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Defining a minimal estrogen receptor DNA binding domain

Sylvie Mader+, Pierre Chambon and John H.White[1,*]
Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité de Génie Génétique et de Biologie Moléculaire de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 Rue Humann, 67085 Strasbourg Cédex, France and [1]Department of Physiology, McIntyre Medical Sciences Bldg, McGill University, 3655 Drummond St., Montreal, Quebec H3G 1Y6, Canada

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ABSTRACT

The estrogen receptor (ER) is a transcriptional regulator which binds to cognate palindromic DNA sequences known as estrogen response elements (EREs). A 66 amino acid core region which contains two zinc fingers and is highly conserved among the nuclear receptors is essential for site specific DNA recognition. However, it remains unclear how many flanking amino acids in addition to the zinc finger core are required for DNA binding. Here, we have characterized the minimal DNA binding region of the human ER by analysing the DNA binding properties of a series of deletion mutants expressed in bacteria. We find that the 66 amino acid zinc finger core of the DBD fails to bind DNA, and that the C-terminal end of the minimal ER DBD required for binding to perfectly palindromic EREs corresponds to the limit of 100% amino acid homology between the chicken and human receptors, which represents the boundary between regions C and D in the ER. Moreover, amino acids of region D up to 30 residues C-terminal to the zinc fingers greatly stabilize DNA binding by the DBD to perfectly palindromic EREs and are absolutely required for formation of gel retardation complexes by the DBD on certain physiological imperfectly palindromic EREs. These results indicate that in addition to the zinc finger core, amino acids C-terminal to the core in regions C and D play a key role in DNA binding by the ER, particularly to imperfectly palindromic response elements. The ER DBD expressed in *E.coli* binds as a dimer to ERE palindromes in a highly cooperative manner and forms only low levels of monomeric protein – DNA complexes on either palindromic or half-palindromic response elements. Conversion of ER amino acids 222 to 226, which lie within region C, to the corresponding residues of the human RARα abolishes formation of dimeric protein – DNA complexes. Conversely, replacement of the same region of RARα with ER residues 222 to 226 creates a derivative that, unlike the RARα DBD, binds cooperatively to an ERE, indicating that this region is important for dimerization in the ER.

INTRODUCTION

The estrogen receptor (ER) is a member of the nuclear receptor family of transcriptional regulators. Nuclear receptors are activated by binding small lipophilic ligands such as steroid and thyroid hormones, retinoids and vitamin D3 (1–5), and recognize specific DNA sequences known collectively as hormone response elements. Several studies have shown that the ER and other nuclear receptors bind to their cognate response elements as dimers (6–11). Sequence comparisons (12) and structure–function analyses (6,13–15) have shown that the receptors are composed of a series of conserved domains. The most highly conserved domain is the DNA binding domain (DBD) located in region C (12,16,17). The 84 amino acid region C, which is 100% conserved between the chicken and human ERs (12,18; Fig. 1), is composed of a 66 amino core containing two zinc fingers (a.a.185 to 250) followed by a 12 amino acid region containing a number of basic residues (a.a. 251–262).

The 66 amino acid zinc finger core of region C is essential for sequence-specific DNA recognition by the ER and the closely related glucocorticoid receptor (GR) (19–21). C-terminal to region C lies region D whose sequence varies widely among the receptors. ER deletion mutants containing region C but lacking region D fail to bind DNA suggesting that at least part of the D region is required for specific DNA binding (6,14,22,23). Amino acids C-terminal to the zinc fingers have also been implicated in DNA binding by orphan nuclear receptors (24). However, residues C-terminal to the zinc fingers are poorly resolved in N.M.R. and X-ray crystallographic studies (25–27) of highly truncated ER and GR DBDs, giving no clues as to the possible role of region D in DNA binding.

Here, we have expressed a series of ER polypeptides containing the DNA binding domain in bacteria and have determined that

* To whom correspondence should be addressed

+ Present address: Department of Biochemistry, McIntyre Medical Sciences Bldg, McGill University, 3655 Drummond St., Montreal, Quebec H3G 1Y6, Canada

the C-terminal end of the minimal ER DBD corresponds to the boundary between regions C and D of the receptor. However, the minimal DBD does not form stable gel retardation complexes on certain physiological imperfectly palindromic EREs. Amino acids of region D up to 30 residues C-terminal to the zinc fingers greatly stabilize DNA binding by the DBD to both perfectly and imperfectly palindromic EREs. The ER DNA binding domain expressed in bacteria binds highly cooperatively as a dimer to estrogen response element (ERE) palindromes, confirming previous studies indicating the presence of a dimerization domain in the DBD (6). We have also generated two chimeric DBDs by converting 5 amino acids in the the C region of ER (a.a. 222−226) to the corresponding residues of human RARα (a.a. 125−129), and by introducing the converse mutation in the human RARα DBD. The ERE DBD mutant containing RARα residues 125 to 129 no longer binds to an ERE cooperatively, whereas the RARα DBD containing ER amino acids 222 to 226 does bind cooperatively, unlike its wild-type counterpart. These results indicate that ER residues 222 to 226 are important for DBD dimerization.

MATERIALS AND METHODS

Plasmid constructions

The β-galactosidase fusion protein expression vector pFP4 was constructed by inserting a sequence encoding a cleavage site for blood coagulation factor Xa [Ile-Glu-Gly-Arg, (28)] followed by KpnI and XhoI sites between the PstI and HindIII sites of vector pUR291 (29) to create pUR291Xa. Subsequently, the KpnI−XhoI fragment encoding the ER DBD from amino acids Met 176 to Ser 282 of HE80 (19) was inserted between the XhoI and KpnI sites of pUR291Xa to create HE81pUR291Xa. The T7 RNA polymerase expression vector pET32 was constructed from pET3C (30) by replacement of gene 10 sequence from gly7 to arg 12 with CAGGGTACCTCTAGACCTCGAGCCTGAG which contains sites for KpnI, XbaI, and XhoI, followed by a stop codon. DNA encoding HE120−HE124 or HE81 was amplified by PCR, generating fragments containing KpnI and XhoI sites at the 5′ and 3′ ends, respectively, which were subsequently inserted into pET32 digested with KpnI and XhoI. HE81RAR-pET32 was constructed by PCR amplification with HE81 primers of DBD sequence from an ER derivative in which nucleotides encoding a.a. 222−226 were replaced by in vitro mutagenesis with sequence encoding the corresponding a.a. from human RARα (his-arg-asp-lys-asn) (31). RARpET32 was constructed by PCR from the human RARα cDNA and contains RARa amino acids (83−187). RARER was created by replacing RAR amino acids (125−129) with ER residues 222−226 (pro-ala-thr-asn-gln) by in vitro mutagenesis. The primary structures of DBD derivatives were confirmed by DNA sequencing.

Expression in *E.coli* and protein purification

400 ml cultures of *E.coli* TG2 containing HE81-pUR291Xa or BL21/pLysS containing pET32 derivatives were grown at 37°C in L broth containing 100μg/ml ampicillin and 30μg/ml chloramphenicol (BL21/pLysS only) to an OD_{595} of 0.6. Expression was then induced by addition of IPTG to a final concentration of 0.5mM. Cells were harvested 1 hr later. Bacterial pellets were resuspended in 10 ml of sonication buffer [25mM Tris−HCl (pH 7.5), 0.2 M NaCl, 1mM DTT, 0.1 EDTA, 10% glycerol and 2.5 μg/ml of protease inhibitors pepstatin, leupeptin, antipain and pepstatin]. Cells were lysed by sonication and insoluble material was removed by centrifugation for 1 hr and 40,000 rpm in a Beckman 50Ti rotor. To isolate the ER DBD from the FP4 fusion protein, extracts were passed over a β-galactosidase TPEG-Sepharose affinity column (32) equilibrated in 50 mM Tris−HCl (pH 7.5), 100 mM NaCl. Bound protein was treated with Factor Xa (Sigma) overnight at 4°C. The eluted protein was 80% pure as judged from Coomassie brilliant Blue-stained SDS polyacylamide gels. DBD derivatives expressed in pET32 were purified from a Heparin Sepharose column by salt step elution in elution in 25mM Tris−HCl (pH 7.5), 1mM DTT, 0.1 EDTA, 10% glycerol. HE81 and HE124 elute in 1M NaCl and are approximately 80% pure as judged by Coomassie brilliant blue staining of SDS polyacrylamide gels. HE123 and HE122 elute in 0.6M NaCl step and are approximately 50% pure (data not shown).

^{35}S-methionine labeling

Cultures of BL21/pLysS cells (20 ml) carrying DBD expression vectors were grown at 37°C to an OD_{595} of 0.5 and aliquots (0.5 ml) were removed and centrifuged for 30 sec. in a microfuge. The pellets were washed with 1.0ml of M9 salts and resuspended in 1 ml of M9 salts containing 0.01% (w/v) of each of 18 amino acids (-cys, -met) and 0.5 mM IPTG. After 30 min of induction at 37°C, 10 μCi of ^{35}S-methionine was added. After 5 min cells were centrifuged for 30 sec in a microfuge and the pellets were resuspended in 40 μl of SDS gel loading buffer and heated at 90°C for 5 min prior to loading on a 12% SDS-polyacrylamide gel.

Gel retardation assays

Extracts were incubated for 15 min on ice in 10 μl of 25 mM Tris−HCl (pH 8.0), 1 mM DTT, 50 mM KCl, 0.1% NP40, 20% glycerol containing 1μg of poly dIdC, and then for a further 20 min at 23°C after addition of 50,000−100,000 cpm (5−10 fmol) of ^{32}P-end-labelled double stranded oligonucleotides. HE15-containing extracts were preincubated for 10 min on ice in the presence of 2μg of poly dIdC. Samples were loaded on 5% polyacrylamide gels equilibrated in 25 mM Tris−HCl/31.3 mM Boric acid/1 mM EDTA (pH 8.0) (0.5×TBE) unless otherwise indicated and electrophoresed at 25mA. Gels were dried prior to autoradiography.

Estimation of the half-life of protein−DNA complexes

Three-fold scale preincubations for gel retardation assays in 20mM KCl containing bromphenol blue tracking dye were performed in a final volume of 30μl. After 15 min on ice, 150,000 cpm (≈30 fmol) of oligonucleotide was added and incubations were continued for a further 20 min at 23°. At this time, 3μl aliquots were taken from each incubation as 0 min timepoints and loaded on a 5% polyacrylamide gel equilibrated in 0.25×TBE prerunning at 8 V/cm with buffer recirculation. 40 pmol of unlabelled V-ERE oligonucleotide was then added to each incubation to trap DBD molecules dissociating from ^{32}P-labelled oligonucleotides. Aliquots (3 μl) were taken 2, 5, 10, 15, 25 min and 35 min later and loaded immediately after withdrawal onto the running gel. Entry time of the sample onto the gel was 45−60 sec so that, for example, a 2 min sample had entered the gel by 3 min and is represented as a 3 min timepoint.

RESULTS

Conversion of ER amino acids 222–226 to the corresponding residues of the human retinoic acid receptor α abolishes dimerization by the isolated ER DBD

Previous studies have shown that ER derivatives with N-terminal truncations to amino acid (a.a.) 178 (HE19) or with C-terminal truncations to a.a. 282 (HE15) bound to ERE-containing oligonucleotides. A C-terminal deletion to a.a. 262 or an internal deletion from a.a. 185 to 251 abolished DNA binding (6,14). These results suggested that the ER DBD lies between a.a. 178–282 which encode the entire region C and part of region D. We have expressed an ER derivative containing a.a. 178–282 (HE81) in *E.coli* as a β-galactosidase fusion protein from the plasmid HE81-pUR291XA (see Materials and Methods).

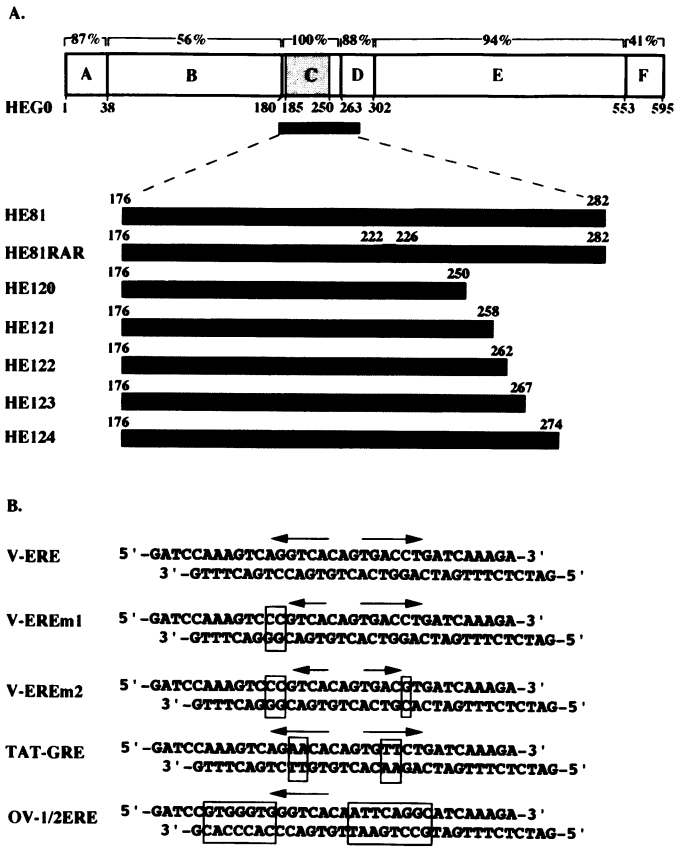

Figure 1. ER DNA binding domain derivatives and oligonucleotides used in gel retardation assays. **(A)** DNA binding domain derivatives. A schematic representation of the human estrogen receptor HEG0 is given above with the six domains of homology between the human and chicken ERs, A to F, indicated (12,14). The percent amino acid homologies between the two receptors are shown above. Region C (amino acids 180–262) contains the 66 amino acid zinc finger core of the DNA binding domain (residues 185–250, lightly shaded box). The ER DNA binding domain derivatives expressed in *E.coli* are shown expanded below with the N- and C-terminal ER amino acids indicated, and with the zinc finger core darkly shaded. In HE81RAR, ER amino acids 222 to 226 (black box) are replaced with the corresponding residues 125 to 129 of the human retinoic acid receptor α. **(B)** Oligonucleotides used in this study. The Xenopus vitellogenin A1 estrogen response element (V-ERE) is shown along with two mutants, V-EREm1, altered in one arm of the palindrome, and V-EREm2, altered in both arms of the palindrome (sequence changes are boxed). Also shown is the rat tyrosine aminotransferase glucocorticoid response element (TAT-GRE), and the half palindrome of the chicken ovalbumin promoter (OV1/2-ERE).

Cleavage of fusion protein bound to a β-galactosidase affinity column with blood coagulation factor Xa liberates HE81 (see Fig. 1A; Materials and Methods; 12). HE81 has an apparent relative molecular mass of 13 kDal on SDS polyacrylamide gels (Figs. 3 and 4, and data not shown).

Incubation of HE81 with a ^{32}P-labelled oligonucleotide containing the Xenopus vitellogenin A2 gene estrogen response element (V-ERE; see Fig. 1B for sequence) lead to the formation of a protein–DNA complex as measured by gel retardation assay (Fig. 2A, lanes 1–4). Stable complex formation was not observed after incubation of the V-ERE with fractions lacking the HE81 (Fig. 2A, lane 1; Fig. 3) nor when the HE81 was incubated with sequences containing point mutations in both arms of the ERE palindrome (V-EREm2, Fig. 1B; Fig. 2A, lanes 8–10). Furthermore, incubation of the HE81 with a glucocorticoid response element (TAT-GRE) lead to the formation of very low amounts of complex only at high DBD concentrations, indicating that complex formation was specific for the ERE sequence (Fig. 1B; Fig. 2A, lanes 11–13).

Studies using ER derivatives expressed in HeLa cells showed that the receptor bound DNA as a dimer and that there is a dimerization domain in the DBD (6). To confirm that the isolated DBD expressed in *E.coli* binds as a dimer we have tested for DBD heterodimerization by incubating HE81 along with extracts of HeLa cells expressing the truncated ER derivative HE15

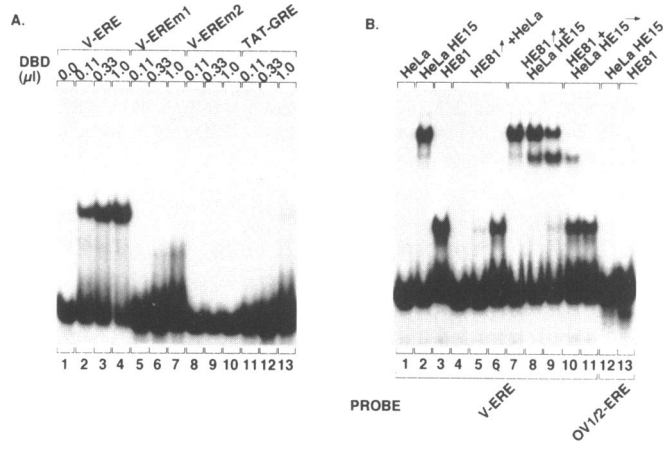

Figure 2. Specific DNA binding by the isolated ER DNA binding domain. **(A)** DNA binding by the ER DBD requires both arms of the ERE palindrome. The gel retardation assay was performed with ER DBD protein HE81 purified by cleavage from a β-galactosidase affinity column (see Materials and Methods). Binding to the Xenopus vitellogenin A1 gene estrogen response element (V-ERE), lanes 1–4; to the V-ERE mutant m1, lanes 5–7; to the V-ERE mutant m2, lane 8–10; and to the rat tyrosine aminotransferase glucocorticoid response element (TAT-GRE), lanes 11–13. Assays were performed with no DBD fraction, or 1μl of DBD diluted 9 times (lanes 2, 5, 8 and 11), 1μl of DBD diluted 3 times (lanes 3, 6, 9 and 12), or 1μl of undiluted DBD fraction (lanes 4, 7, 10 and 13). See Fig. 1B for sequences of oligonucleotides, and description of HE81. **(B)** The isolated ER DBD forms heterodimers with C-terminally truncated ER derivative HE15. The gel retardation assay was performed with 3μl of whole cell extracts from untransfected HeLa cells (lanes 1, 4, 5 and 6) or from HeLa cells transfected with an HE15 expression vector (lanes 2 and 7–11), and in lanes 3 to 13, a fraction (2μl) containing a dilution of ER DBD peptide HE81 eluted from a β-galactosidase affinity column (see Materials and Methods). HE81 was used undiluted (lanes 3, 6, 9–11, and 13), diluted 3-fold (lanes 5 and 8), or diluted 9-fold (lanes 4 and 7). HE15-containing extract was used undiluted except in lanes 10 and 11 where 3- and 9-fold dilutions were used, respectively. V-ERE (lanes 1–11) and OV-1/2ERE oligonucleotides (lanes 12, 13) were used as probes.

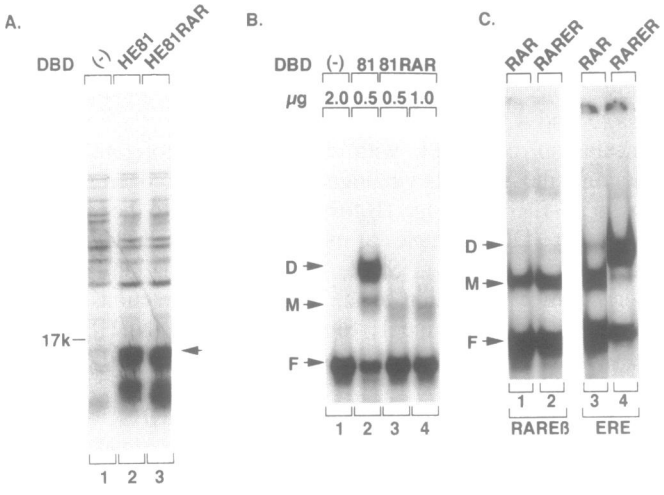

Figure 3. ER amino acids 222−226 are important for cooperative DNA binding by the ER DBD. **(A)** Expression of HE81 and HE81RAR in *E.coli*. ^{35}S-methionine-labelled extracts of *E.coli* BL21 expressing either no DBD (lane 1), HE81 (lane 2), or HE81RAR (lane 3) were run on a 12% SDS polyacrylamide gel which was dried and autoradiographed. Expressed DBD is indicated by the arrowhead. The position of a 17kD molecular weight marker is indicated at the left. **(B)** HE81RAR does not form dimeric protein-DNA complexes. Extracts expressing no DBD (2.0μg, lane 1), HE81 (0.5μg, lane 2), or HE81RAR (0.5μg, lane 3; 1.0μg, lane 4; 2.0μg) were incubated with ^{32}P-labelled V-ERE oligonucleotide in a gel retardation assay (see Materials and Methods). Incubations were run on a 5% polyacrylamide gel equilibrated in 0.5× TBE. **(C)** Introduction of ER amino acids 222−226 into the RARα DBD creates a derivative that binds cooperatively to the V-ERE. The RAR DBD (lanes 1 and 3) or RARER DBD (lanes 2 and 4) were tested for binding to the retinoic acid response element of the human RARβ gene (RAREβ; ref 36) (lanes 1 and 2) or the V-ERE (lanes 3 and 4). Free DNA, complexes corresponding to DBD monomers and dimers are indicated by F, M and D, respectively.

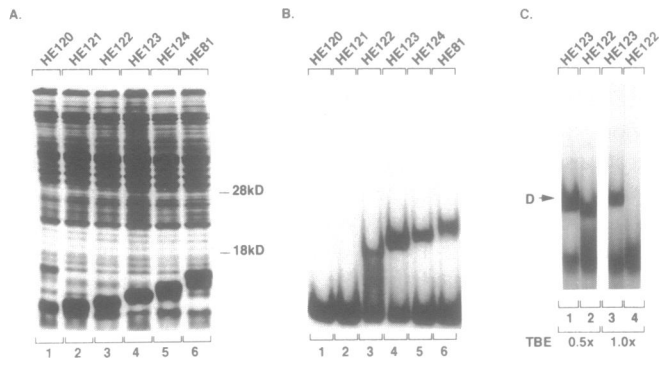

Figure 4. Determination of the minimal ER DNA binding domain by gel retardation assay. **(A)** Expression of ER DBD derivatives HE81 and HE120−124 in *E.coli*. ER DBD derivatives expressed in *E.coli* using the bacteriophage T7 expression system (30) were labelled with ^{35}S-methionine as described in Materials and Methods. Aliquots (500μl) of cells were lysed in SDS gel loading buffer and loaded on a 12% SDS polyacrylamide gel. After running, the gel was dried and autoradiographed. Bands corresponding to HE120−124 and HE81 run near the bottom of the gel. See Fig. 1 for descriptions of ER DBD derivatives. **(B)** Gel retardation assay of ER DBD derivatives. Quantities of cleared bacterial lysates containing similar amounts of HE120−124 or HE81 as indicated were incubated under gel retardation assay conditions (see Materials and Methods) in the presence of ^{32}P-labelled V-ERE (see Fig. 1) and run on a 5% polyacrylamide gel equilibrated in 0.5× TBE. **(C)** HE122-V-ERE complexes dissociate during migration in gels equilibrated in 1× TBE. Gel retardation assays with HE122 (lanes 1 and 3) or HE123 (lanes 2 and 4) were performed with 5% polyacrylamide gels equilibrated in 0.5× TBE (lanes 1 and 2) or 1X TBE (lanes 3 and 4).

(Fig. 2B), which corresponds to ER amino acids 1−282 and lacks the ligand binding domain (14). HE15 generated a dimeric complex with the V-ERE (Fig 2B, lane 2; ref. 6) which was much more highly retarded than that of HE81 (Fig. 2B, lane 3). Incubation of a control HeLa cell extract not containing HE15 with increasing quantities of the ER DBD gave rise to a retarded complex corresponding to the DBD (Fig. 2B, lanes 4−6). In contrast, incubation of HE15-containing extract together with the ER DBD gave rise to three complexes corresponding to the dimers of the ER DBD, dimers of HE15, and a complex of intermediate mobility corresponding to a heterodimer between the DBD and HE15 (Fig. 2B, lanes 7−11). No binding was observed by either HE81 or HE15 to the half ERE palindrome of the chicken ovalbumin gene (OV-1/2ERE; Fig. 2B, lanes 12 and 13) strongly suggesting that none of the observed complexes corresponded to monomers. Taken together the above results indicate that the isolated ER DBD expressed in *E.coli* binds highly cooperatively to both halves of the ERE palindrome as a dimer. This conclusion is also supported by methylation interference experiments (data not shown). These results also show that no eukaryotic-cell specific post-translational modifications are necessary for ER DBD dimerization.

The ER DBD formed very low levels of an unstable complex of high mobility on V-ERE sequences mutated in one arm of the palindrome (Fig. 2A, lanes 5−7), but not on the half palindrome of the chicken ovalbumin promoter (Fig. 2B, lanes 12, 13; see also Figs. 3, 5, and 7). Formation of this complex was seen to varying degrees on the V-ERE depending on the experiment (Figs. 2−5 and 7). We consider this complex to correspond to an ER DBD monomer by the following criteria. It migrated more rapidly than the complex formed by the dimeric DBD (Figs. 2,3,5 and 7). It was formed only in the presence of the ER DBD (Figs. 2B and 3B, and data not shown). It was not formed on oligonucleotides lacking one or more ERE half sites (Fig. 2A, lanes 8−10, and data not shown). Finally, it was the only ER DBD-dependent complex formed on oligonucleotides containing one ERE half-site or two ERE half-sites not arranged in the form of an ERE palindrome (data not shown).

N.M.R. analysis of the solution structure of the ER DBD has suggested that amino acids 222 to 226 could be closely apposed upon dimerization (25). We have therefore changed these residues to those of the human retinoic acid receptor α (RARα, residues 125−129) by in vitro mutagenesis to create HE81RAR (see Fig. 6). Expression of HE81RAR in *E.coli* gave rise to a 13 kDal polypeptide by SDS polyacrylamide gel electrophoresis which was of identical molecular mass to that of the ER DBD and which was not found in control extracts (Fig. 3A). However, unlike the ER DBD which formed a dimeric protein−DNA complex (D) in a gel retardation assay, HE81RAR formed only low levels of a complex corresponding to a DBD monomer (M) (Fig. 3B, compare lanes 2 with lanes 3, 4). No such complexes were observed in extracts not expressing a DBD (Fig. 4B, lane 1). The mobilities of the complexes corresponding to monomers of HE81 and HE81RAR were not identical. Note that three independent isolates HE81RAR gave rise to monomeric complexes that migrated slightly faster than those of HE81 (data not shown).

We have also replaced the corresponding amino acids of human RARα DBD with residues 222 to 226 of the ER to create

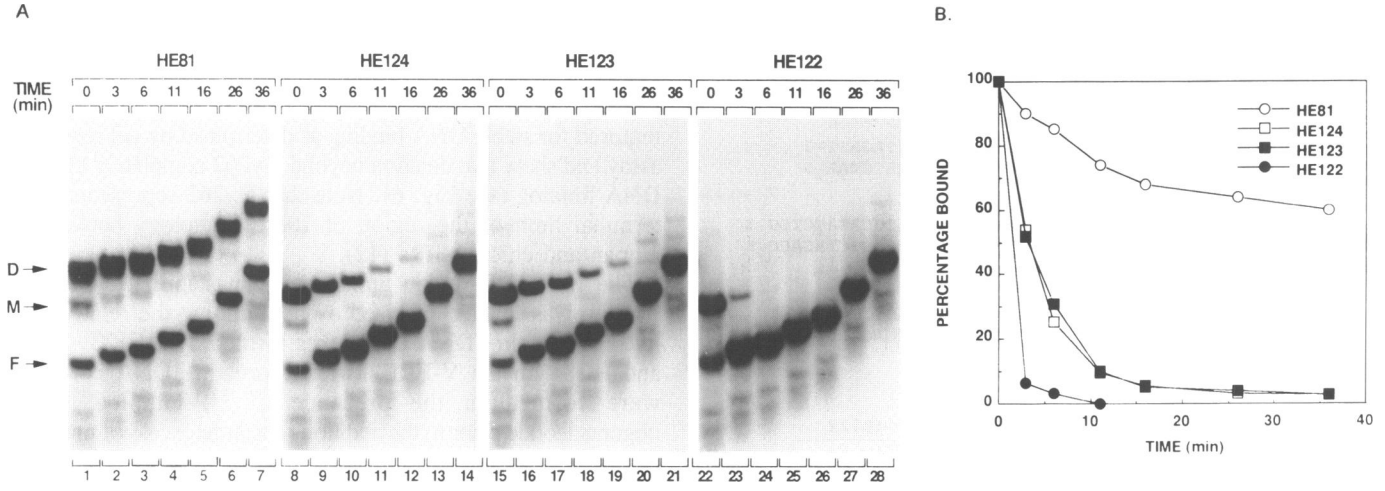

Figure 5. D-region amino acids are required for stable DNA binding by the isolated ER DBD. **(A)** Decay of DBD-V-ERE complexes formed by HE81 (lanes 1−7), HE124 (lanes, 8−14), HE123 (lanes 15−21), or HE122 (lanes 22−28) was monitored by gel retardation assay. A large excess (40 pmol) of unlabelled V-ERE was added to preformed protein-^{32}P-labelled DNA complexes at time 0 min to prevent reformation of dissociated complexes and 3 μl aliquots of incubations were loaded on a running 5% polyacrylamide gel equilibrated in 0.25× TBE at the times indicated (see Materials and Methods for details). **(B)** Graphic representation of the results of A. Bands were excised from dried gels and radioactivity was determined by liquid scintillation counting. Results are normalized to the amount of complex formed at 0 min which is given a value of 100%.

RARER. The RAR DBD and the derivative RARER bind at similar levels predominantly as monomers (M) to the directly repeated half-sites of the RARβ gene retinoic acid response element (RAREβ; ref 36) (Fig. 3C, lanes 1 and 2). In contrast, whereas the RAR DBD bound predominantly as a monomer to the V-ERE (Fig. 3C, lane 3), RARER bound cooperatively as a dimer (D) to the same sequence (Fig. 3C, lane 4). The above results indicate that that ER amino acids 222−226 are important for dimerization and that dimerization is essential for stable DNA binding by the isolated ER DBD.

Amino acids in region D stabilize DNA binding by the isolated ER DBD

Previous studies with HeLa cell extracts have shown that whereas HE15 (a.a. 1−282) binds DNA, HE16 (a.a. 1−262) fails to bind to EREs (6) indicating that D region amino acids contribute to ER DNA binding. The structure of this region has not been defined in X-Ray crystallographic and N.M.R. studies of either the estrogen or glucocorticoid receptors. To further define the role of D region amino acids in ER DNA binding, we have determined the minimal number of C-terminal residues required for stable protein−DNA complexes as measured by gel retardation assay. A series of C-terminal deletion mutants of the ER DBD, HE120-HE124 and HE81 (Fig. 1A), were expressed in *E.coli* using the bacteriophage T7 expression system (30). Their expression produced of a series of polypeptides from 8 to 13 kDa as estimated by SDS polyacrylamide gel electrophoresis (Fig 4A). These derivatives were expressed at similar levels and were soluble in cleared lysates of sonicated cells.

Gel retardation assays were performed with the V-ERE probe to analyse DNA binding (Fig 4B). No retarded complex formation was observed with cleared lysates of cells expressing HE120, or with HE121 (Fig. 4B, lanes 1 and 2). A complex was formed with HE122 which apparently partly dissociated during the migration of the gel (Fig. 4B, lane 3). The stability of HE122−DNA complexes was strongly dependent on the ionic strength of the running buffer used in gel retardation complexes. Complexes that were stable in 0.5×TBE (see Materials and Methods) did not survive migration in 1×TBE (Fig 4C, lanes 1 and 3). In contrast, stable complexes were observed with

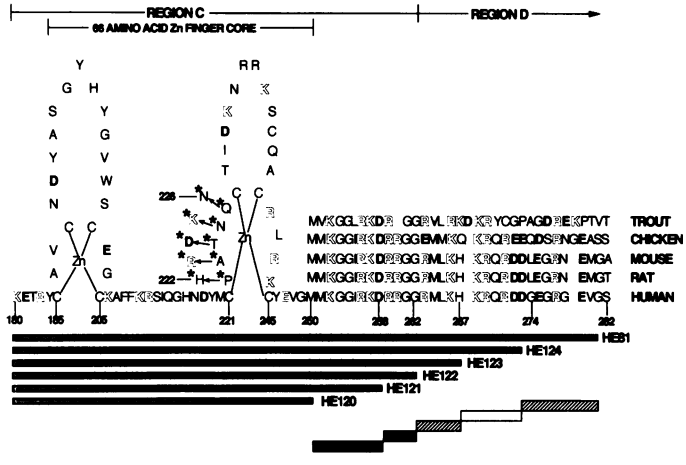

Figure 6. Summary of the results of Figs. 3 to 5. All of region C and region D to a.a. 282 are shown. The 66 amino acid zinc finger core (a.a. 185−250) is indicated above. The chicken and human ERs are 100% conserved in region C. In region C (from human ER residue 250) and region D, the corresponding amino acids of the rat, mouse, chicken and trout ERs (12,43,44), are shown above. Note the gaps to maximize homology between the different sequences. Positively and negatively charged residues are indicated by outlined and bold letters, respectively. The amino acid sequences of HE81 and HE120−HE124 are delineated below by the gray bars. The bars at the bottom summarize the effect of C-terminal deletions on DNA binding. The white bar indicates that deletion of the corresponding sequence results in no change in the stability of DNA binding, whereas the light striped bar indicates that a deletion is accompanied by a decrease in the half-life of the resulting DBD-ERE complex (see Fig. 5). The dark striped bar indicates that deletion abolishes DNA binding as measured by gel retardation assay (see Fig. 4). Also indicated are the changes in sequence from human ER to human RARα in human ER residues 222 to 226 which disrupt dimerization (see Fig. 3).

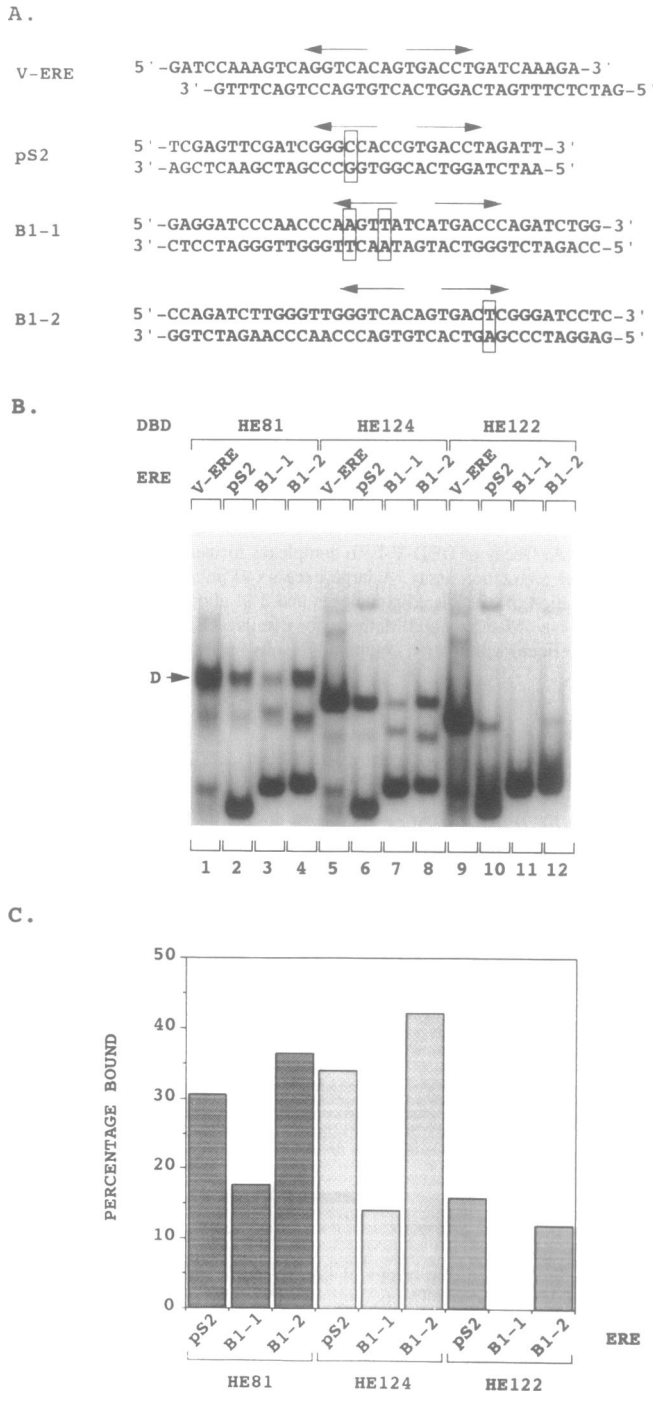

Figure 7. Region D amino acids are required for stable binding to imperfectly palindromic EREs. **(A)** ERE oligonucleotides used: the two imperfectly palindromic EREs of the Xenopus vitellogenin B1 gene (37), and the imperfectly palindromic human pS2 gene ERE (38) are shown (oligonucleotides B1-1, B1-2, and pS2, respectively) along with the sequence of the V-ERE. The ERE palindrome is indicated by the divergent arrows and deviations from the consensus are boxed. **(B)** Gel retardation assay of complex formation by HE81 (lanes 1–4), HE124 (lanes 5–8) and HE122 (lanes 9–12) on V-ERE (lanes 1, 5, and 9), pS2 ERE (lanes 2, 6 and 10), Xenopus B1 ERE 1 (B1-1; lanes 3, 7 and 11) and ERE 2 (B1-2; lanes 4, 8 and 12) is shown. The position of complexes formed by HE81 dimers (D) is indicated. **(C)** Graphic representation of the results of B. Free DNA and retarded bands corresponding to complexes formed by HE81, HE124 and HE122 or the pS2, and both Xenopus vitellogenin B1 EREs were excised and radioactivity was determined by liquid scintillation counting. Radioactivity in each dimeric complex was plotted as a percentage of the sum of ^{32}P in dimeric complexes and free oligonucleotide.

HE123, HE124 and HE81 in 0.5×- and 1×TBE (Fig. 4B, lanes 4 and 5; Fig. 4C, lanes 2 and 4) and at buffer concentrations as high as 4×TBE (data not shown). These results define a region between gly262 and his267 as a C-terminal limit of the ER DBD required for stable DNA binding as determined by gel retardation assay and show that deletion beyond gly262 completely abolishes DNA binding (see Fig. 6). Note that gly262 represents the C-terminal limit of the region of 100% homology between the human and chicken ERs (12).

The role of D-region amino acids in stabilizing ER DNA binding was further analysed by determining the relative half-lives of protein–DNA complexes formed by HE122–HE124 and HE81 on the V-ERE. Briefly, protein–DNA complexes were formed in the presence of ^{32}P-labelled V-ERE oligonucleotide prior to the addition of a large excess of unlabelled competitor oligonucleotide (40 pmol) sufficient to completely abolish binding to a radiolabelled ERE in a competition experiment (data not shown). Aliquots were then removed and loaded onto a running gel equilibrated in 0.25× TBE at the timepoints indicated (Fig 5A; see Materials and Methods for details). Deletion from ser282 (HE81) to gly274 (HE124) reduced the half-life of the ER DBD–DNA complex more than 10-fold from greater than 35 min to approximately 3.5 min (Figs 5A, compare lanes 1–7 and 8–14; Fig. 5B), suggesting that a structural domain stabilizing ER DNA binding was disrupted. Interestingly, deletion to his267 (HE123) had no additional effect on stability (Fig. 5A, lanes 15–21; Fig. 5B), suggesting that secondary structure disrupted by deletion from ser282 to gly274 was not further affected by deletion to his267. Consistent with the results of Fig. 4, deletion to gly 262 (HE122) further reduced the half-life of protein–DNA complexes to less than 1 min (Figs, 5A, lanes 22–28, Fig. 5B). These results indicate that D-region amino acids are responsible for stabilizing ER DNA binding by the isolated ER DBD, and raise the possibility that independent structural determinants located both N-terminal to his267 (HE123) and C-terminal to gly274 (HE124) contribute to binding stability. The results of the above C-terminal deletion studies are summarized in Fig. 6.

D region amino acids are required for formation of gel retardation complexes on certain imperfectly palindromic EREs

Elimination of D region amino acids also affected the affinity of the ER DBD for imperfectly palindromic EREs. Binding of HE81, HE124 and HE122 to the V-ERE, the two imperfectly palindromic EREs of the Xenopus vitellogenin B1 gene (37), and the imperfectly palindromic human pS2 gene ERE (38) was examined by gel retardation assay (Fig. 7A, oligonucleotides B1-1, B1-2, and pS2, respectively). HE81, like the full length ER (39,40), bound to all of the ERE sequences tested (Fig. 7B, lanes 1–4; Fig. 7C). HE124 binding to the ERE sequences tested was similar to that of HE81 (Fig. 7B, lanes 5–8; Fig. 7C). On the other hand, HE122 bound to the pS2 and B1-2 EREs only weakly relative to its affinity for the V-ERE, and did not form a stable complex on the B1-1 ERE at all (Fig. 7B, lanes 9–12; Fig. 7C). Note that 16-fold dilutions of the extracts used in this experiment did not saturate the V-ERE and gave very similar levels of binding to the V-ERE (data not shown). These results are consistent with our analyses of the half-lives of protein–DNA complexes formed by ER DBD derivatives (Fig. 5) and indicate that D region amino acids are essential for stable binding to weak EREs.

DISCUSSION

We have characterized the DNA binding properties of the isolated human estrogen receptor DNA binding domain expressed in *E.coli*. The ER DBD binds DNA highly cooperatively as a dimer. Heterodimeric complexes are formed between HE81 and the truncated ER derivative HE15 on the V-ERE (Fig. 2). Gel retardation assays with mutant ERE sequences (Figs 2 and 3) as well as methylation and ethylation interference experiments (data not shown) confirm that the ER DBD dimers contact both arms of the ERE palindrome. These results are consistent with those obtained with the ER expressed in transiently transfected HeLa cells (6), and with the Xenopus ER DBD (41). Several DNA binding experiments performed here indicate that DBD monomers bind with only low affinity to ERE half-sites as judged by the formation of variable levels of a complex migrating faster than that formed by DBD dimers. Unlike the complex formed by ER DBD dimers, the monomer complex is also formed on certain oligonucleotides containing only one ERE half-site or half-sites arranged in configuration other than that of an ERE palindrome (Fig. 2 and data not shown).

Conversion of human ER amino acids 222 to 226 (pro-ala-thr-asn-gln) to those of the human retinoic acid receptor α (his-arg-asp-lys-asn) abolishes cooperative DNA binding by the ER DBD (Fig. 3). In the converse experiment, replacement of the corresponding RAR residues 125 to 129 with ER amino acids 222 to 226 creates an RAR DBD derivative, RARER, that binds cooperatively to an ERE. These results are in agreement with the N.M.R. solution structure of the ER DBD which suggests that residues 222 to 226 can be closely apposed and thus act as a dimerization domain if adjacent DBDs are juxtaposed to permit binding to an ERE palindrome (25).

Conversion of the corresponding residues of the glucocorticoid receptor DBD to those of the human thyroid hormone receptor β also abolished cooperative DNA binding (42). However, in apparent contrast, monomers of the resulting GR DBD derivative readily formed stable complexes with GRE half-sites (42). HE81RAR formed only very low levels of monomeric protein DNA complexes and only at high protein concentrations (Fig. 3B, and data not shown). Similarly, HE81 bound only very weakly as a monomer to ERE half-sites (Figs 2, 5, and 7). These results suggest that monomers of the GR DBD bind more stably to response element half-sites than do monomers of the ER DBD. This may provide an explanation for the observation that EREs generally conform more closely to the palindromic consensus than do GREs. Typical imperfectly palindromic EREs contain one consensus half-site and only one or two nonconsensus bases in the other arm of the palindrome (see Fig. 7, for example), whereas GREs, such as the MMTV or Hepatitis B response elements (43,44), can be more arrays of half-sites than palindromes. One half of a GR DBD dimer bound stably to a consensus half-site may thus stabilize binding of the other half to an adjacent sequence widely divergent from a GRE consensus. In the case of the ER, multiple contacts at both half-sites are apparently necessary for stable DNA binding.

Stable binding to an imperfectly palindromic ERE by the DBD requires ER sequences in addition to the zinc finger core. The minimal DBD required for formation of stable complexes on the consensus V-ERE, HE122, contains C-terminal sequence to gly262 (Figs. 4 and 6). This residue corresponds to the limit of 100% homology between the the human and chicken ERs (12; Fig. 6) which marks the boundary between the C and D regions of the ER, and is 12 amino acids C terminal to the zinc finger core. However, HE122 binding to the V-ERE is very sensitive to ionic strength (Fig. 4). In addition, HE122, unlike longer DBD derivatives, does not form stable gel retardation complexes under any of our conditions on the Xenopus vitellogenin B1-1 ERE which contains two nonconsensus bases in one arm of the palindrome (Fig. 7). Similarly, HE122 binding to the pS2 and Xenopus vitellogenin B1-2 nonconsensus EREs is very weak relative to its affinity for the V-ERE (Fig. 7).

D region amino acids up to ser282 (HE81) greatly stabilize binding of the ER DBD to perfectly and imperfectly palindromic EREs alike (Figs. 5 and 7). The apparent half-life of HE81 on the the V-ERE is over 35-fold greater than that of HE122 under identical conditions (Fig. 5). Interestingly, HE124 and HE123, which are truncated at gly274 and his267, respectively, have very similar intermediate half-lives on the V-ERE (Fig. 5). These results raise the possibility that there may be two domains of secondary structure in region D contributing to DNA binding stability; one of which is disrupted in deleting from ser282 to gly274, and a second which is disrupted in deleting from his267 to gly 262.

What are the possible roles of region D in DNA binding by the isolated ER DBD? One possibility which we do not favour is that region D amino acids are required solely to maintain the structural integrity of the zinc finger core of the DBD. N.M.R. studies, which generated the solution structure of the ER zinc finger core, were performed with a DBD derivative corresponding to HE122 (25), indicating that the conformation of the zinc fingers is stable in this derivative. It is possible that region D serves to make additional nonspecific contacts with the DNA that stabilize binding to the ERE. However, the methylation interference patterns of HE81 and HE122, the longest and shortest forms of the DBD studied, are identical, suggesting that there are no differences in critical purine contacts made by the two derivatives (data not shown). In addition, ethylation interference experiments performed on protein−DNA complexes formed by HE122, HE123, HE124 and HE81 isolated from gels equilibrated in 0.5×TBE did not reveal any difference between the interference patterns of HE122 and longer DBD derivatives. Moreover, the patterns obtained with HE123, HE124 and HE81 are not altered if protein−DNA complexes are isolated in 2×TBE or 4×TBE (data not shown), conditions under which no stable binding is observed with HE122. There are two possible explanations for these results; either longer derivatives do not make phosphate contacts additional to those made by HE122, or that several additional contacts are made by these derivatives none of which alone is essential for DNA binding at higher ionic strength. The possibility that numerous phosphate contacts are made is consistent with the presence of a number of basic residues C-terminal to the zinc fingers running from amino acid 252 through the C-terminus of HE122 (gly262) to residue 271. These charged residues are highly conserved among ERs of different species (see Fig. 6). A helical wheel diagram of this region shows that many of these basic residues can be aligned on the same face of an α helix (data not shown). In this regard, basic residues in the D region of the orphan nuclear receptor NGFI-B have been implicated in receptor DNA binding (24). Finally, we cannot rule out the possibility that D region amino acids stabilize DNA binding by forming a supplemental dimerization domain or acting to stabilize an existing domain. However, if this were the case, and the D region has no role in stabilizing binding of monomers, then all DBD derivatives from HE122 to HE81 should bind as

monomers to the same degree. In this regard, HE123, HE124 and HE81 bind, albeit weakly, as monomers to an ERE, whereas no such binding is observed by HE122 (Figs. 2, 3, 5 and 7, and data not shown).

In summary, these studies demonstrate a critical role of D region amino acids in stabilizing DNA binding by the ER DBD. Moreover they point to a need for further structural studies performed with larger DBD derivatives to analyse their interaction not only with the ERE but also with its flanking sequences.

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