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Isolation of estrogen receptor-binding sites in human genomic DNA

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ABSTRACT

Total genomic DNA digested by restriction enzymes was mixed with the DNA-binding domain of the estrogen receptor (ER-DBD) that was expressed in *Escherichia coli* and the fragments that bound to it were selected by nitrocellulose filter. These fragments were cloned into a plasmid vector and amplified. This selection process was repeated six times and five fragments ranging from 0.2 to 2 kb were isolated. Interestingly, each of these fragments had a perfect palindromic estrogen responsive element (ERE) (GGTCANNNTGACC). More surprisingly, one of the fragments was found to be derived from the same locus as a fragment obtained by another similar but independent experiment. The results indicate that the ER-DBD region can bind by itself specifically to the perfect palindromic ERE with a 3 base pair spacing but it does not bind strongly enough to the half palindromic EREs or to the imperfect palindromic EREs. Chloramphenicol acetyltransferase assay has shown that some of these fragments have estrogen-dependent enhancer activity, suggesting the existence of a target gene near these fragments. The method described here may be generally applicable for screening and isolation of other transcription factor-binding sites in genomic DNA.

INTRODUCTION

In contrast to the wide variety of estrogen action on different organs and tissues, relatively few genes are known that are responsive to estrogen receptor (ER). Estrogen responsive genes that have been identified so far include those of vitellogenin (1), prolactin (2), pS2 (3), ovalbumin (4) and progesterone receptor (5). More important genes which should regulate the growth of female organs such as mammary gland and uterus in response to estrogen, for example, have not yet been found. Moreover, ERs have also been identified in various nuclei of female as well as male central nervous system (6–9) and in bone cells (10, 11) implicating some important roles of estrogen responsive genes in a number of extragonadal organs. Under these circumstances, identification of more estrogen responsive genes appears crucial for more understanding of the molecular physiology of estrogen action. In this work we have attempted to screen and isolate estrogen responsive genes by a simple procedure.

Steroid hormone receptors bind to their responsive elements and regulate the transcription of target genes. The receptors consist of separate functional domains and the DNA-binding domain is essential to bind to their specific responsive elements. This domain contains the 'zinc finger' motifs that are critical for the interaction of these proteins to their cognate sequences (12, 13). The domain-swap and site-directed mutagenesis experiments have shown that the DNA-binding activity and specificity exist exclusively in this domain (12) and relatively few amino acids in the domain can determine the specificity of its binding (14–17). The DNA-binding domains of the glucocorticoid (18, 19), progesterone (20) and estrogen (21) receptors expressed in *Escherichia coli (E. coli)* are sufficient to bind to their responsive elements.

We expected that the binding specificity of the DNA-binding domain of the ER (ER-DBD) could be utilized to isolate the DNA elements in genomic DNA that flank the estrogen responsive genes. Therefore, we produced a protein of the ER-DBD in *E. coli* and screened human genomic fragments by selction cycles with filter binding. Five ER-binding fragments containing perfect palindromic estrogen responsive element (ERE) sequences were isolated and some of these fragments showed estrogen-dependent enhancer activities.

MATERIALS AND METHODS

Isolation of the ER-binding fragments

High molecular weight DNA from HeLa cells was prepared according to the standard protocol (22). The DNA (10 μg) was digested with PstI and BamHI or with other restriction enzymes. The filter binding selection was performed as follows. The genomic DNA fragments were incubated with the ER-DBD protein (10 pmol) for 30 min on ice. The binding reaction was carried out in a volume of 400 μl of binding buffer containing 40 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol and 0.1 mg/ml BSA. Thereafter, this solution was passed slowly through a pre-soaked nitrocellulose filter (Schleicher & Schuell, BA85, 0.45 μm, 25

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mm) . The filter was then washed with 500 μl of washing buffer containing 40 mM Tris-HCl (pH 7.5) and 1 mM EDTA five times. The DNA trapped by the filter was eluted from the filter in a volume of 400 μl of elution buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20 mM NaCl and 0.1% SDS. The DNA trapped by the filter (≈ 10 ng) was cloned into the plasmid vector pUC18 (PstI−BamHI) and transformed in JM109 high frequency competent cells (STRATAGENE). The cells were cultured on a LB-ampicillin plate for 24 hr and the plasmid DNA was prepared by alkaline treatment followed by centrifugation in cesium chroride-ethidium bromide gradient (22). Then, the plasmid DNA (10μg) was again incubated with the ER-DBD protein (10 pmol) and the selection cycle was repeated. In an alternative approach, whole genome PCR method was performed according to Kinzler et al. High molecular weight DNA digested with Sau3AI was ligated to the catch linker (23). The fragments bound to the ER-DBD protein were selected by filter binding procedure described above and amplified by polymerase chain reaction (PCR) method. This cycle was repeated five times.

Plasmid constructions

An expression vector of ER-DBD, pKK/ER-DBD, was prepared in the following manner. C-terminal deletion mutant of rat cER (ERcDNA) (24), rERCΔ281, was prepared in which C-terminal 282 amino acids were deleted and a linker containing a BglII site was attached (Imakado,M; unpublished data). The sticky ends of the BclI-BglII fragment of rERCΔ281 was filled with the large fragment of DNA polymerase I. This fragment encodes amino acids 181 −278 which correspond to amino acids 176−273 of human cER (25, 26). It contains whole DNA-binding domain of human cER (amino acids 185−250) and was cloned into SmaI site of pKK223-3 (Pharmacia). AT rich oligonucleotide linker of 20-base-pair(bp)-long containing ATG start codon was inserted into the EcoRI site of pKK223-3 to express the ER-DBD protein efficiently. The sequences of oligonuclotides are 5′-AATTATGAAAGCAATTTTCG-3′ for sense strand and 5′-AATTCGAAAATTGCTTTCAT-3′ for anti-sense strand. Multiple termination linker, which was synthesized as 5′-AGCTAGTCTAGACT-3′ for both strands, was inserted into HindIII site of pKK223-3. The pKK/ER-DBD expresses 98 amino acids of human cER and 22 amino acids derived from rat cER and vector sequence. The plasmid pUC/vitERE was constructed as follows. The oligonucleotides containing the wild type ERE of *Xenopus* vitellogenin gene A2 enhancer (vitERE) (−338/−310) (27) was synthesized as 5′-CAAAGTCAGGTCACAGTGACCTGATCAAAGAGCT-3′ and 5′-CTTTGATCAGGTCACTGTGACCTGACTTTGAGCT-3′. These oligonucleotides were annealed and inserted at the SacI site of pUC18.

The wild type rat ER expression vector pSV2RcER was described previously (24). The E1, E2 and E3 fragments was inserted into HindIII site and BamHI site of pBLCAT2 (28) which carried Herpes simplex virus thymidine kinase (tk) promoter (−105/+51). The ERE2, ERE3 and vitERE fragments were synthesized as oligonucleotides and inserted into HindIII site and SalI site of pBLCAT2. The ERE2 and ERE3 were also inserted into pUC18 (HindIII−SalI) to prepare pUC/ERE2 and pUC/ERE3. The sequences are:

1. for ERE2 5′-TCGACTTGGGGTCAGCCTGACCTGGATCCTGA-3′ and 5′-AGCTTCAGGATCCAGGTCAGGCTGACCCCAAG-3′,
2. for ERE3 5′-TCGACAAGGTCAAAGTGACCTTTTGGATCCTGA-3′ and 5′-AGCTTCAGGTCCAAAAGGTCACTTTGACCTTG-3′,
3. for vitERE 5′-TCGAGTCAGGTCACAGTGACCTGATCGGATCCTGA-3′ and 5′-AGCTTCAGGATCCGATCAGGTCACTGTGACCTGAC-3′.

Preparation of the ER-DBD protein

E. coli JM109 cells transformed with the pKK/ER-DBD expression vector were grown to an O.D. ≈0.6 in 200 ml of LB-ampicillin medium, and the culture was induced by 1 mM IPTG for 10 hr. The induced cells were harvested, rinsed with the lysis buffer containing 30 mM Tris-HCl (pH 7.5), 30 mM NaCl and 0.05 mM $ZnCl_2$, suspended in 20 ml of the same buffer and disrupted by sonication. The sonicated lysate was centrifuged at 10,000 *g* for 30 min and the precipitate was suspended in 20 ml of 1 M sucrose and centrifuged at 10,000 *g* for 30 min. Then, the precipitate was suspended in 40 ml of 2% Triton X-100−10 mM EDTA for 12 hr and centrifuged again at 10,000 *g* for 30 min. The precipitate was dissolved in 5 ml of 8 M urea and subsequently dialyzed against dialysis buffer containing 4 M urea, 30 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.05 mM $ZnCl_2$, and 1 mM DTT for 12 hr, and then against dialysis buffer containing 30 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.05 mM $ZnCl_2$, and 1 mM DTT for 12 hr. Homogeneity was confirmed by SDS-polyacrylamide gel electrophoresis (29).

Nitrocellulose filter binding assay

The plasmid pUC/vitERE was digested with HpaII and EcoRI. The fragments were labeled with [α-^{32}P]dCTP and large fragment of DNA polymerase I. The ER-binding fragments (PstI−BamHI), pUC/vitERE (PstI−BamHI) and pUC18 (PstI−BamHI) were labeled with [α-^{32}P]dGTP in the same way. The labeled DNA fragments (10,000 cpm−100,000 cpm) were incubated with the ER-DBD protein (0−5 pmol) for 30 min on ice. The binding reaction was carried out in a volume of 200 μl of binding buffer in the presence of 1 μg of poly[d(I-C)] as carrier DNA. Other competitor DNA was added when required. The filter binding procedure was performed as described in 'Isolation of the ER-binding fragments' except that 10 μg of carrier DNA was added in elution buffer. The resulting DNA fragments were analyzed on a 5% polyacrylamide gel or counted by liquid scintillation counter to determine the recovery.

DNase I footprinting and sequencing

The plasmid pUC/vitERE was digested with XbaI. The ends were dephosphorylated by bacterial alkaline phosphatase C75 (BAP) and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Single-end-labeled fragment was recovered from 1% agarose gel after EcoRI digestion. The DNA fragments (5 fmol) were incubated in a volume of 50 μl of the binding buffer containing 25 mM Tris-HCl (pH 7.5), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 0.5 mM DTT, 10% glycerol, 2% polyvinyl alcohol, 25 μM $ZnCl_2$ and 1 μg of poly[d(I-C)] with the ER-DBD protein (0−2 pmol) for 30 min on ice. Thereafter 50 μl of the buffer containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ solution and 3 μl of the buffer containing 4 ng of DNase I were added and the incubation continued for 60 sec at room temperature and then stopped by addition of 3 μl of stop solution containing 0.5 M EDTA (30). The resulting fragments were analyzed on a 5% denaturing polyacrylamide gel.The sequence ladders of DNA probes for footprinting were prepared by Maxam and Gilbert method (31). The sequencing for the ER-binding fragments was carried out by dideoxy method (32).

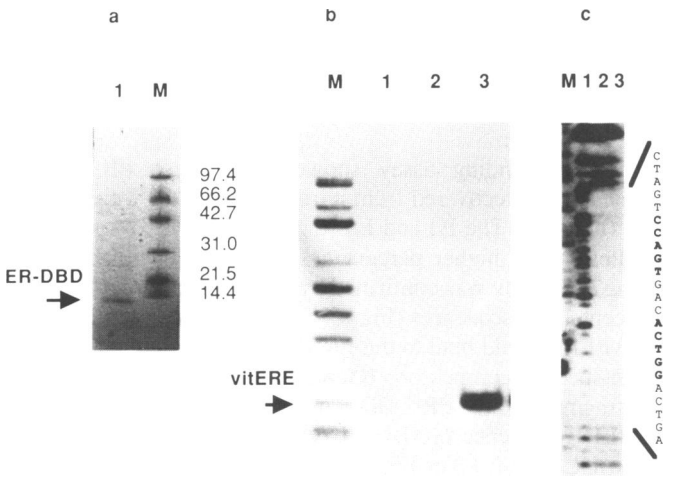

Fig. 1. The purity and binding specificity of the ER-DBD protein. (a) SDS-polyacrylamide gel electrophoresis of the purified ER-DBD protein. The ER-DBD protein (lane 1) and the molecular weight marker proteins (BIORAD)(lane M). The arrow shows the ER-DBD protein and the numbers refer to the molecular mass of reference proteins (kDa). (b) Filter binding assay with the ER-DBD protein. The pUC/vitERE was digested with HpaII and EcoRI and labeled with [α-^{32}P]dCTP and Klenow fragment. An aliquot of the labeled fragments was loaded on lane M (5,000 cpm). The remainder (100,000 cpm) was incubated without the ER-DBD protein (lane 1) or with 0.2 pmol (lane 2) or 5 pmol (lane 3) of the ER-DBD protein. The fragments trapped by the nitrocellulose filter were analyzed on 5% polyacrylamide gel. The arrow shows the fragment containing vitERE. (c) DNase I footprinting with the ER-DBD protein. The single-end labeled DNA fragment containing vitERE was incubated without the protein (lane 1) or with 1 pmol (lane 2) or 2 pmol (lane 3) of the ER-DBD protein. After DNase I digestion, the resulting fragments were analyzed on 5% denaturing polyacrylamide gel. The sequence ladder A(>C) (lane M) and the sequence protected by the ER-DBD protein are shown.

Southern blot analysis

High molecular weight DNA (10 μg) from HeLa cells was digested with either BamHI, BglII, EcoRI, HindIII or XbaI. Digested DNA were resolved on 0.7% agarose gels, transferred to a nitrocellulose filter and hybridized to the probes (33). Probes used were the E2 and E3 fragments.

Chloramphenicol acetyltransferase (CAT) assay

COS-7 cells were routinely maintained in Eagle's minimal essential medium (MEM) (Nissui) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Laboratory). One day prior to transfection, 1×10^6 cells were plated in 60 mm petri dishes and maintained in MEM containing 10% FBS. One hour prior to transfection, the medium was replaced with MEM containing 10% dextran-coated charcoal-treated FBS (34). Phenol red was omitted from the medium (35). The transfections were performed by calcium-phosphate precipitation method (36). Cells were transfected with 0.1 μg of pSV2RcER, 2 μg of reporter plasmids, 2 μg of pCH110 β-galactosidase expression vector (Pharmacia), used as an internal control to normalize for variations in transfection efficiency. The total amount of DNA transfected was made up to 20 μg with carrier DNA pGEM3Zf(−) (Promega). After 12 hr incubation the cells were divided into two dishes and cultured further in the absence or presence of 1×10^{-7}M 17β-estradiol in MEM containing 10% dextran-coated charcoal-treated FBS for 24 hr. The cell extracts were assayed for protein concentration (37) and CAT activities (38).

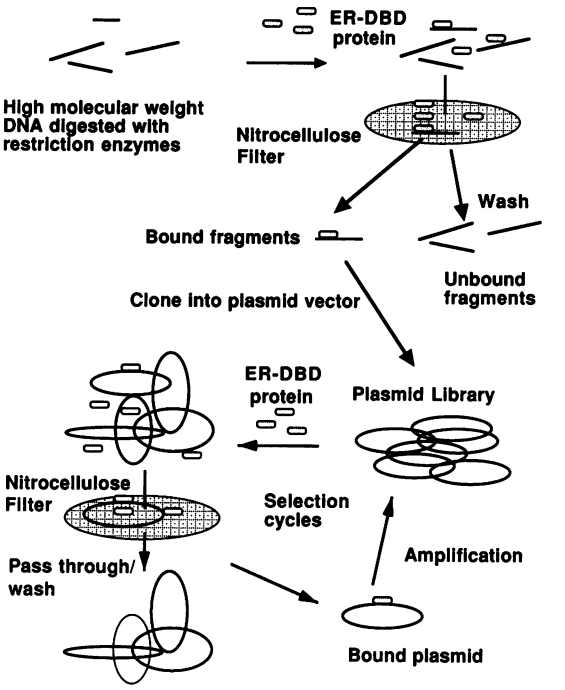

Fig. 2. The strategy used for the screening of ER-binding fragments in genomic DNA. The model diagram of selection cycles is shown. First, the genomic DNA fragments digested with restriction enzymes were selected by nitrocellulose filter and cloned into a plasmid vector. Next, from the library of cloned plasmids, the ER-binding fragments were trapped by nitrocellulose filter and concentrated by repeating the selection cycles.

RESULTS

Expression and purification of the ER-DBD protein in *E.coli*

A DNA fragment containing the whole ER-DBD was expressed in *E. coli*. The protein produced was accumulated in the inclusion bodies in *E. coli* cells. The cells were disrupted by sonication and the inclusion bodies were collected from the sonicated lysate by sucrose gradient centrifugation. They were denatured and solubilized in 8 M urea and renatured by step dialysis in the presence of 0.05 mM $ZnCl_2$. The concentration of the soluble protein was 600 nM. SDS-polyacrylamide gel electrophoresis showed that the recombinant protein was purified to near homogeneity (Fig. 1a). The protein migrates with a Mr of 14 kDa which corresponds to the calculated Mr (14,039 Da) of the ER-DBD protein.

The binding specificity of the ER-DBD protein

We confirmed the DNA-binding specificity of the ER-DBD protein by filter binding assay and footprinting. The vitellogenin A2 enhancer sequence containing an ERE was cloned into plasmid pUC18. The plasmid (pUC/vitERE) was digested with HpaII and EcoRI and 3'-end labeled with [α-^{32}P]dCTP. The labeled fragments were mixed with the ER-DBD protein and bound fragments were trapped by nitrocellulose filter. Only the fragment containing the ERE of vitellogenin enhancer was trapped (Fig. 1b: lane2, 3). Other fragments derived from plasmid pUC18 sequence failed to bind the protein and passed through the filter. The sequence protected by the ER-DBD protein was then determined by DNase I footprinting. The single-end labeled DNA fragment containing the ERE of vitellogenin A2 enhancer was prepared from the pUC/vitERE plasmid, incubated with the ER-DBD protein and then digested with DNase I. The protected

sequence was the consensus sequence of ERE (Fig. 1c: lane 2, 3) and corresponded to the binding sequence of the intact ER (39).

Isolation of the ER-binding fragments in genomic DNA

We tried to screen the ER-binding sites in human genomic DNA as described in 'MATERIALS AND METHODS'. The fragments bound to the ER-DBD protein were selected by nitrocellulose filter. The selection process shown in Fig. 2 was repeated six times and five independent clones were isolated. These plasmids contained inserts ranging from 200 bp to 2 kb and were named E1−E5. Sequence analysis has shown that all of these fragments contain perfect palindromic ERE sequences termed here ERE1−ERE5, respectively (Fig. 3). Southern blot analysis was performed for the E2 and E3 fragments (Fig. 4a) which showed that these fragments were present in single copy or at most a few copies in human genome. In addition to the E1−E5 fragments, we also isolated seven ER-binding fragments from genomic DNA digested with Sau3AI by the whole genome PCR method (23). Only one of them contained the perfect ERE sequence and was named Epcr. Interestingly, the Epcr was a part of the E3 fragment (Fig. 4b) as demonstrated by the presence of identical ERE3 and neighboring sequences. (Fig. 4c). These findings indicate that we have isolated the ER-binding fragment derived from the same locus in the genome by two independent experiments with similar but slightly different procedure (see 'DISCUSSION').

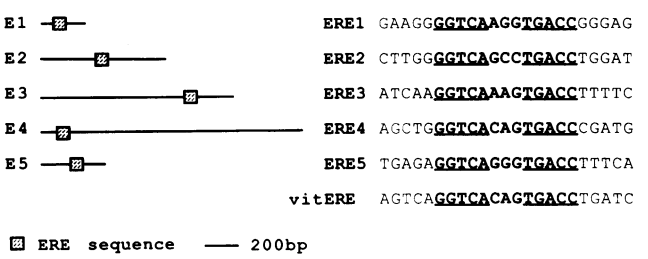

Fig. 3. The ER-binding fragments and their ERE sequences. The five ER-binding fragments (E1−E5) were sequenced. The position of the perfect palindromic ERE is shown by the box. The palindromic sequences (ERE1−ERE5) are shown with underlines and compared with vitERE.

The filter binding assay showed that these ER-binding fragments were recovered from nitrocellulose filter with various ratios (Fig. 5a). The E3 and E5 fragments was recovered from the filter with a higher percentages than the E2 and E4. The binding specificity was confirmed by the specific competition by ERE containing sequences (Fig. 5b). The E1−E5 fragments and pUC/vitERE could bind to the ER-DBD protein in the presence of non-specific competitor (100×pUC18: A). The binding of these fragments to the ER-DBD protein was competed specifically by vitERE sequence (100×pUC/vitERE: B), ERE2 sequence (100×pUC/ERE2: C) or ERE3 sequence (100×pUC/ERE3: D).

Enhancer activity of the ER-binding fragments

We wanted to know if these ER-binding fragments have any estrogen-dependent enhancer activity. The whole ER-binding fragments, E1−E3, were inserted into a reporter vector pBLCAT2 having a Herpes simplex virus tk promoter to construct the E1-tk-cat, E2-tk-cat, and E3-tk-cat, respectively. The reporter plasmids were co-transfected with the ER expression vector pSV2RcER (24) into COS-7 cells. As controls, the reporter plasmids without insert (tk-cat) and with the ERE of vitellogenin enhancer (vitERE-tk-cat) were assayed simultaneously. 12 hr after transfection, the cells were divided into two dishes and cultured in the presence or absence of estradiol. After 24 hr of culture, the CAT activities were assayed (Fig. 6). The ER expression vector was able to stimulate vitERE-tk-cat in the presence of estradiol but not tk-cat itself. In the absence of estradiol the stimulation by ER was always less than in the presence of estradiol. The estrogen-dependent enhancer activity was demonstrated clearly for the E1-tk-cat, although some background activity was noted even without the ligand. For E2-tk-cat and E3-tk-cat, the enhancer activity was undetectable. To examine

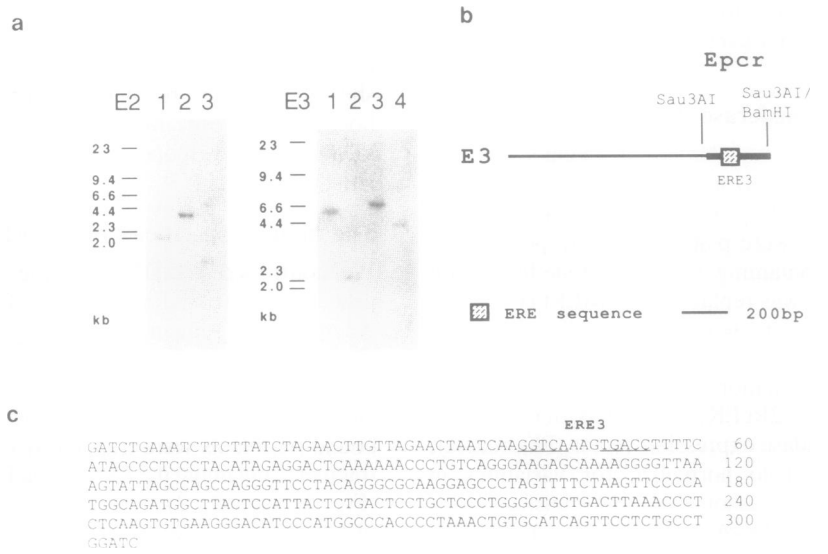

Fig. 4. Further characterization of E2 and E3. (a) Southern blot analysis of the E2 and E3 fragments. High molecular weight DNA (10 μg) from HeLa cells was digested with either BamHI (E3, lane1) BglII (E3: lane2), EcoRI (E2:lane 1, E3: lane3), HindIII (E2: lane2, E3: lane4) or XbaI (E2: lane 3). Digested DNA samples were resolved on 0.7% agarose gels, transferred to a nitrocellulose filter and hybridized to the probes (E2,E3). (b) The Epc fragment corresponds to the ERE3 containing fragment (Sau3AI-Sau3AI) derived from the E3 fragment. (c) The sequence of the Epcr is shown. The palindromic ERE (ERE3) is marked by underlines.

the possibility that the surrounding sequences may interfere with the enhancer activity, the short sequences containing the ERE2 and ERE3 which are the perfect palindromic ERE in the E2 and E3 fragments were cloned into the reporter vector pBLCAT2 (ERE2-tk-cat and ERE3-tk-cat) and tested for the enhancer activity. These minimum ERE sequences indeed showed certain estrogen-dependent enhancer activities, suggesting that some surrounding sequences have an interfering or silencing activity in COS-7 cells.

DISCUSSION

In this work we have isolated from human genomic DNA ER-binding fragments having perfect palindromic ERE sequences by affinity binding to the ER-DBD protein. Only perfect palindromic ERE sequences were cloned suggesting their special affinity to the ER-DBD protein. The *Xenopus* vitellogenin gene A2 estrogen-inducible enhancer (27, 40) has this sequence to which the ER expressed in HeLa cells binds tightly in the presence of estrogen (41). To date, however, no perfect palindromic ERE sequence has ever been identified as estrogen-dependent enhancer in humans. In this study, it is now established that the sequences containing perfect palindromic ERE with 3 bp spacing exist in human genome and also the DNA-binding domain of the estrogen receptor alone could specifically recognize and bind to the consensus ERE (GGTCANNNTGACC) without other factors such as an ER DNA binding stimulatory factor (DBSF) (42) or Fos-Jun complex (43) which may exist in cellular extracts and modify the binding of the ER to the ERE.

Previously, a whole genome PCR method has been used for the screening of *Xenopus* transcription factor TFIIIA-binding sites (23). We also tried to isolate ER-binding fragments by a similar procedure but with filter binding for selection instead of immune precipitation. We could isolated a 300-bp-long ER-binding fragment (Epcr) after five selection cycles (Fig. 4b, c). However, only short DNA fragments of less than a few hundred base pairs were obtained by this method with the shorter fragments being selected more efficiently. In addition, the misreading that might occur during polymerase chain reaction is always the cause of some uncertainty. In contrast, when using the method described here, the size of the fragments does not apparently affect the efficiency of the selection process and false positives caused by PCR method can be avoided. We could obtain relatively long fragments of up to 2−3 kb, which were readily cloned into a plasmid vector. These cloned fragments are suitable for sequencing, chromosome walking, analyzing for enhancer activity or Northern blotting.

An isolated fragment by the whole genome PCR method was incidentally a part of the E3 fragment containing ERE3 and neighboring sequences. Assuming a random appearance of the 10 bp sequence, there may be one perfect palindromic ERE sequence (GGTCANNNTGACC) in about every 1000 kb ($\approx 4^{10}$)bp. Thus human genome ($\approx 3\times10^9$ bp) could potentially have several thousand ERE sequences. The fact that we isolated the ER-binding fragment derived from the same locus in the genome by two independent experiments strongly suggests that we are selecting by this method specific fragments that bind to the ER-DBD protein with extraordinary affinity. Indeed, the E3 fragment was one of the ER-binding fragments recovered from

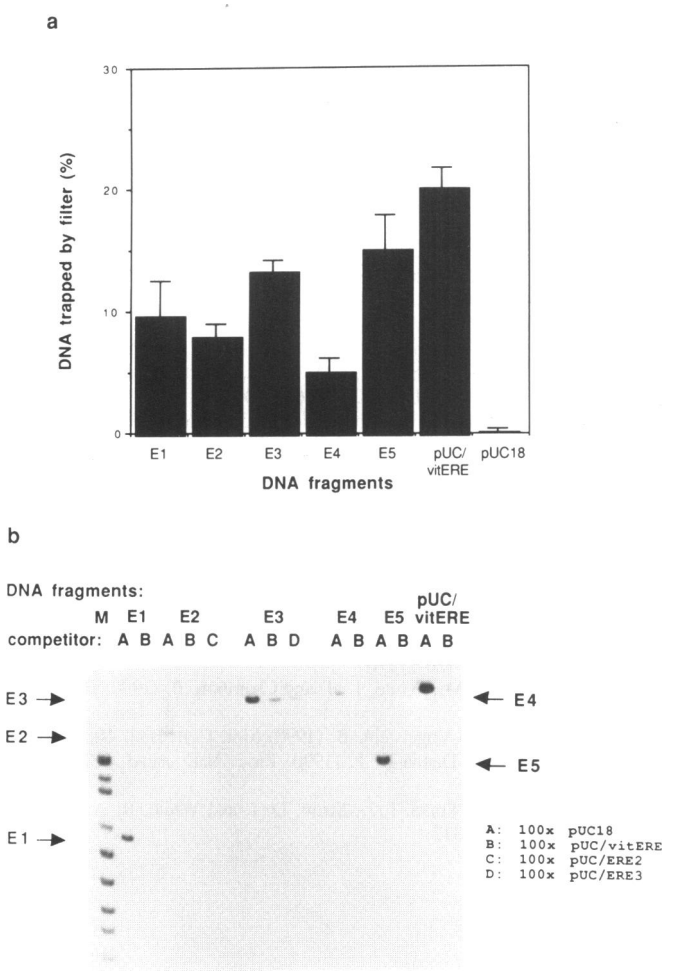

Fig. 5. The binding specificities of the ER-binding fragments. (a) The recovery percentages of the ER-binding fragments by filter binding assay. The E1−E5 fragments (PstI-BamHI), pUC/vitERE (PstI-BamHI), and pUC18 (PstI-BamHI) were end labeled by [α-^{32}P]dGTP and Klenow fragment. The labeled fragments (10,000 cpm each) were incubated with 1 pmol of the ER-DBD protein. Filter binding assay was performed and the recovery ratio was determined. Results are presented as the mean ± S. D. of triplicate determination. (b) Filter binding assay with competitors. Filter binding assay was performed for the fragments described above (10,000 cpm each) with a non-specific competitor (100×pUC18: A) or specific competitors (100×pUC/vitERE: B, pUC/ERE2: C and pUC/ERE3: D). The resulting fragments were analyzed on 5% acrylamide gel. The size of the ER-binding fragments (E1−E5) and the size markers (pUC18 digested with HpaII: lane M) are shown.

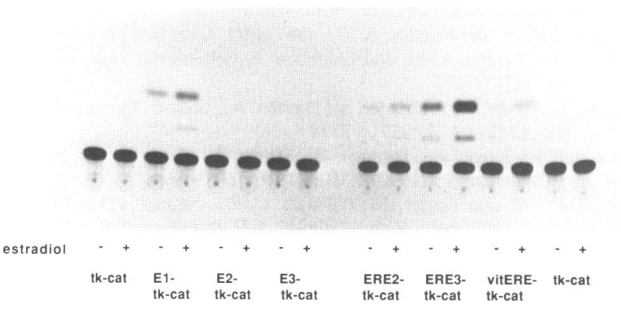

Fig. 6. The estrogen-dependent enhancer activity of the ER-binding fragments and their EREs. The reporter plasmids containing E1-tk-CAT, E2-tk-cat, E3-tk-cat, ERE2-tk-cat, ERE3-tk-cat, vitERE-tk-cat and tk-cat were transfected into COS7 cells with the ER expression vector. After culturing in the absence or presence of 1×10^{-7}M 17β-estradiol, CAT assay was performed.

the filter with a higher percentage (Fig. 5a). The recoveries were different among the ER-binding fragments albeit their all having just one perfect palindromic ERE.

It has been reported that imperfect palindromic EREs or half-palindromic EREs are active as the estrogen-dependent enhancer *in vivo*. The enhancers of rat prolactin (2) and human pS2 (3) gene have imperfect palindromic ERE. The enhancer of chicken ovalbumin gene (4) has GGTCA half-palindromic EREs. It has also been shown that two imperfect palindromic EREs in *Xenopus* vitellogenin gene B2 enhancer act together synergistically to confer high estrogen-dependent enhancer activity (39, 44). We could not isolate these sequences in this work. It is possible that strong binding of these sequence to the ER may require some other protein factor(s) that is functionally significant. To obtain enhancer sequences which bind to the ER-DBD protein more weakly, one might stop the selection cycles after only a few repeats and combine this with a screening assay for estrogen-dependent enhancer activity. Alternatively, one can screen the fragments that bind to multiple transcription factors such as estrogen receptor and progesterone receptor. Indeed some enhancer regions have multiple binding sites for different transcription factors within 1−2 kb. In such a case, there would be a high probability of isolation. Some of the long fragments obtained here (-2kb) may also contain such a region.

We isolated here the binding sites of a transcription factor (ER) in human genomic DNA and showed their possible enhancer activity. Recently, some attempts to isolate the binding sites of DNA-binding proteins have been reported (23, 45, 46) and target genes of *Drosophila* transcription factor Ultrabithorax (Ubx) have been located near the Ubx-binding sites in genomic DNA (47). We are now trying to identify a target gene of the ER near these ER-binding fragments. Furthermore, this method may also be useful for the isolation of other transcription factor-binding sites in genomic DNA.

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