Purified Estrogen Receptor DNA Binding Domain Expressed in Escherichia coli Activates Transcription of an Estrogen-responsive Promoter in Cultured Cells*

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The region of the Xenopus laevis estrogen receptor responsible for interaction with DNA, the DNA binding domain (DBD), has been cloned and overexpressed in Escherichia coli using a T7 RNA polymerase expression system. Extracts from cells transformed with the DBD expression vector contain a single protein which reacts with polyclonal antibodies to estrogen receptor and exhibits sequence-specific binding to a DNA fragment containing a consensus estrogen response element. The DBD protein has been purified to near homogeneity. Determination of the rotational relaxation time of the dansylated DBD by fluorescence polarization and size fractionation by Superdex column chromatography indicate that the DBD is a monomer in solution. The DBD forms a single protein-estrogen response element complex in gel mobility shift assays at DBD concentrations of 18–3,600 nM, suggesting that the DBD is bound to both halves of the palindromic estrogen response element.

To investigate the ability of the DBD expressed in bacteria to activate gene expression, we have developed a simple liposome-based system for delivery of protein into cultured cells. Transfected DBD protein elicited large, concentration-dependent increases in transcription of an estrogen receptor regulated reporter gene. These data demonstrate that the bacterially expressed DNA binding domain, which represents a small portion of the Xenopus laevis estrogen receptor, retains significant ability to activate transcription of an estrogen-responsive promoter in vertebrate cells.

The estrogen receptor and other members of the nuclear receptor superfamily of ligand-regulated transcription factors share common structural features. Discrete regions of these nuclear receptors are responsible for ligand binding, DNA binding, nuclear localization, transcription activation, and dimer formation. Members of the nuclear receptor family contain two regions which exhibit a high degree of functional conservation and amino acid sequence homology, the ligand and DNA binding domains. The moderately conserved ligand binding domain is responsible for specific binding of the hormone and contains sequences important in dimerization (1, 2) and transcription activation (3–7). The highly conserved, cysteine-rich DNA binding domain contains two zinc finger motifs (8) and mediates sequence-specific DNA binding (3, 7).

Deletion analysis of glucocorticoid (9, 10) and progesterone (11) receptor mutants demonstrated that the DNA binding domain and a short region of adjacent sequence is sufficient for significant hormone-independent activation of transcription from responsive genes. Although extensive studies have not been done to determine the ability of the estrogen receptor DNA binding domain to activate transcription, mutant receptors encoding the amino-terminal and DNA binding domains have been reported to have little or no ability to activate transcription of powerful synthetic estrogen receptor-responsive promoters (3, 5, 6).

To investigate the role of the DNA binding domain in DNA binding and transcription activation, significant quantities of this protein are required. Although expression of full length steroid hormone receptors in Escherichia coli has proven difficult (12), the DNA binding domains of several steroid hormone receptors have been successfully expressed in E. coli (8, 13–15). However, since the bacterially expressed estrogen receptor DNA binding domain might lack necessary post-translational modifications such as phosphorylation (16, 17), its ability to function as a sequence-specific DNA binding protein and transcription activator was unknown.

To evaluate the activity of the DBD, we developed a simple liposome-based protein transfection system to introduce purified Xenopus laevis estrogen receptor DNA binding domain and an estrogen-responsive reporter gene into a Xenopus liver cell line. The purified, bacterially expressed DNA binding domain of the estrogen receptor retained substantial ability to activate transcription of the estrogen-responsive reporter gene.

EXPERIMENTAL PROCEDURES

Construction of the DNA Binding Domain Expression Vector pT7DBD—The plasmid pXER, which encodes the entire protein coding sequence of the X. laevis estrogen receptor (18), was used to make sequential site-directed deletions of the regions encoding amino acids 1–159 and 282–596 and to insert a translation stop site (TGA) followed by an EcoRI site immediately after the region coding for amino acid 281. The resulting plasmid, pXER160/281, was digested with NcoI and EcoRI to form a 337-base pair fragment, which encodes the DNA binding domain of the Xenopus estrogen receptor and a short amino-terminal acidic region (amino acids 171–281). The 253-base pair fragment was fractionated on polyacrylamide gels and electrosephoresis.

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† The abbreviations used are: DBD, DNA binding domain; ER, estrogen receptor; ERE, estrogen response element; FPLC, fast performance liquid chromatography; IPTG, isopropyl-1-thio-β-d-galactopyranoside; SDS, sodium dodecyl sulfate.
luted, and the 3' ends were filled in with Klenow fragment. A BamHI adapter was formed by annealing a 5'-biotinylated phosphocellulose column (5' GATGTCGCAGA 3') with a 5'-biotinylated phosphocellulose column (5' GATGTCGCAGA 3'). The column was filled in with Klenow fragment and loaded onto a 7% polyacrylamide gel. The gel was run at 200-250 mM NaCl. Fractions containing ERE binding activity and a dose-response to E2 were combined, dialyzed against 50 mM phosphate buffer, pH 7.6, and then added to 5000 cells per 60-mm dish. After 24 h, the cells were washed twice with 0.6 X TBS (3.5 mM Tris, 84 mM NaCl, pH 7.5) and once with 0.6 X serum-free Hiuchi's medium. For transfection of the DBD protein, 50-200 µg of purified protein, 2 µg of VITB1/EER reporter construct, and 1 µg of the luciferase vector pT109 (used as an internal standard) were added to 1.5 ml of Hiuchi's medium and gently mixed. Purified DBD was dialyzed against 5 mM phosphate buffer, pH 7.6, containing 100 mM NaCl prior to use. For transfections of the plasmid encoding the full length estrogen receptor, 2 µg of the Xenopus estrogen receptor expression plasmid, TKXERO (27), was combined with 2 µg of VITB1/EER and 1 µg of pT109 in 1.5 ml of 0.6 X Hiuchi's serum-free medium. 50 µl of Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) was added to 1.5 ml of 0.6 X serum-free medium and gently inverted three times. The DNA-lipofection mixtures were cultured for 5 min. The mixture was gently added to the cultured cells. The transfected cells were incubated at 20 °C. After 24 h, 3 ml of 0.6 X Hiuchi's medium containing 20% charcoal-stripped fetal bovine serum with appropriate ethanol or 17β-estradiol additions were added. Cells were incubated for an additional 24 h. Medium was removed by aspiration, and 5 ml of 0.6 X Hiuchi's medium containing 10% charcoal-treated calf serum was added. Plates transfected with TKXERO (27) were incubated for an additional 16 h at 20 °C. Cell extracts were prepared, and CAT assays were performed as previously described (31).

RESULTS

Expression and Characterization of the Estrogen Receptor DNA Binding Domain—BL21 (DE3) pllys S cells (20) were transformed with the DBD expression plasmid, pT7DBD, and grown in the presence or absence of 0.5 mM IPTG. Cell extracts were prepared and fractionated on SDS-polyacrylamide gels, blotted onto nitrocellulose, and incubated with polyclonal antibodies to a 20-amino acid segment of the estrogen receptor DNA binding domain (24). The estrogen receptor specific antibodies reacted with a protein present in extracts of cells that had been induced with IPTG (Fig. 1, panel A, f). Very low levels of this protein were also detected in extracts of uninduced cells that had been transformed with pT7DBD (Fig. 1, panel A, c). The faint higher molecular weight bands present in extracts from both uninduced and induced cells appear to be a nonspecific reaction with abundant E. coli proteins.

Although the apparent molecular weight of the IPTG-induced protein was greater than the predicted molecular weight of the BD (13,700), the highly charged nature of the DBD could result in aberrant electrophoretic mobility. The DBD contains 24 lysine and arginine residues (20% basic amino acids) as well as 15 glutamic acid and aspartic acid residues (13% acidic amino acids). The DNA binding domain...
Fig. 1. Characterization of the DNA binding domain produced in E. coli. Panel A, BL21(DE3) cells transformed with the pT7DBD expression vector were either induced with IPTG for 3 hours (I) or maintained without IPTG (U). Cells were harvested, and extracts were prepared. The resulting cellular proteins were separated on SDS-polyacrylamide gels, blotted onto nitrocellulose, and detected by estrogen receptor-specific polyclonal antibodies as described under “Experimental Procedures.” Lines indicate the positions of the molecular weight standards. Panel B, crude extracts prepared from cells that had not been transformed (lane 1) or had been transformed with pT7DBD (lanes 2 and 3) were incubated with the 51-base pair 32P-labeled DNA fragment containing a single ERE and 6 μg of poly(dI/dC) in the absence (lanes 1 and 2) or presence (lane 3) of a 50-fold excess of the unlabeled ERE-containing DNA fragment. Incubations and electrophoresis were carried out as described under “Experimental Procedures.”

Fig. 2. Purification of the Xenopus estrogen receptor DNA binding domain. Crude extracts from cells that had been induced with IPTG were loaded onto a Bio-Rex 70 column and eluted with a 150–500 mM NaCl gradient. Fractions containing DBD were pooled, dialyzed, loaded onto a phosphocellulose column, and eluted with a 200–800 mM NaCl gradient. Crude extract (CRUDE), partially purified DBD after Bio-Rex 70 fractionation (BIOREX), and purified DBD eluted from the phosphocellulose column (PCELL) were fractionated by electrophoresis on a 5–20% acrylamide gradient gel. The protein standards (M) were the same as those used in Fig. 1.

of the glucocorticoid receptor expressed in E. coli also exhibits a substantially higher apparent molecular weight than is predicted from its amino acid sequence (14). It was unlikely that the expressed DBD protein was larger than anticipated since sequencing of pT7DBD revealed that the appropriate translation initiation and termination codons were intact.

We used gel mobility shift assays to test the ability of the bacterially expressed DBD to bind to the estrogen response element (ERE). When crude cell extracts containing the DBD were incubated with a 51-base pair 32P-labeled DNA fragment containing a consensus ERE, a protein-DNA complex was formed (Fig. 1, panel B, lane 2). The shifted band disappeared on addition of a 50-fold excess of the unlabeled ERE-containing DNA fragment (Fig. 1, panel B, lane 3). Extracts from nontransformed cells, however, were incapable of forming a complex with the ERE-containing DNA fragment (Fig. 1, panel B, lane 1). We also showed that the unlabeled consensus ERE was a strong competitor for binding in a more extensive set of competition gel mobility shift assays using crude DBD and the ERs of the vitellogenin B1 promoter. In these assays, the NF1 binding sequence was a very poor competitor, and the imperfect vitellogenin B1 EREs exhibited an intermediate ability to compete. These data demonstrate that the bacterially expressed DBD exhibits sequence-specific binding to the ERE.

Purification of the DBD—Because detailed studies of DBD-DNA interaction required the purified protein, the DBD was purified from extracts of transformed, induced cells. An excellent purification of the highly basic DBD protein was achieved by chromatography of crude extracts on a Bio-Rex 70 cation exchange column (Fig. 2, BIOREX). Chromatography on phosphocellulose removed most of the remaining contaminants yielding a nearly homogeneous preparation.

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related to the molecular weight (33). Rotational relaxation times of 15 ns for a 14,000-dalton anhydrous monomer and 30 ns for a 28,000-dalton anhydrous dimer were predicted. We obtained a polarization of 0.134 at 25 °C for a 0.5 mg/ml solution of the dansylated DBD. With the observed limiting polarization of 0.420 and a fluorescence lifetime of 10.9 ns, according to the equation of Perrin (33), the rotational relaxation time was $r = 13.2$ ns. This is in good agreement with the value of 15 ns expected for a monomer and is quite different from the value of 30 ns expected for a DBD dimer. Thus, two independent techniques indicate that the DBD is a monomer in solution.

**Binding of Purified DBD to the ERE Palindrome**—Since the DBD was monomeric in solution, it was of interest to determine whether the DBD could bind to a single half-site of the ERE palindrome. Gel mobility shift assays were carried out in which varying amounts of purified DBD were incubated with the 51-base pair $^3P$-labeled DNA fragment containing a single consensus ERE. In order to identify the gel-shifted band which corresponds to EREs with DBD molecules on both halves of the ERE palindrome, we incubated the labeled ERE fragment with 1000 ng of DBD, a 9,700-fold excess of DBD over ERE half-sites. Since the mobility of all of the DNA was altered at this 3600 nm concentration of DBD, it seems reasonable to conclude that the single shifted band contains EREs with DBD monomers bound to both halves of the palindrome (Fig. 4). A single gel-shifted band, which co-migrated with the gel-shifted band seen at the highest concentration of DBD protein, was observed at all DBD concentrations tested, including 5 ng of DBD, which produced only a faint gel-shifted band. Higher percentage acrylamide gels and overexposure of autoradiograms failed to provide any evidence for additional gel-shifted bands (data not shown). Thus, although the DBD was a monomer in solution, the DBD-ERE complex appeared to be a species in which both arms of the ERE palindrome were occupied by the DBD.

**DBD Expressed in E. coli Activates Transcription from a Vitellogenin Promoter**—Since the DBD protein expressed in bacteria retained the capacity for sequence-specific DNA binding, we examined whether the protein also had the capacity to activate transcription of an ERE-containing reporter gene. In cell-free transcription assays, the progesterone (34) and glucocorticoid (35) receptor DNA binding domains were shown to be transcriptionally active, but large amounts of DNA binding domain were required for activation. To test the ability of the DBD protein to activate transcription in an *in vivo* assay, we developed a simple liposome-based transfection system in which purified DBD protein was introduced into cultured *Xenopus* liver cells. A liposome-DNA-DDB mixture was prepared and incubated with cultured *Xenopus* liver cells. As cells were exposed to increasing amounts of purified DBD protein, there was a concomitant rise in the level of CAT expression from an estrogen-responsive reporter gene (Fig. 5). When cells were transfected with 50, 100, or 200 μg of purified DBD, CAT activity increased by 7-, 10-, and 16-fold, respectively (Fig. 5). The specificity of the DBD activation of gene expression was demonstrated in control liposome transfer experiments in which neither IgG, bovine serum albumin (BSA), or immunoglobulin G (IgG) was used.
DBD. In the same transfection, expression of the vitellogenin promoter was increased 2.2-fold by the presence of 50 μg of DBD and 28-fold in the presence of 200 μg of DBD.

In other control experiments in which 2 μg of the estrogen receptor expression vector TKXERO and the VITB1/ERE reporter plasmid were co-transfected into the cells, a 9-fold increase in CAT activity was observed in the presence of 10^{-7} M 17β-estradiol (data not shown). Although a direct comparison of the efficiency of activation of the VITB1/ERE promoter by the DBD and by full length estrogen receptor encoded by transfected DNA is not possible, these data demonstrate that the DBD elicited large increases in expression of the estrogen-regulated reporter gene.

**DISCUSSION**

The DNA Binding Domain Binds to the ERE as a Monomer—The estrogen receptor is a dimer in solution (36) and binds to the ERE palindrome as a dimer (1, 2). Mouse estrogen receptor mutants that are unable to form dimers do not show detectable binding to an ERE-containing DNA fragment (2). These data suggest that, for the full length estrogen receptor, dimerization is a necessary prerequisite for DNA binding. However, we find that the purified DNA binding domain of the Xenopus estrogen receptor is a monomer in solution. Fractionation of the purified DBD on a high resolution gel filtration column reveals the presence of a single protein peak that is too small to be a receptor dimer. Although the apparent molecular weight of the DBD determined by gel filtration (18,600) is greater than is predicted from its amino acid sequence (13,700), the extended nonglobular structure of the DBD (8) could result in an increase in apparent molecular weight on gel filtration columns. In addition, fluorescence measurements of the rotational relaxation time of the dnasylated DBD (ρ = 13.2 ns) strongly support the view that the receptor is monomeric in solution. These data indicate that the weak constitutive dimerization domain present in the DNA binding domain is insufficient to induce dimerization and support the view that the strong ligand-induced dimerization domain present in the hormone binding domain of the estrogen receptor plays a critical role in dimerization (1, 2). Our conclusion is in agreement with a recent NMR study, which concluded that the somewhat smaller protein encoding the DNA binding domain of the human estrogen receptor is a monomer in solution (8).

Estrogen receptor dimers normally occupy the two equivalent binding sites on the estrogen response element palindrome. However, the estrogen receptor dimer does not bind to an ERE half-site (37). Since the DBD is monomeric, we investigated the possibility that the DBD monomer might be able to interact with an individual half-site of the ERE palindrome. Gel mobility shift assays in the presence of a vast (nearly 10,000-fold) excess of DBD result in a complete shift of the labeled DNA to a single slower migrating band (Fig. 4), which should correspond to a species in which both halves of the ERE palindrome are saturated with the DBD. The fact that the same shifted band was the only one observed at all DBD concentrations tested suggests that even at rather low concentrations of DBD, monomeric DBD molecules occupy both halves of the ERE palindrome. Additional evidence that a single DBD monomer does not exhibit stable binding to an ERE half-site comes from a study in which we examine the ability of the imperfect EREs in the vitellogenin B1 promoter to compete for binding to the consensus ERE. The consensus ERE palindrome is at least 30-fold more effective as a competitor than ERE 1, which contains a consensus ERE half-site on one side of the palindrome and differs from the consensus sequence by two nucleotides on the other half of the palindrome. DNAse I footprinting experiments performed with the DNA binding domain of the human estrogen receptor also suggest that both ERE half-sites are occupied (8).

The most plausible explanation for our data is that binding of the monomeric DBD to the ERE half-sites is stabilized by protein-protein interaction between the bound DBD monomers. Structural studies of the glucocorticoid and estrogen receptor DNA binding domains have defined a discrete region which may be important in forming protein-protein contacts when DBD monomers are bound to the two ERE half-sites (8, 38). This region is completely conserved in the human and Xenopus estrogen receptors. Thus, once two DBD monomers are bound to the two ERE half-sites, stabilization of the protein-DNA interaction could occur by formation of DBD dimers on the ERE. More detailed studies will be required to distinguish between these possible modes of DBD-ERE interaction. (a) Interaction of a single DBD monomer with an ERE half-site results in the formation of a complex which rapidly dissociates, unless stabilized by the nearly simultaneous binding of a second DBD monomer. (b) Binding of the first DBD monomer to one half-site results in highly cooperative binding of a second DBD monomer to the other half-site. (c) Binding of the monomeric DBD to an ERE half-site results in a protein-DNA complex which is too unstable to survive electrophoresis and thus goes undetected.

Although we cannot exclude the possibility that a minute amount of DBD dimer is present in our preparations and is responsible for the binding we observe in gel mobility shift assays, this seems improbable. No evidence supporting the presence of any level of DBD dimer was obtained in the gel filtration or fluorescence experiments. In addition, the fact that the mobility of approximately one-half the labeled DNA is shifted with 90 nM DBD (62.5 ng, Fig. 4) suggests that the binding is due to the major protein component of the solution, rather than a rare contaminant.

**The DBD Activates Transcription of a Vitellogenin-derived Reporter Gene**—The domains of the estrogen receptor responsible for transcription activation are not as precisely defined as those in the glucocorticoid receptor (9, 10, 39, 40). None of the common transactivation motifs such as acid blob, helix loop helix, proline, or glutamine-rich sequences which have been reported in other systems (41) have been identified in the estrogen receptor. Instead, long extended regions of the amino-terminal and hormone binding domains have been implicated in transcription activation. Using synthetic promoters, two groups have reported that mutants which lack these sequences have little or no ability to activate transcription (3, 5, 6). However, in a study of the weak prolactin ERE, which differs from the consensus ERE by two nucleotides, Waterman et al. (7) found that human ER mutants which lack the hormone binding domain retain full capacity to activate transcription. To clarify the transcriptional activity of the DNA binding region of the estrogen receptor, we employed the Xenopus vitellogenin B1 promoter (42, 43) containing an additional consensus ERE at −359, cultured Xenopus liver cells, and the purified DNA binding domain of the Xenopus ER. To establish that the protein synthesized in bacteria retains biological activity, we used a liposome delivery technique to introduce the purified DBD protein into cells. The liposome-based system we describe, which uses commercially available cationic liposomes (44), is simpler to use than the system recently described by Debs et al. (45) in which the liposomes were prepared by sonication.

Introduction of the bacterially produced DBD into Xenopus liver cells always resulted in an efficient dose-dependent...
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The level of induction, however, varied with individual DBD preparations and, in other experiments, was considerably higher than the 16-fold we report here. We used high concentrations of DBD protein because this highly basic protein (20% of the amino acids are lysine and arginine residues) might interact poorly with cationic liposomes. Interaction with the liposomes and transcription activation may be facilitated by a short acidic tract (amino acids 267–279 of the Xenopus estrogen receptor; Ref. 18) at the carboxyl terminus of the DBD. The acidic nature of this region is conserved among all cloned estrogen receptors. In the Xenopus estrogen receptor, 6 of 13 amino acid residues are glutamate or aspartate. Although this sequence cannot form an amphipathic α helix, it is distantly related to the acidic transcription activation sequences observed in other proteins (46–49). However, plasmids encoding Xenopus estrogen receptor mutants, in which two additional copies of this acidic sequence are present, exhibit transactivation activity only 2-fold greater than that of the wild type receptor. Although these data indicate that this sequence does not play a major role in transactivation by the full length estrogen receptor, which contains two other transcription activation domains (6), it is possible that this region makes a significant contribution to transactivation by the DBD, in which the other transactivation domains are absent.

The efficiency with which the DBD activates transcription of the reporter gene in transient transfection assays may stem from a combination of several factors. Since the DBD and the reporter gene are combined prior to the addition of liposomes, the DBD may be present at extremely high local concentrations and bind to the ERE before transfection into the cells. In contrast, when the expression plasmid encoding the intact XER is utilized, the newly synthesized receptor must locate the ERE in the nucleus. Since the DBD contains an efficient nuclear localization sequence (50, 51), it may facilitate transfer of the transfected DBD-DNA complex into the cell nucleus. Although the level of DBD present in cells was not quantitated, it is possible that the level of transfected DBD protein may be much higher than the level of intact receptor in cells transfected with the Xenopus estrogen receptor expression plasmid.

A DBD-dependent increase in CAT activity is observed at all concentrations of DBD transfected into cells. The failure to observe a plateau suggests that even at this very high concentration of DBD, the ERs may not be fully saturated with DBD protein. The mutant vitellogenin B1 reporter promoter used contains three functional ERs. The native promoter contains two imperfect ERs, ER2 and ER1, which differ from the consensus ER by one and two nucleotides, respectively. Both of these ERs appear to be essential for efficient estrogen-dependent transcription activation (52). To increase the activity of this promoter in transfactions, we added a consensus ER at −359 (27). The imperfect ER (AGTTATCATGACC), which differs from the consensus ER (GGTCAnnnTGACC) by two nucleotides, may not be occupied by the DBD unless extremely high concentrations of DBD are present. Debs et al. (45) employed both the natural mouse mammary tumor virus promoter and a synthetic promoter containing a glucocorticoid response element and also failed to observe a plateau in activation with either promoter, even at very high levels of transfected glucocorticoid receptor DNA binding domain.

The vitellogenin promoter is a complex promoter which is transcriptionally inactive in vivo in the absence of estradiol-XER complex. Nevertheless, the promoter contains binding sites for several upstream activator sequences including a CAAT box, an NF1-related activator element (27), and a liver-specific silencer (53). The impressive ability of the DBD to activate transcription of the vitellogenin-based reporter gene may be due to its ability to facilitate or stabilize the interaction of transcription factors with these sequences. It seems plausible that the strong transactivation domains present in the full length estrogen receptor might be required to directly facilitate formation of a transcription complex around the TATA box, but that proteins with much weaker transactivation domains, such as the DBD, could still effectively recruit other transcription factors which in turn facilitate formation of a transcription complex. Somewhat similar conclusions have been reached by Tassett et al. (6).

Purified progesterone (34) and glucocorticoid (35) receptor DBDs are weak activators of transcription in cell-free systems. In addition, purified glucocorticoid receptor DBD is also transcriptionally active in liposome transfer experiments (45). Our demonstration that the estrogen receptor DBD retains the ability to activate an ER-responsive gene lends credence to the idea that the DNA binding domains of steroid receptors contain sufficient information to induce at least weak, constitutive expression of hormone responsive genes.

In this work we show that the bacterially expressed monomeric estrogen receptor fragment, containing only 111 of the 586 amino acids found in full length receptor retains the ability to interact specifically with the estrogen response element and is a potent transcriptional activator when introduced into cultured cells.

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