT2 DBD cannot form dimers (lane 4). pGT3 (and GT3 DBD) has TR sequence through the interfinger region and the first five amino acids of the second finger; pGT3 can activate a TRE and GT3 DBD associates as dimers on the TRE *in vitro* (lane 6). These added sequences in pGT3 correspond to the D box shown previously (19) and are a requirement for TRE activation. The chimeric DBDs GT1, GT2, and GT3 are all capable of binding to an ERE in the form of dimers (lanes 1, 3, and 5) and as intact receptors are transcriptionally active on an ERE. The ability to form dimers as well as to generate a functional response on an ERE is lost when the remaining portion of the second finger is substituted with TR sequence (i.e., with an entire DBD TR) (Figs. 1*A* and 3*B*). This suggests

Table 1. Quantitation of methylation interference

ER DBD binding to	AGGTCA bound/free × 100, %	TGACCT bound/free × 100, %	Lanes
^{32}P-ERE	6	11	1, 2
	6	8	3, 4
^{32}P-TRE	44	52	5, 6
	43	60	7, 8
	44	66	9, 10

For quantitation, the dried gel shown in Fig. 4 was scanned by an Ambis radionucleotide imaging system. Areas outside the response element were used to standardize the bound and free lane of each sample in order to eliminate any variation in loading. After subtracting the background, a ratio was calculated by dividing the cpm in the indicated guanosine residues (underlined) in the bound lane by the cpm of the comparable area in the free lane of each sample pair.

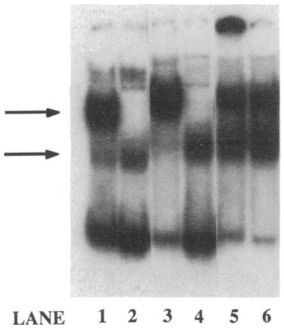

FIG. 5. Mobility-shift assay examining chimeric DBDs. Extracts containing chimeric receptor DBDs were incubated with ^{32}P-ERE or ^{32}P-TRE. Lanes: 1, 3, and 5, ^{32}P-ERE probe; 2, 4, and 6, ^{32}P-TRE probe; 1 and 2, GT1 extract; 3 and 4, GT2 extract; 5 and 6, GT3 extract. Arrows indicate receptor-specific bands.

that the ability of an ER DBD to form dimers on an ERE is determined by the C-terminal half of the second zinc finger.

DISCUSSION

Recent studies examining the solution structure of GR (23) and ER (24) DBDs provide insights into the structural relationship of amino acids critical for activation of cognate HREs. Since this work was completed, the crystallographic structure of the GR DBD bound to DNA has been published (25), revealing that dimers are formed through contact of the D-box amino acid residues of the second finger. Our finding that the function of the D box in TR is to facilitate dimer formation on a TRE is in agreement with this model and suggests that TR and possibly ER form complexes with their HREs in a fashion similar to that of the GR.

There are now a number of reports providing direct evidence that members of the steroid receptor family bind to their HREs as dimers (8–12). Studies therein and the data provided here indicate that there are at least two dimerization "domains" within the various receptors—one located in the C-terminal or ligand binding domain and one in the DBD. Dimerization through the C-terminal portion has been postulated to occur within a consensus sequence found in several members of the steroid receptor superfamily (10). This domain is not essential since mutant GRs lacking it are still capable of activating transcription to wild-type levels (3, 26). Other studies have reported that TR inhibition of transcription from an ERE is dependent on the C terminus of TR (16, 27). The explanation for this finding is not yet clear but it may relate to the fact that receptors have two dimerization domains, each of which may function in an independent fashion (i.e., the TR may be forming a nonactivating but stable receptor–DNA complex). Nevertheless, our data together with the crystallographic data (25) support the hypothesis that the dimerization site within the DBD is essential for positive transcriptional activation from a HRE.

It is interesting to note that DNA binding experiments with chimeric receptors clearly indicate that the same amino acids required for TRE activation by TR are also essential for dimer formation of the TR DBD on a TRE. The aptly named D box could be referred to as the dimerization box for TR. We emphasize, however, that in this study the TR DBD was examined in the context of the GR. It is possible that in the context of the entire TR, the D box possesses distinct properties that are not displayed by the GR and ER. This notion may be supported by the observation that TR can recognize both direct and inverted repeats (28) and is capable of binding to DNA as a heterodimer (11).

In regard to the ER, functional activity and dimerization on an ERE are dependent on the distal part of the second finger. The data supporting this conclusion are that pGT2 and pGT3, which can both partially activate an ERE, have DBDs that can dimerize on an ERE. If the remainder of the TR second finger is added, the resultant TR DBD can no longer substantially activate an ERE or form dimers on an ERE.

The picture that is emerging suggests that dimerization of receptors, in particular via the DBD, is an essential component of the hormone response pathway and that the second finger plays a predominant role in this capacity. Of particular note in this study is the finding that dimerization *per se* provides an added dimension whereby receptors can distinguish among their specific HREs.

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