MOLECULAR AND CELLULAR BIOLOGY, Jan. 1989, p. 43–49 Vol. 9, No. 1
0270-7306/89/010043-07$02.00/0
Copyright © 1989, American Society for Microbiology

Specific Binding of Estrogen Receptor to the Estrogen Response Element

LUDGER KLEIN-HITPASS,[1] SOPHIA Y. TSAI,[1] GEOFFREY L. GREENE,[2] JAMES H. CLARK,[1] MING-JER TSAI,[1] AND BERT W. O'MALLEY[1]*

Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030,[1] and Ben May Institute, University of Chicago, Chicago, Illinois 60637[2]

Received 11 July 1988/Accepted 29 September 1988

Gene transfer studies have shown that estrogen regulation of specific genes is mediated by estrogen response elements (ERE). We report that binding of the estrogen receptor to the ERE can be detected by a gel retardation (band shift) assay. This binding interaction was highly sequence and receptor specific. Methylation interference analysis showed that the ERE contact sites of estrogen receptor displayed a perfect twofold rotational symmetry. This is compatible with estrogen receptor binding to the ERE as a head-to-head dimer.

Steroid hormone regulation of gene transcription (for reviews, see references 6, 26, and 41) is mediated by specific intracellular receptors in target cells of mammary glands, liver, uterus, and oviduct. Upon activation by hormone binding, these receptors interact specifically with target genes, resulting in increased transcription initiation rates. Molecular cloning of various receptor cDNAs revealed that steroid receptors are a family of hormone-dependent transcription factors having a common structural organization (6, 11). Their carboxy terminus harbors the ligand-binding site, which is characterized by a high content of hydrophobic amino acids. Removal of this domain can render the molecule a less effective but constitutive transcription factor (9, 14). This observation has led to the suggestion that binding of hormone might induce a conformational change in the molecule which unmasks or creates the specific binding characteristics of the receptor. The domain responsible for DNA binding includes a highly conserved region that putatively forms two "zinc finger" structures similar to those first proposed for TFIIIA (30). It is thought that these fingers interact directly with DNA. Despite extensive efforts, separation of DNA-binding and transactivation function has not been achieved for steroid receptors as it has for yeast transcription factors Gal4 (19) and GCN4 (15).

By gene transfer into mammalian cells, the *cis*-acting DNA sequences involved in steroid hormone regulation have been identified in several genes (for a recent review, see reference 6). These sites of receptor interaction, called hormone response elements (HREs), are usually located upstream of the gene promoter and display enhancer properties (3, 21, 27, 31). Regulatory estrogen response elements (EREs) have been localized by functional analyses in the 5'-flanking region of the vitellogenin genes of *Xenopus laevis* (21, 22, 27, 34), the chicken vitellogenin II gene (2, 21), and the rat prolactin gene (28, 40). The EREs of the *Xenopus* vitellogenin A1 and A2 genes and the chicken vitellogenin II gene are palindromic sequences with 5-base-pair (bp) stems separated by a 3-bp spacer. Single copies of these elements matching the consensus sequence GGTCANNNTGACC, where N is any nucleotide, are able to confer significant estrogen inducibility on reporter genes (21, 23). EREs of the *Xenopus* vitellogenin B1 and B2 genes contain one or two nucleotide substitutions with respect to this consensus which almost completely destroy the regulatory capacity (21, 27). However, by synergistic action of two imperfect ERE copies, *cis*-linked genes acquire high estrogen inducibility (20, 27).

In vitro estrogen receptor-binding experiments complementing the gene transfer studies have used more indirect and laborious methods, such as DNA cellulose competition assays (21), electron microscopy (37), exonuclease III footprinting (28), and an avidin-biotin-conjugated DNA-binding assay with in vitro-synthesized estrogen receptor (40). More powerful high-resolution footprinting techniques such as methylation interference analyses have not been applied to studies of estrogen receptor binding.

In this paper we demonstrate that binding of crude as well as highly purified estrogen receptor can be detected in a convenient band shift assay. We show that binding of estrogen receptor to the ERE of the vitellogenin A2 gene is highly specific. We determined the sites at which estrogen receptor contacts the ERE by methylation interference analysis. Our results suggest that estrogen receptors are likely to bind to the ERE as a dimer.

MATERIALS AND METHODS

Cell transfections. CV-1 cells were grown in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). DNA was introduced into 40 to 70% confluent cells in serum-free medium with Polybrene (5 μg/ml) (18), followed by a 25% glycerol shock 4 to 6 h later (24). Cells were cultured in medium containing 100 nM 17-β-estradiol. Cell extracts were prepared 48 h posttransfection and assayed for chloramphenicol acetyltransferase (CAT) activity (10).

Plasmids. ERETKCAT plasmids were constructed by insertion of synthetic oligonucleotides (21-mer) containing vitellogenin A2 sequences from −332 to −318 (39) and *Bgl*II overhangs into pBLCAT2 (25). The orientation of the inserted fragment was determined by sequencing (32). pBLCAT2 contains the promoter of the herpes simplex virus thymidine kinase (TK) gene coupled to the CAT gene of *Escherichia coli* and RNA processing signals of simian virus 40. Estrogen receptor cDNA expression vector ΔHER has been described elsewhere (1).

Oligonucleotides. Synthetic oligonucleotides were purified by gel electrophoresis (20% polyacrylamide, 8 M urea).

* Corresponding author.

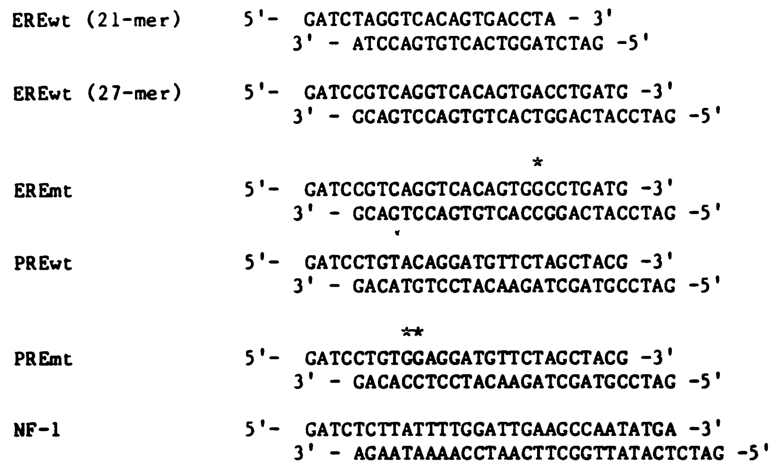

FIG. 1. Oligonucleotides used. Asterisks indicate mutations.

Complementary strands were annealed in 10 mM Tris (pH 8.0)–200 mM NaCl–1 mM EDTA by heating to 95°C and cooling to room temperature over a period of 3 h. The oligonucleotides shown in Fig. 1 were used.

Band shift assay. Binding reactions (10-μl volume) were carried out at room temperature for 20 min, and reaction mixtures contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 10% glycerol, 2% Ficoll 400 (Pharmacia), 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol (DTT), 0.2 μg of bovine serum albumin, 100 ng of pBR322 digested with *Hin*fI (if not otherwise indicated), and 0.1 to 0.2 ng (1×10^4 to 2×10^4 cpm) of specific probe (ERE 27-mer) labeled with [α-^{32}P]dGTP by using the Klenow fragment of DNA polymerase. Protein-DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis (8) in 4% polyacrylamide (19:1, acrylamide-bisacrylamide) gels. Gels were run at room temperature in 50 mM Tris–50 mM boric acid–1 mM EDTA at a constant voltage of 160 V, dried, and autoradiographed with intensifying screens.

Methylation interference assays. The ^{32}P-labeled DNA fragments were partially methylated as described previously (38). Briefly, about 20 ng of DNA fragment was incubated in a 200-μl reaction mixture containing 50 mM dimethyl sulfate, 50 mM sodium cacodylate, and 1 mM EDTA, pH 8.0, at room temperature for 8 min and then purified by repeated ethanol precipitation. About 3 ng of methylated DNA was incubated with 8 ng of purified MCF-7 estrogen receptor in the presence of 1 μg of *Hin*fI-digested pBR322 fragments in a final volume of 35 μl under the conditions described for the band shift assay. Protein-DNA complexes and free DNA were isolated from a preparative band shift gel and further processed as described before (38).

Preparation of protein fractions. Nuclear extract from MCF-7 cells grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics was prepared essentially as described by Dignam et al. (5). The dialyzed extract contained about 3 mg of protein per ml and was stored in small portions at −80°C.

Calf uterine cytosol was prepared from prepubertal calf uteri (Pelfreeze Biologicals, Rogers, Ark.). About 50 g of the frozen tissue was powdered in liquid nitrogen and slowly added to 150 ml of ice-cold buffer (20 mM Tris [pH 7.4], 5 mM DTT, 20 mM sodium molybdate) with stirring in a Polytrone homogenizer. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was recentrifuged at 105,000 × *g* for 1 h at 4°C. The high-speed supernatant (cytosol) contained about 10 mg of protein per ml and was stored at −80°C.

Estrogen receptor from MCF-7 cytosol was prepared by estradiol- and heparin-Sepharose chromatography as described by Greene (12).

RESULTS

As a probe for our band shift experiments, we chose the ERE of the vitellogenin A2 gene. This element has been shown to have high regulatory potential in transient transfections of ERETKCAT reporter constructions into estrogen-responsive MCF-7 cells (21, 23). We synthesized two different ERE oligonucleotides containing vitellogenin A2 sequences from positions −335 to −315 (ERE 27-mer) and −332 to −318 (ERE 21-mer). To ensure that these particular elements were functional, we assayed their ability to confer estrogen receptor-dependent stimulation of transcription to an indicator gene. We inserted the ERE oligonucleotides upstream of the TK-CAT fusion gene of vector pBLCAT2 (25). These pBLCAT2 derivatives were then cotransfected with estrogen receptor expression vector ΔHER (1) into the monkey kidney cell line CV-1, which was cultured in the presence of estrogen. As shown in Fig. 2, CAT activity detected in cells cotransfected with ΔHER and TK-CAT constructions carrying the ERE 21-mer in either orientation was 5- to 10-fold higher than in the control, in which expression vector lacking estrogen receptor cDNA insert was cotransfected. In contrast, expression of pBLCAT2 was not significantly changed by coexpression of estrogen receptor. Essentially the same result was obtained with clones containing the ERE 27-mer (data not shown). These results demonstrate that the ERE oligonucleotides used in this study are able to confer estrogen receptor-dependent regulation of gene transcription and thus represent sites of estrogen receptor interaction. Cotransfections performed in medium containing charcoal-stripped serum showed that induction of ERETKCAT expression by estrogen receptor required the presence of estradiol (data not shown).

To detect estrogen receptor binding to the ERE oligonucleotide (ERE 27-mer), we first performed band shift assays with estrogen receptor purified from the human breast cancer cell line MCF-7 as described by Greene (12). Our

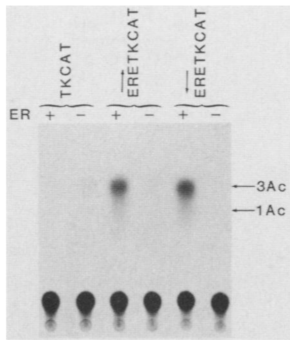

FIG. 2. ERE 21-mer mediates estrogen receptor-dependent activation of a TK-CAT reporter gene. CV-1 cells were transiently cotransfected with 5 μg of the indicated reporter gene construction and 5 μg of estrogen receptor (ER) cDNA expression vector (+) or its vector control (−). CAT assays were performed with 100 μg of protein. The positions of the reaction products (1- and 3-acetylated [Ac] chloramphenicol) on the autoradiogram of the thin-layer chromatogram are indicated. The spot with the lowest mobility represents the nonconverted substrate [^{14}C]chloramphenicol. The orientation of the ERE in the constructs with respect to the TK promoter is indicated by arrows. Plasmid TKCAT is identical to pBLCAT2 (25).

preparation was apparently homogeneous for 66-kilodalton (kDa) receptor protein as judged by the presence of a single silver-stained band on a sodium dodecyl sulfate–15% polyacrylamide gel (data not shown). As shown in Fig. 3, we observed protein-ERE complexes with the estrogen receptor preparation from MCF-7 cells. Typically, a sharp band (CI) within a zone of smeared DNA-protein complexes was observed. Since MCF-7 cells reportedly contain a nonreceptor protein that binds to the ERE of the chicken vitellogenin II gene (7), we wanted to demonstrate that our complexes indeed contained estrogen receptor. We therefore added rat monoclonal estrogen receptor antibody H222 (13), which is directed against the hormone-binding domain of human estrogen receptor, to certain of the binding reaction mixes. If the observed protein-ERE complexes contain estrogen receptor, binding of antibodies to the receptor should either inhibit complex formation by disturbing protein-DNA interaction or increase the mass of the complex and further retard it. As shown in Fig. 3 (lanes 3 to 5), the latter case was observed; i.e., addition of antibody H222 yielded a discrete decrease of the electrophoretic mobility of the DNA-protein complexes (CII). Monoclonal antibody PR22, which reacts with both forms of the chicken progesterone receptor (36), had no effect (Fig. 3, lane 7). H222 antibodies alone did not cause any complex formation, as expected (Fig. 3, lane 1). These results prove that the complexes observed contained estrogen receptor.

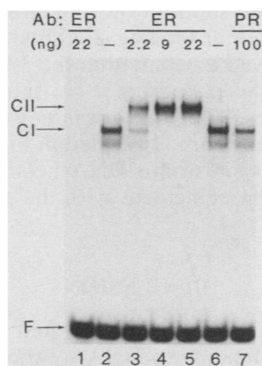

FIG. 3. Binding of highly purified estrogen receptor from MCF-7 cells to the ERE. Binding reaction mixes (lanes 2 to 7) contained about 3 ng of receptor and 0.1 ng of probe (ERE 27-mer). Protein-DNA complexes were separated from protein-free DNA by polyacrylamide gel electrophoresis and visualized by autoradiography. Monoclonal antibody (Ab) H222 (13) against estrogen receptor (ER) or antibody PR22 (36) against progesterone receptor (PR) was included in the binding reaction mixes. The amount of purified antibodies added is indicated above the lanes. CI, Estrogen receptor-ERE complex; CII, antibody-estrogen receptor-ERE complex; F, protein-free probe.

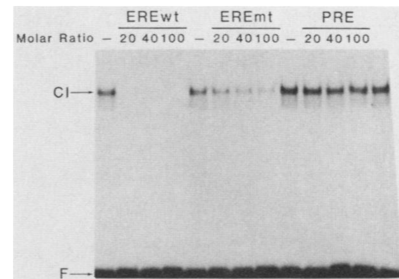

FIG. 4. Sequence-specific binding of estrogen receptor to the ERE. In band shift competition analysis, unlabeled competitor oligonucleotides were included in the binding reaction mix prior to addition of purified estrogen receptor (0.4 ng). The molar ratio of competitor over the labeled probe (ERE 27-mer, 0.1 ng) is indicated above each lane. The sequences of EREwt (27-mer), EREmt, and PRE oligonucleotides are given in Materials and Methods. CI, Estrogen receptor-ERE complex; F, protein-free probe.

To further characterize the estrogen receptor-ERE complexes, we performed band shift competition assays with unlabeled synthetic oligonucleotides as competitor DNA. A 20-fold molar ratio of homologous ERE oligonucleotide (EREwt) over the labeled probe almost completely prevented estrogen receptor binding (Fig. 4). In contrast, an oligonucleotide containing the distal progesterone/glucocorticoid response element (PRE) of the rat tyrosine aminotransferase (TAT) gene (35) did not significantly compete for estrogen receptor binding even at a 100-fold molar ratio. Similarly, no competition at a 100-fold molar ratio was observed with a mutated PRE containing two base exchanges that destroy function completely (35) or with an oligonucleotide containing the nuclear factor I-binding site (4) of the adenovirus major late promoter (data not shown). A mutated ERE oligonucleotide (EREmt) containing a single A-to-G conversion within one stem of the palindromic ERE competed significantly for estrogen receptor binding, although 5- to 10-fold less efficiently than the wild-type ERE. These results demonstrate that estrogen receptor ERE binding is highly sequence specific.

Next we examined whether the band shift assay allows us to detect estrogen receptor in crude extracts. We therefore prepared a nuclear extract from MCF-7 cells and a cytosol extract from calf uteri. Both fractions formed several protein-ERE complexes (Fig. 5A). In the calf uterine cytosol (lanes 4 and 5), addition of a saturating amount of monoclonal antibody H222 to the binding reaction converted complex CI and a complex migrating between CI and CIII to more slowly migrating forms, confirming that they both contained estrogen receptor. The decrease in complex III formation upon addition of antibodies is probably an unspecific effect of the immunoglobulin fraction, since an overall decrease in complex formation was observed. We assume

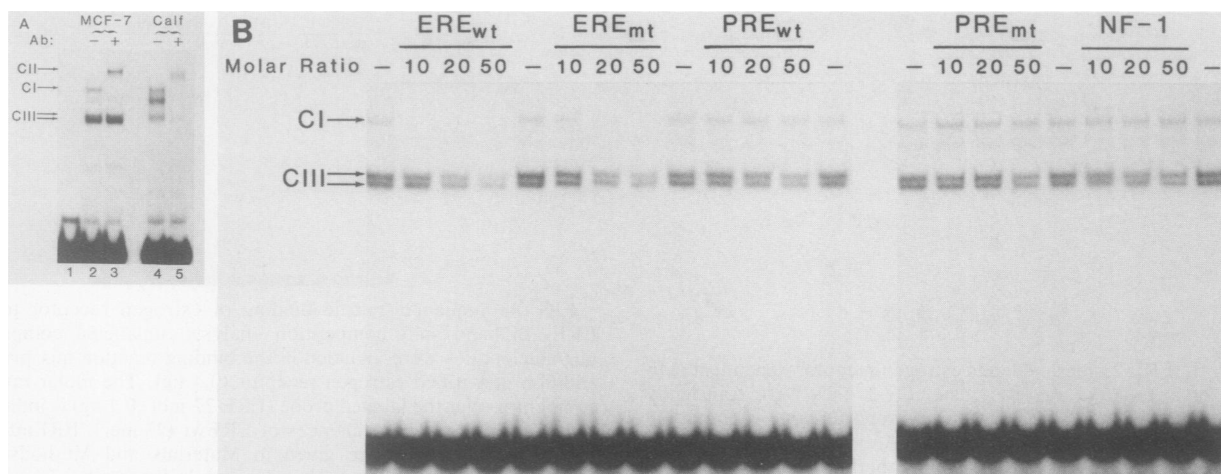

FIG. 5. Detection of estrogen receptor in MCF-7 nuclear extract and calf uterine cytosol. (A) Identification of estrogen receptor-ERE complexes in MCF-7 nuclear extract (MCF-7) and calf uterine cytosol (calf) by using specific antibodies. MCF-7 nuclear extract (3 μg) (lanes 2 and 3) and calf uterine cytosol (10 μg) (lanes 4 and 5) were analyzed by band shift analyses. Binding reaction mixes contained 0.2 ng of probe (ERE 27-mer) and 500 (lanes 1 to 3) or 100 ng (lanes 4 and 5) of *Hin*fI-digested pBR322 nonspecific competitor. In lanes 3 and 5, monoclonal antibody H222 (22 μg) was included in the binding reaction mix. Lane 1 shows protein-free probe. CI and CII, See legend to Fig. 2; CIII, major protein-ERE complexes detected in MCF-7 cells which did not react with estrogen receptor antibody H222. (B) Band shift competition analysis was performed as described in the legend to Fig. 4, with 6 μg of MCF-7 nuclear extract protein in the presence of 500 ng of nonspecific competitor and 0.2 ng of probe (ERE 27-mer). The molar ratio of competitor over the labeled probe is indicated. The sequences of the competitor oligonucleotides are given in Fig. 1. EREwt, Wild-type ERE; EREmt, mutated ERE; PREwt, progesterone response element of the *tat* gene of rat (35); PREmt, mutated PRE (35); NF-1, nuclear factor 1-binding site of adenovirus major late promoter (4).

that the receptor-ERE complexes migrating faster than complex CI are formed by degraded or modified receptor molecules. In the case of the MCF-7 nuclear extract (lanes 2 and 3), the antibody affected only the mobility of the most slowly migrating complex (CI); it left four other, more quickly migrating complexes unchanged. This suggests either that the latter complexes are not formed by estrogen receptor or that the receptor is degraded to such a degree that the antibody epitope has been deleted.

To further investigate the specificity of the different complexes detected in the MCF-7 nuclear extract, a band shift competition assay was performed (Fig. 5B). Formation of complex CI was sequence specific, since binding could be efficiently inhibited by a 10-fold molar excess of ERE oligonucleotide, whereas PRE, PREmt, and NF-I oligonucleotides did not inhibit at all. As with purified MCF-7 receptor, the mutated ERE (EREmt) showed reduced competition efficiency compared with EREwt. Formation of the higher-mobility complexes (CIII) by proteins present in the MCF-7 nuclear extract could be partially inhibited by a 50-fold molar ratio of EREwt and EREmt. However, a 50-fold molar ratio of PREwt, PREmt, and NF-I also inhibited binding slightly, indicating that the interaction of these proteins with the ERE probe is not highly specific. Similar results were obtained with calf uterine cytosol (data not shown).

We defined precisely the DNA contact sites involved in estrogen receptor binding by using a methylation interference assay. Our probes were the 128-bp *Hin*dIII-*Ava*II fragments of pBLCAT2 derivatives containing the ERE 21-mer in both orientations, end-labeled in their lower strand at the *Hin*dIII restriction site by end-filling with Klenow enzyme. In addition to the ERE, these probes contain binding sites for SP1 and CTF transcription factors (16) within the thymidine kinase promoter sequences of the fragments. However, band shift experiments with these probes and our purified estrogen receptor preparations revealed only the estrogen receptor-DNA complex (data not shown). Probes were partially methylated at the N3 position of A residues and at the N7 position of G residues by dimethyl sulfate and incubated with purified estrogen receptor from MCF-7 cells. DNA present in receptor-DNA complexes as well as free probe were isolated from a preparative band shift gel, cleaved at methylated A and G residues by NaOH treatment, and resolved on a sequencing gel. Positions at which methylation of G and A residues interferes with receptor binding should not be cleaved in the probe recovered from the protein-DNA complex band. Comparison of the cleavage pattern of free and complexed probe revealed that methylation of all G residues of both strands within the conserved stems of the palindromic ERE strongly interfered with receptor binding (Fig. 6A). Only weak interference was observed at G's within the 3-bp spacer separating the stems of the palindrome. No interference was detected at A residues. The purine residues that are in contact with estrogen receptor are illustrated in Fig. 6B. The projection of the contact sites onto DNA helices reveals a symmetrical distribution coincident with the dyad symmetry of the ERE.

DISCUSSION

The data presented here are the first demonstration of estrogen receptor binding to DNA by the band shift assay. The presence of estrogen receptors in the complexes was proven by using monoclonal anti-estrogen receptor antibodies, which caused a mobility shift of the protein-DNA complexes. Thus, our experiments clearly show that the affinity of estrogen receptor for its specific regulatory element is high enough to maintain stable protein-ERE complexes during nondenaturing gel electrophoresis.

Compared with other estrogen receptor-DNA binding assays, this direct binding assay offers several advantages: (i) it is very simple and fast and thus allows analysis of many

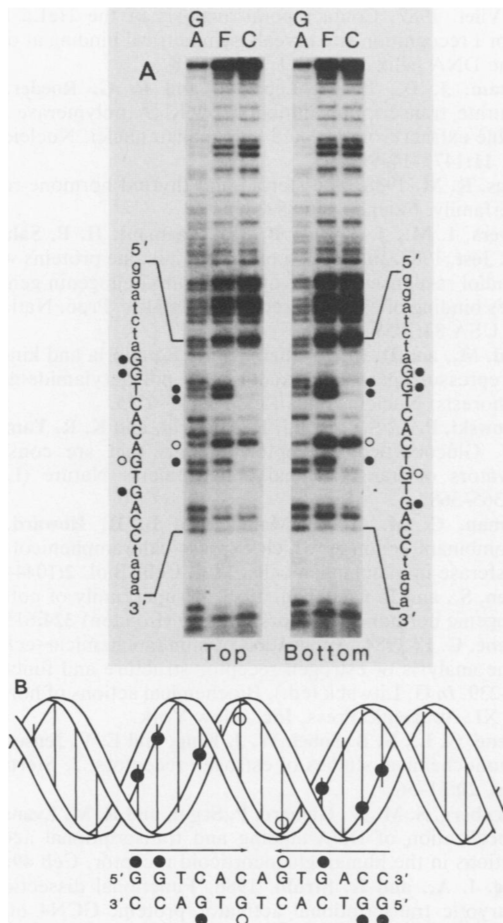

FIG. 6. Purine contact sites of estrogen receptor on the ERE. (A) Methylation interference analysis of estrogen receptor binding. As probes, the *Hin*dIII-*Ava*II fragments (128 bp) of the two different ERETKCAT constructs shown in Fig. 2 were both labeled at the *Hin*dIII site by end-filling with Klenow enzyme. Note that the sequences of the two fragments differ at only one position (the center of the ERE) due to the palindromic structure of the ERE. The left and right panels show analyses of the top and bottom strands of the ERE, respectively, with respect to its natural orientation in the vitellogenin A2 gene. The relevant portion of the sequence is given. Solid and open circles depict strong and weaker methylation interference sites, respectively. G/A, G and A sequencing reaction (29); F, protein-free probe; C, probe present in estrogen receptor-DNA complex. (B) Summary of the methylation interference data; projection of the purine contact sites that are important for estrogen receptor binding to DNA helices.

different samples in parallel; (ii) it allows the use of methylation interference analysis to identify the precise location of estrogen-binding sites within genes; (iii) it does not require labeling of receptor with radioactive ligand; (iv) specific and nonspecific ERE binding proteins can be differentiated by competition analysis; and (v) it allows the identification of receptors in complex mixtures of DNA-binding proteins by the use of specific antibodies. The band shift assay is sensitive enough for tracing estrogen receptor during purification. As shown in Fig. 5, we can detect estrogen receptor in 3 μg of nuclear protein prepared from estrogen receptor-expressing MCF-7 cells as well as in 10 μg of calf uterine cytosol protein. We estimate that the detection limit of the assay under our standard conditions is about 1 fmol of receptor in the binding reaction mix. However, by decreasing the amount of nonspecific competitor and by increasing the probe concentration, an even higher sensitivity could be achieved.

Our competition experiments with synthetic oligonucleotides demonstrate that estrogen receptor binds specifically to the ERE and has no detectable affinity for a PRE. This confirms results obtained by DNA-cellulose competition assays (21). Compared with EREwt, the mutated ERE (corresponding to EREmt1 in reference 21) had a 5- to 10-fold-lower but still significant affinity for estrogen receptor in our assay (Fig. 4 and 5B). This result correlates well with results of gene transfer experiments which showed that EREmt1 has a clearly reduced regulatory capacity compared with EREwt (21).

Due to the presence of estrogen during the purification and in the cell culture medium, the purified MCF-7 receptor (Fig. 3 and 4) and the crude nuclear extract from MCF-7 cells (Fig. 5) most likely contain hormone-occupied receptors. Therefore, it is not surprising that we can detect receptor binding without addition of hormone. However, this interpretation is not compatible with the results obtained with calf uterine cytosol (Fig. 5A), in which occupied receptors could not be detected; labeling of receptor with radioactive hormone under nonexchange (4°C) and exchange (30°C) conditions gave identical results (data not shown). Thus, the cytosolic receptor prepared as described in this paper is able to bind to the ERE in the absence of added hormone. However, it is not clear whether this is a consequence of heat or salt activation of the receptors during their preparation and analysis or whether the complexes observed represent a minority of in vivo-activated and hormone-occupied estrogen receptors.

We demonstrated that highly purified estrogen receptor from MCF-7 cells lacking any detectable contamination with other DNA-binding proteins is able to bind to the ERE. This is in contrast to results reported by Feavers et al. (7), who postulated an essential role of a nonhistone ERE-binding protein in estrogen receptor binding. This protein (NHP-I), which is distinct from estrogen receptor, probably accounts for one of the more quickly migrating complexes (CIII) detectable in MCF-7 nuclear extract and calf uterine cytosol (Fig. 5). Since complex III proteins (i) are not detectable in our highly purified estrogen receptor preparation (Fig. 1), (ii) do not significantly stimulate complex I formation when added to the purified preparation (data not shown), and (iii) do not bind in a sequence-specific manner to the ERE that is comparable to the specificity of estrogen receptor (CI) complexes (Fig. 4B), it seems likely that complex III proteins are not essential for estrogen receptor binding. Such proteins, however, may contribute to the biology of estrogen action by contacting and stabilizing specific binding of the receptor with its enhancer element. Finally, the receptor-DNA contact sites which we determined by methylation interference analysis (Fig. 6) differ from the NHP-I contact sites similarly deduced by Feavers et al. (7), indicating that NHP-I is not present in complex I. We therefore assume that estrogen receptor binds directly to the ERE and that binding specificity is an inherent property of the receptor itself. However, we should note that our experiments cannot exclude formally the possibility that a non-DNA-binding protein contaminating our preparations might be involved somehow in receptor binding. Stabilization of protein-DNA complexes by protein-protein interaction with non-DNA-binding proteins has been reported recently (38).

As shown in Fig. 6, all strong methylation interference sites of estrogen receptor binding were located within one

and a half successive turns of the major groove and accessible from one face of the DNA helix. Consistent with an exonuclease footprint (28), our experiments show that both halves of the ERE palindrome are in contact with the receptor complex. This suggests that a DNase I footprint that covered only the proximal half of the chicken vitellogenin II ERE and additional neighboring sequences (17) was not produced by estrogen receptor. Within the three central nucleotides of the palindrome, methylation of one G in each strand interfered only weakly with binding, indicating that contacts in this region of the ERE are not essential for high-affinity binding. However, since the ERE of the vitellogenin A1 gene, which does not contain a G residue in that region of the upper strand, showed slightly reduced regulatory capacity compared with the vitellogenin A2 ERE (21), we speculate that contacts in this nonconserved region might have a positive effect on the binding affinity of the regulatory element. Interestingly, the ERE mutation (EREmt), which decreased competition efficiency (Fig. 3 and 5) (21) as well as regulatory capacity (21), did not affect a nucleotide whose methylation interfered with binding.

The DNA contact sites of estrogen receptor display a perfect twofold rotational symmetry which is consistent with the dyad symmetry of the ERE sequence. This finding is compatible with the estrogen receptor binding to the ERE as a head-to-head dimer, where each monomer contacts only one of the half-sites of the ERE. Analysis of contact sites of glucocorticoid (33) as well as certain ligand-independent transcription factors, such as nuclear factor I (4), similarly revealed a symmetrical distribution and suggested dimer binding. Recent results obtained in our laboratory indeed proved that glucocorticoid receptors bind to glucocorticoid response elements as dimers and that binding of dimers involves a cooperative mechanism (37a). Our result, that a point mutation in one-half of the symmetrical ERE decreased competition efficiency at least fivefold, which is much more than would be expected if both half-sites bind receptor independently, might indicate that assembly of estrogen receptor dimers on a single ERE also involves a cooperative mechanism.

ACKNOWLEDGMENTS

We thank G. Schütz and Bruno Luckow, Institute of Cell and Tumor Biology, German Cancer Research Center, Heidelberg, and D. O. Toft, Department of Biochemistry and Molecular Biology, Mayo Medical School, Rochester, Minn., for providing plasmid pBLCAT2 and monoclonal antibody PR22, respectively. In addition, we thank Jennifer Liao, Cindy Porter, and David Miller for their technical assistance, Beth Lynn Maxwell for providing CV-1 cells, and Cheryl McCarthy for typing the manuscript.

This work is supported by Public Health Service grants HD 08188 and HD 07857 (to B.W.O.) and HD 08436 (to J.H.C.) from the National Institutes of Health and grants from NCI (CA 02897 to G.L.G.) and ACS (BC 86 to G.L.G.).

LITERATURE CITED

1. **Bradshaw, M. S., M.-J. Tsai, and B. W. O'Malley.** 1988. A far upstream ovalbumin enhancer binds nuclear factor-1-like factor. J. Biol. Chem. **263:**8485–8490.
2. **Burch, J. B. E., M. I. Evans, T. M. Friedman, and P. J. O'Malley.** 1988. Two functional estrogen response elements are located upstream of the major chicken vitellogenin gene. Mol. Cell. Biol. **8:**1123–1131.
3. **Chandler, V. L., B. A. Maler, and K. R. Yamamoto.** 1983. DNA sequences bound specifically by glucocorticoid receptor *in vitro* render a heterologous promoter hormone responsive *in vivo*. Cell **33:**489–499.
4. **de Vries, E., W. van Driel, S. J. L. van den Heuvel, and P. C. van der Vliet.** 1987. Contact point analysis of the HeLa nuclear factor I recognition site reveals symmetrical binding at one side of the DNA helix. EMBO J. **6:**161–168.
5. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1981. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
6. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240:**889–895.
7. **Feavers, I. M., J. Jiricny, B. Moncharmont, H. P. Saluz, and J. P. Jost.** 1987. Interaction of two nonhistone proteins with the estradiol response element of the avian vitellogenin gene modulates binding of estradiol-receptor complex. Proc. Natl. Acad. Sci. USA **84:**7453–7457.
8. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**6505–6525.
9. **Godowski, P. J., S. Rusconi, R. Miesfeld, and K. R. Yamamoto.** 1987. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature (London) **325:**365–368.
10. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
11. **Green, S., and P. Chambon.** 1986. A superfamily of potentially oncogenic hormone receptors. Nature (London) **324:**615–617.
12. **Greene, G. L.** 1984. Application of immunochemical techniques to the analysis of estrogen receptor structure and function, p. 207–239. *In* G. Litwack (ed.), Biochemical actions of hormones, vol. XI. Academic Press, Inc., New York.
13. **Greene, G. L., N. B. Sobel, W. J. King, and E. V. Jensen.** 1984. Immunochemical studies of estrogen receptors. J. Steroid Biochem. **20:**51–56.
14. **Hollenberg, S. M., V. Giguere, P. Segui, and R. M. Evans.** 1987. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell **49:**39–46.
15. **Hope, I. A., and K. Struhl.** 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell **46:**885–894.
16. **Jones, K. A., K. R. Yamamoto, and R. Tjian.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter *in vitro*. Cell **42:**559–572.
17. **Jost, J.-P., M. Seldran, and M. Geiser.** 1984. Preferential binding of estrogen-receptor complex to a region containing the estrogen-dependent hypomethylation site preceding the chicken vitellogenin II gene. Proc. Natl. Acad. Sci. USA **81:**429–433.
18. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4:**1172–1174.
19. **Keegan, L., G. Gill, and M. Ptashne.** 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science **231:**699–704.
20. **Klein-Hitpass, L., M. Kaling, and G. U. Ryffel.** 1988. Synergism of closely adjacent estrogen-responsive elements increases their regulatory potential. J. Mol. Biol. **201:**537–544.
21. **Klein-Hitpass, L., G. U. Ryffel, E. Heitlinger, and A. C. B. Cato.** 1988. A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucleic Acids Res. **16:**647–663.
22. **Klein-Hitpass, L., M. Schorpp, U. Wagner, and G. U. Ryffel.** 1986. An estrogen-responsive element derived from the 5'-flanking region of the *Xenopus* vitellogenin A2 gene functions in transfected human cells. Cell **46:**1053–1061.
23. **Klock, G., U. Strähle, and G. Schütz.** 1987. Oestrogen and glucocorticoid responsive elements are closely related but distinct. Nature (London) **329:**734–736.
24. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyltransferase gene by DEAE-dextran mediated DNA transfection coupled with dimethyl sulfoxide or glycerol shock. Nucleic Acids Res. **12:**5707–5717.
25. **Luckow, B., and G. Schütz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of

eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15**:5490.
26. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237–1245.
27. **Martinez, E., F. Givel, and W. Wahli.** 1987. The estrogen-responsive element as an inducible enhancer: DNA sequence requirements and conversion to a glucocorticoid-responsive element. EMBO J. **6**:3719–3727.
28. **Maurer, R. A., and A. C. Notides.** 1987. Identification of an estrogen-responsive element from the 5′-flanking region of the rat prolactin gene. Mol. Cell. Biol. **7**:4247–4254.
29. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
30. **Miller, J., A. D. McLachlan, and A. Klug.** 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J. **4**:1609–1614.
31. **Ponta, H., N. Kennedy, P. Skroch, N. E. Hynes, and B. Groner.** 1985. Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc. Natl. Acad. Sci. USA **82**:1020–1024.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Scheidereit, C., H. M. Westphal, C. Carlson, H. Bosshard, and M. Beato.** 1986. Molecular model of the interaction between the glucocorticoid receptor and the regulatory elements of inducible genes. DNA **5**:383–391.
34. **Seiler-Tuyns, A., P. Walker, E. Martinez, A.-M. Merrilat, F. Givel, and W. Wahli.** 1986. Identification of estrogen-responsive DNA sequences by transient expression experiments in a human breast cancer cell line. Nucleic Acids Res. **14**:8755–8770.
35. **Strähle, U., G. Klock, and G. Schütz.** 1987. A DNA sequence of 15 base pairs is sufficient to mediate both glucocorticoid and progesterone induction. Proc. Natl. Acad. Sci. USA **84**:7871–7875.
36. **Sullivan, W. P., T. G. Beito, J. Proper, C. J. Krco, and D. O. Toft.** 1986. Preparation of monoclonal antibodies to the avian progesterone receptor. Endocrinology **119**:1549–1557.
37. **ten Heggeler-Bordier, B., R. Hipskind, A. Seiler-Tuyns, E. Martinez, B. Corthesy, and W. Wahli.** 1987. Electron microscopic visualization of protein-DNA interactions at the estrogen responsive element and the first intron of the *Xenopus laevis* vitellogenin gene. EMBO J. **6**:1715–1720.
37a. **Tsai, S. Y., J. Carlstedt-Duke, N. L. Weigel, K. Dahlmann. J.-Å. Gustafsson, M.-J. Tsai, and B. W. O'Malley.** 1988. Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell **55**:361–369.
38. **Tsai, S. Y., I. Sagami, H. Wang, M.-J. Tsai, and B. W. O'Malley.** 1987. Interactions between a DNA-binding transcription factor (COUP) and a non-DNA binding factor (S300-II). Cell **50**:701–709.
39. **Walker, P., J.-E. Germond, M. Brown-Luedi, F. Givel, and W. Wahli.** 1984. Sequence homologies in the region preceding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDL II genes. Nucleic Acids Res. **12**: 8611–8626.
40. **Waterman, M. L., S. Adler, C. Nelson, G. L. Greene, R. M. Evans, and M. G. Rosenfeld.** 1988. A single domain of the estrogen receptor confers deoxyribonucleic acid binding and transcriptional activation of the rat prolactin gene. Mol. Endocrinol. **2**:14–21.
41. **Yamamoto, K.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209–252.