A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor

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Received October 30, 1987; Revised and Accepted December 9, 1987

ABSTRACT

Sequences located upstream of the transcription initiation site of the *Xenopus vitellogenin A2* (vit A2) gene contain a hormone dependent enhancer that confers estrogen control to the heterologous thymidine kinase (tk) promoter. As a minimal functional estrogen responsive element (ERE), we have defined the 13 bp palindrome GGTCACAGTGACC. This ERE binds estrogen receptor preferentially in vitro. Although the ERE shares some structural features with the glucocorticoid responsive element (GRE) it is distinct from this element since it neither binds glucocorticoid receptor in vitro nor does it confer glucocorticoid inducibility to a fusion gene. Point mutations within the ERE decrease its affinity for the estrogen receptor and result in a complete loss of estrogen inducibility.

INTRODUCTION

Transcription of genes is thought to be controlled by trans-acting factors which bind to DNA sequences in the vicinity of regulated promoters. Among the different known regulatory factors, the steroid hormone receptors are one of the most extensively studied group. By functional analyses of various transfected cell lines 5'flanking regions of genes involved in glucocorticoid (1-9) regulation have been identified. By footprinting techniques it has been demonstrated, that glucocorticoid and progesterone receptors interact with relatively small binding sites within functionally defined regions (2,7,9,16-18). In the most of the glucocorticoid responsive genes multiple binding sites for glucocorticoid receptor have been identified (7,9,16-18) which in the chicken lysozyme gene and in the mouse mammary tumour virus (MMTV) long terminal repeat region even overlap with progesterone receptor binding sites (17). Consensus sequences have been derived by sequence comparison of glucocorticoid receptor binding sites and from functional analyses. These consensus sequences range from six (16) to seventeen (2) nucleotides. However, it is not evident in any of these cases whether such a minimal regulatory element separated from its natural flanking regions suffices for significant hormonal control. Furthermore, information is lacking on how

deviations from these consensus sequence change the regulatory activity and the receptor binding properties of the responsive elements.

Using the gene transfer approach we have recently localized two different regulatory elements within the 5'flanking region of the *Xenopus laevis* vitellogenin A2 (vit A2) gene (13). The vit A2 gene is a member of a small gene family encoding the yolk precursor proteins whose expression is strictly dependent on estrogen (for review see refs.19-21). Functional analyses of constructs of vit A2 5'flanking regions linked to the thymidine kinase (tk) promoter revealed an element which increased the basal level of transcription. More importantly, a short cis-acting element (18 bp) could be identified which confers estrogen inducibility to the heterologous tk promoter. A single 13 bp palindrome within these 18 bp proved to be essential for hormonal regulation. Homologous palindromic elements are present in 1 to 3 copies within the 5'flanking sequences of various estrogen regulated genes (22). Recent results obtained from analyses of the vitellogenin B1 and B2 genes (14,15) confirmed the important role of these palindromic elements in estrogen regulation.

In the present communication we determine the minimum sequence requirement for a functional estrogen responsive DNA element (ERE) and characterize its functional properties. These data are correlated with in vitro studies of estrogen receptor binding. We further demonstrate in in vitro receptor binding studies and functional assays that the estrogen responsive element is distinct in function and in receptor binding properties from a glucocorticoid responsive element although these two elements share some structural similarities.

MATERIAL AND METHODS

Materials

Plasmid Constructions

pBL-CAT8⁺ was constructed by replacing the HindIII/Bgl II fragment of pA2 (-821/-87) tk-CAT8⁺ with the Hind III/Bgl II fragment of pBL-CAT2 (13) containing the pUC18 multiple cloning site upstream of the thymidine kinase promoter (Position -105 to +51). To construct pBL-CAT9⁺, pA2(-821/-87) tk-CAT8⁺ was linearized with Bam HI and digested with S1 nuclease to remove the sticky ends. After ligation of Hind III linkers (8mer), the DNA was digested with Cla I and Hind III and the isolated Cla I-Hind III fragment containing tk-CAT-SV40 sequences was ligated with the Cla I-Hind III fragment from pEMBL9⁺ (23) containing the multiple cloning site of pUC9. Sequencing of the polylinker region revealed that probably by S1 overdigestion in addition to the Bam HI sticky end two bp of double stranded DNA had been removed, resulting in the sequence 5'-AAGCTTGGCCCGCCCC-3' (Hind III site and SP1 binding site are underlined) at the border of the polylinker and the tk promoter fragment.

Synthetic oligonucleotides were synthesized with the Pharmacia Gene Assembler. Removal of protecting groups and cleavage of the oligonucleotide-support linkage was done as recommended by Pharmacia. For annealing about

0.01 A260-units of each of the two complementary oligonucleotides were mixed, heated to 100°C and allowed to cool slowly to room temperature over a period of several hours. 2 µl of the annealing reaction were ligated with 100 ng Hind III/Bam HI digested vector pBL-CAT⁸ in a final volume of 40 µl. Resulting recombinants were checked by sequencing according to the method of Sanger et al. (24) or by super-coil sequencing (25).

Transient Transfection and Cell Culture

The human breast carcinoma cell line MCF-7 was cultured as previously described. Transient transfections of MCF-7 cells were performed either by the DEAE-dextran technique as reported (13) or by the calcium phosphate precipitation technique according to Ott et al. (26). Briefly, one day prior to the transfection, MCF-7 cells were plated at a density of 2 to 3x10⁶ cells per 10 cm dish into medium containing 10% calf serum (CS, Gibco) and 1 µM tamoxifen. Two hours before addition of the precipitate the medium was renewed. 475 µl of the precipitate containing 9.5 µg of plasmid DNA was added to each petri dish (10 ml medium). After 5 h the cells were rinsed, shocked for two min with 15% glycerol in phosphate buffered saline (PBS), rinsed with PBS and cultured in 10 ml medium containing 10% charcoal-treated fetal calf serum with 1 µM tamoxifen. Hormone treatment, preparation of cell free extracts, and CAT assays were done as described (13). Rat XC cells were cultured and transiently transfected by the DEAE-dextran method as reported in Cato et al. (11). Mouse Itk-clones were transfected as reported (27).

Preparation and SP6 Mapping of Cellular RNA

To prepare total RNA, cells from two petri dishes were rinsed and scraped into ice-cold PBS. After centrifugation for 5 min at 4°C, the cell pellet was resuspended in 1 ml PBS. Cells were lysed by addition of 1 ml 0.2 M Tris-HCl pH 7.5, 25 mM EDTA pH 8.0, 0.3 M NaCl, 2% SDS. High molecular DNA was sheared by repeated passing through a syringe. After addition of 50 µl proteinase K (20 mg/ml) samples were incubated for 30 min at 37°C. Subsequently, nucleic acids were extracted with 2 ml phenol/chloroform/isoamylalcohol (25:24:1). RNA was selectively precipitated by addition of one volume of 4 M LiCl at 4°C. After precipitation 20 µg aliquots of RNA were digested with 0.2 µg of DNase I (Boehringer) in the presence of 5U RNasin (Genofit, Geneva) to remove contaminating DNA. After phenol/chloroform extraction and ethanol precipitation RNA was analyzed by SP6 mapping (28,29) with the SP-pTK-CAT probe described by Miksicek et al. (7).

Steroid Hormone Receptor Binding Assays

Nuclei from 6x10⁸ MCF-7 cells cultured for a week in Dulbecco's medium supplemented with 3% charcoal-treated fetal calf serum were extracted with 50 mM Tris-HCl pH 8.7, 10 mM EDTA, 1.5 mM DTT, 500 mM KCl, 10% glycerol, 0.1 mM phenylmethylsulfonylfluoride (PMSF). Upon centrifugation at 105,000 xg for 1 h at 0°C, the supernatant was labeled with 50 nM ³H -17β-estradiol (89 Ci/mmol) for 5 h at 0°C. The nuclear extract was passed through Sephadex G100 pre-equilibrated with 50 mM Tris-HCl pH 8.7, 5 mM EDTA, 1.5 mM DTT, 100 mM KCl, 0.1 mM PMSF to remove unbound estradiol. Fractions containing labeled estrogen receptor were pooled and used in the binding experiments. Receptor preparations routinely used for the binding assay contained about 400 fmol receptor/mg protein as measured by the dextran-coated charcoal receptor assay (30). Estrogen receptor binding was measured by the DNA cellulose competition assay according to the method of Pfahl (31). The DNA cellulose (1.5 mg of calf thymus DNA/g of cellulose) was prepared as described (32). The reaction mixture contained 15 µg calf thymus DNA bound to cellulose, the various DNA fragments to be tested and about 50 fmol receptor in the buffer used in the gel filtration procedure. The samples were incubated for 45 min at room temperature and processed as described by Pfahl (31). Glucocorticoid receptor binding was also measured by the DNA cellulose assay essentially as described by Pfahl (31) using ³H -triamcinolone acetone labeled rat liver cytosol,

which had been prepared according to the procedure of Westphal and Beato (33). The various DNAs to be tested in the binding assays were either digested with Sma I or with another restriction enzyme followed by treatment with S1 nuclease to generate blunt ends.

RESULTS

The ERE of the *Xenopus vitellogenin A2* gene acts in both orientations

Our previous study had shown that a 5'flanking element of vit A2 confers estrogen regulation to the thymidine kinase promoter (13). Since the ability to act on heterologous promoters is a characteristic feature of enhancers (34) we examined whether the ERE shares one of the other properties of enhancers in that they can act independently of orientation. We used four different fragments of the vit A2 5'flanking region linked to the tk promoter driving the transcription of the CAT gene. These fragments when linked to the tk promoter in their natural orientation mediate estrogen induction of CAT activity when transfected into MCF-7 cells (13). We now test these fragments in the reverse orientation relative to the tk promoter. Two of the four fragments (-482/-87; -331/-87) contain in addition to the ERE the activator element (see Figure 1B) which increases the basal activity of the tk promoter (13). Figure 1C shows that in extracts obtained from cells transiently transfected with clones -191/-331 and -297/-331 and cultured without estrogen only very low CAT activity can be detected. Upon estrogen treatment at least 10 (-191/-331) to 50 fold (-297/-331) induced CAT activity can be found. Induced CAT activity in cells containing clones with the inverted fragments did not differ significantly from clones carrying the fragments in the normal orientation (-331/-191; -331/-297). Estrogen inducibility is also maintained in clones containing the inverted ERE and activator (-87/-482; -87/-331; Figure 1B). However, CAT activity produced both in uninduced and induced cells after transfection of the constructs -87/-482 and -87/-331 was consistently lower than that detected in cells transfected with constructs -482/-87 and -331/-87. This result indicates that the strength of the basal activator element is decreased, when the fragments are reversed relative to the start of transcription of the tk promoter. Since in these constructs both the orientation and the distance of the activator element relative to the tk promoter are different, we cannot decide whether the reduction in basal level of expression is a result of this distance and/or orientation variation of the activator element. Regardless of this observation, it is evident that the ERE acts in an orientation independent manner.

The ERE can act over long distances

To examine whether the ERE like other enhancers can act over long distances in either orientation, we increased the distance between the ERE and the tk

Figure 1: The ERE can act in an orientation independent manner.

tk-CAT plasmids containing the indicated vit A2 fragments cloned into pBL-CAT8⁺ or pBL-CAT9⁺ were transfected transiently into MCF-7 cells (DEAE-dextran technique). Protein extracts from control and estrogen treated cultures were prepared and assayed for CAT activity.

(A) The plasmid pBL-CAT8⁺ contains the HSV thymidine kinase (tk) promoter controlling expression of the chloramphenicol acetyltransferase (CAT) gene cloned into the vector pEMBL8⁺ (23) together with the splicing and polyadenylation signals of SV40. The unique Hind III (H) and Bam HI (B) restriction sites within the pUC18 multiple cloning site (m) upstream of the tk promoter are indicated. pBL-CAT9⁺ is identical to pBL-CAT8⁺ except for the multiple cloning site derived from pUC9. The restriction sites Bgl II (Bg), Sma I (S) and Cla I (C) are given.

(B) and (C) Autoradiograms of standard thinlayer chromatographic assays of CAT activity (39) are shown. Each CAT assay was performed with 250 ug protein from control (-) and estrogen treated (+) cells. The lower spot corresponds to nonacetylated substrate. Transfected plasmids contain the vitellogenin A2 5'flanking sequences indicated above the chromatogram cloned as Hind III-Bam HI fragments into pBL-CAT8⁺ and pBL-CAT9⁺ in the natural or inverse orientation, respectively. The schemes below indicate the positions of the ERE and activator (A) in the respective plasmids relative to the tk promoter.

promoter by the insertion of different phage λ DNA fragments into the unique Bam HI and Hind III sites of clones (-331/-87)8⁺ and (-87/-331)9⁺, respectively. As summarized in Figure 2, all clones containing an ERE in the normal orientation at distances up to 2.6 kb upstream of the tk promoter are 13 to 79 fold estrogen inducible. Even when inverted, the ERE at a distance of 2.3 kb (clone (-87/-331) λ 2322.9⁺) confers 36 fold estrogen induction to the tk promoter. The different basal activities of the constructs presented in Figure 2 as well as their varying degrees in estrogen inducibility could reflect distance and / or orientation dependence of the activator element and also influences of the inserted λ DNA. Control clones containing the large

fragments only ($\lambda 2392.8^+$; $\lambda 2322.9^+$) showed a 3 to 4 fold induction of CAT activity comparable to those observed with the tk-CAT construct (pBL-CAT8⁺, pBL-CAT9⁺). These increases are non-specific and most probably arose as a result of the mitogenic effect of 17 β -estradiol on MCF-7 cells.

The ERE controls transcript initiation at the tk promoter

To prove that the hormonal effect observed in CAT assays is reflected at the mRNA level, total RNA was prepared from transiently transfected MCF-7

		CAT activity		Induction Factor
		-E ₂	+E ₂
pBL-CAT8 ⁺	0.04	0.12	3.0
	(-331/-87)8 ⁺	9.6	145.9	15.2
	(-331/-87) $\lambda 651.8^+$	0.1	7.9	79.0
	(-331/-87) $\lambda 2392.8^+$	2.8	36.6	13.1
	$\lambda 2392.8^+$	0.05	0.13	2.6
pBL-CAT9 ⁺	0.67	1.94	2.9
	(-87/-331)9 ⁺	1.3	104.5	80.4
	(-87/-331) $\lambda 564.9^+$	2.6	51.9	20.0
	(-87/-331) $\lambda 2322.9^+$	1.0	35.8	35.8
	$\lambda 2322.9^+$	0.33	1.31	4.0

Figure 2: The ERE acts over long distances

Plasmids were transiently transfected into MCF-7 cells by CaPO₄/DNA coprecipitation and kept either without (-E₂) or with estrogen (+E₂). Depending on the activity of the extracts, 10 to 500 ug of protein were added to the CAT enzyme assay to obtain less than 50 percent conversion of substrate in order to be in the linear range of the CAT assay. Radioactive spots from the thin layer chromatogram were excised and counted. CAT activity is expressed as percentage chloramphenicol acetylated per 500 ug of protein per 2 hr incubation.

The structure of the recombinant plasmids is given schematically. The arrow represents vitellogenin A2 sequences (-331/-87) and thin lines represent λ DNA. To construct (-331/-87) $\lambda 651.8^+$ and (-331/-87) $\lambda 2392.8^+$, plasmid (-331/-87)8⁺ (13) was linearized by Bam HI digestion, treated with alkaline phosphatase and ligated with the 651 bp and 2392 bp Bgl II fragments of the phage λ DNA, respectively. Clone $\lambda 2392.8^+$ was obtained by ligating the 2392 bp Bgl II restriction fragment with Bam HI digested pBL-CAT8⁺. (-87/-331) $\lambda 564.9^+$ and (-87/-331) $\lambda 2322.9^+$ were derived from (-87/-331)9⁺ by inserting either a 564 bp or 2322 bp Hind III fragment of λ DNA into the unique Hind III site upstream of the tk promoter. $\lambda 2322.9^+$ is a control clone containing the 2322 bp Hind III fragment only. In both control clones ($\lambda 2392.8^+$ and $\lambda 2322.9^+$) the orientation of the respective fragment is the same as in the corresponding ERE containing plasmids. Abbreviations: Hind III, H; Bam HI, B.

cultures 16 hours after the addition of estrogen and assayed by an RNase protection assay (28,29). Using a labeled antisense RNA covering the 298 bp Eco RI/Pvu II fragment of pTk-CAT (7) a protected mRNA of 210 nucleotides is expected for transcripts correctly initiated at the tk cap site. As shown in Figure 3, in the absence of vit sequences (pBL-CAT⁸⁺) no transcripts starting at the tk cap site can be detected (lanes 1 and 2). When the vit 5'flanking fragment from position -331 to -87 is present in its normal orientation (clone (-331/-87)⁸⁺), a basal level of correctly initiated transcripts is detectable which is strongly increased upon estrogen treatment (compare lanes 3 and 4). When the vit sequences are inverted (clone (-87/-331)⁹⁺) basal transcription is reduced to about 20 percent, but high inducibility is maintained (lanes 5 and 6). These results prove that the estrogen effects observed at the CAT activity level reflect changes of CAT mRNA concentrations. In general, the read through transcripts (298-signal) are also stimulated by estrogen. This is expected, since the ERE acts as an enhancer over long distances (see preceeding paragraph) and has been documented in other hormone regulated systems (11). The mRNA analysis also confirms the hormone independent transcriptional activation by the activator, and that the effect is reduced when the fragment is reversed (compare lanes 3 and 5). More importantly, only 35 bp from position -331 to -297 are sufficient for high estrogen inducibility (lanes 7 and 8, Figure 3). Even when separated by about 2.5 kb of λ DNA from the tk promoter the hormonal

Figure 3: The ERE activates transcripts initiated at the tk promoter

20 ug of total RNA prepared from transiently transfected MCF-7 cultures was analyzed by a ribonuclease protection assay. As a probe we used a labeled RNA synthesized in vitro by SP6 polymerase complementary to the 298 bp Eco RI/Pvu II fragment of plasmid pTK-CAT. The tk-CAT sequences in plasmid pTK-CAT (7) are identical to the tk-CAT sequences in our constructs. Cells were transfected with the plasmids indicated and incubated for 16 h without (-) or with 10^{-7} M estrogen (+). The construction of the various plasmids is explained in the legends of Figure 2 and 4. Lane M shows labeled Hae III restricted pBR322 size markers. The lane marked tRNA shows the products of a control hybridization with 20 ug tRNA from E.coli. On the autoradiogram correctly initiated tk-CAT transcripts (210 nt) and read through transcripts (298 nt) are marked by arrow heads.

effect mediated by the ERE both in the normal and reverse orientation (lanes 9-12, Figure 3) is still detectable at the mRNA level, although induction factors are lower than those observed in the CAT assay (Figure 2). Taken together these data prove that the ERE is a cis-acting regulatory element, that is able to act on initiation of transcription at a heterologous promoter in an orientation and relatively distance independent manner.

A thirteen basepair element is sufficient for estrogen regulation

We analyzed the 5'flanking regions of other genes containing a palindromic sequence related to the one found in the vit A2 gene to determine whether they are also capable of conferring estrogen regulation to an indicator gene in transfection experiments. We inserted in vitro synthesized oligonucleotides corresponding to sequences 5'upstream of the start of transcription of *Xenopus* vit A1 (positions -335/-317) and chicken vit II (-625/-591) genes into the vector pBL-CAT8⁺. As shown in Figure 4, both regions contain elements identical to the vit A2 palindrome except for one or two nucleotide changes in the central positions. Determination of the estrogen inducibility of these constructs after transfection into MCF-7 cells revealed that both oligonucleotides confer significant estrogen regulation to the tk-CAT hybrid gene. Clone A1(-335/-317) mediates 10 to 16 fold estrogen induction of CAT activity, whereas with ch(-625/-591) even higher induction factors (25 to 57 fold) were obtained (Figure 4).

Strong homology between the functional vit A2, A1 and ch II sequences is restricted to the palindromic elements. This suggests that the palindrome itself might be sufficient for estrogen control. We therefore synthesized oligonucleotides containing only the 13 bp palindrome of vit A2 and placed them upstream of the tk promoter. To avoid an unfavourable spacing of regulatory elements i.e. the ERE and tk promoter elements, we have positioned the 5'end of the palindrome exactly as it is in the shortest vit A2 deletion construct (-331/-313, Figure 4) that showed substantial estrogen inducibility in transfection experiments. As shown in Figure 4, the 13 bp palindrome of vit A2 (ERE wt) confers about 20 fold estrogen induction to the tk-CAT gene. RNA analysis by SP6 mapping also showed (Figure 3, lanes 13 and 14) that estrogen treatment of MCF-7 cells transfected with ERE wt increases the amount of correctly initiated tk-CAT transcripts 5 to 10 fold. These results demonstrate that the palindrome 5'-GGTCACAGTGACC-3' devoid of its natural flanking regions is sufficient to confer estrogen induction of transcription.

Single base substitutions within the ERE destroy its function

To examine how deviations from the ERE consensus sequence influence its functional properties, we introduced point mutations into both stems of the vit A2 palindrome (positions -331 to -319) by in vitro mutagenesis. Since

the most 5' nucleotide of the palindrome is not conserved in other potential ERE elements identified in the vit B1 and B2 genes by sequence comparison (22) we changed that position (-331) either to an A or a C (ERE mt2 and mt3, respectively; Figure 4). Furthermore we prepared an ERE element, in which position -321 (A) was substituted by a G residue (ERE mt1; Figure 4). As summarized in Figure 4, none of the ERE mutants showed induction factors significantly above the values obtained with the vector lacking an ERE

		Induction Factor				
		MCF-7			Le33	Le42
pBL-CAT8+		3.3	1.8	3.0	1.2	1.9
A2(-331/-297)	gGGTTACAGTGAATGTAACCTC	63.2			12.3	28.1
A2(-331/-313)	gGGTTACAGTGAATGATCA	32.4	16.8	16.5		
A1(-335/-317)	gGGTTACAAATCAACA	12.6	16.0	9.7		
ch(-625/-591)	gGGTTACGCGGAGCTGAAAGAACACATTGATCC	57.3	34.4	25.3	8.4	22.0
ERE wt	gGGTTACAGTGAATgctagA	17.4	13.6	25.2	6.6	6.8
ERE mt 1	gGGTTACAGTgGgctagA	2.5	1.9	2.4	2.5	4.0
ERE mt 2	gGGTTACAGTGAATgctagA	2.9	2.1	2.6	2.5	1.7
ERE mt 3	gGGTTACAGTGAATgctagA	1.4	1.4	1.4	1.4	1.3
consensus	GGTTANNNTGAAT					

Figure 4: A 13 bp palindrome is sufficient to confer estrogen regulation to the tk promoter.

Transient transfection of MCF-7 cells with the indicated constructs was carried out as described in the legend of Figure 2. Estrogen-responsive Ltk⁻ clones Le33 and Le42 were transfected according to Drüge et al. (27). The CAT activity and induction factors were calculated from CAT assays as described in Figure 2. The results of three independent MCF-7 transfections are shown. The sequences of the upper strands of the synthetic oligo-nucleotides of the regulatory elements present in different tk-CAT constructs are given. All elements were cloned with a Hind III restriction site (AAGCTT) on the left and a Bam HI site (GGATCC) on the right into pBL-CAT8⁺. Sequences have been aligned to show maximal homology of the palindromic element. The positions of the homologous sequences relative to the natural cap sites of the respective genes are indicated in brackets and refer to published sequences (22,40,41). Below the sequence of the oligonucleotides representing the wildtype (wt) ERE and ERE mutants (mt) is given. Nucleotides of the ERE constructs which are homologous to the vitellogenin A2 gene (see clone A2 -331/-297) are represented by capitals. Nucleotides represented by white letters on a black background are all sequences that share homology to the conserved stems of the palindromic consensus. The derived consensus sequence is indicated. A2 and A1 refer to the Xenopus A2 and A1 vitellogenin genes, whereas ch stands for the chicken vitellogenin II gene.

element (pBL-CAT8⁺) when analyzed in MCF-7 cells. This indicates that the integrity of both stems of the palindrome is important for its function. We observed similar results (Figure 4) by transient transfection of these ERE mutants into two different mouse Ltk⁻ clones (Le33 and Le42) which have acquired estrogen-responsiveness by stable transfection of an estrogen receptor expression vector. (27). Compared to the vector pBL-CAT8⁺, ERE mt3 in most experiments is even inhibited by estrogen treatment. We assume, that the induction of CAT activity of clone pBL-CAT8⁺ is an unspecific effect, since 2 to 3 fold stimulation of CAT activity is observed with most CAT plasmids tested under our transfection conditions.

The estrogen receptor is the trans-acting factor that binds preferentially to the ERE

To analyze whether estrogen receptor binds in vitro to the functionally defined ERE, nuclear extracts of estrogen-responsive MCF-7 cells were labeled with ³H -17 β -estradiol and used in a DNA cellulose competition assay (31). In this assay the ability of soluble DNA to compete with DNA immobilized to cellulose for binding the receptor is determined. (Fig.5).

In Table I a summary of the estrogen receptor binding affinities of various vit A2-tk-CAT constructs including the point mutants described above is presented. All inducible constructs show 5 to 11 fold higher binding

Figure 5: DNA cellulose competition experiments with vitellogenin -tk-CAT constructs reveal preferential estrogen receptor binding.

Competition experiments were carried out as described in materials and methods. The data are given as half reciprocal plots of percent bound receptor against amount of competitor DNA. Percent bound represents the fraction of ³H -17 β -estradiol-receptor-complex bound to DNA cellulose in the presence of competing DNA compared to the total bound receptor without competitor DNA. Constructs (-331/-87)8⁺ and (-324/-87)8⁺ contain the indicated vitellogenin A2 sequences cloned into the vector pBL-CAT8⁺.

affinities for estrogen receptor than the plasmid pBL-CAT8⁺ or salmon testes DNA. Non-inducible constructs lacking either half (-324/-87) or the entire ERE (-317/-87); -260/-87) have low relative binding affinities of 1.4 to 2.2. Similar low values (1.6 to 3.4) are obtained with the constructs containing single base substitutions within the ERE (ERE mt1, 2 and 3) that destroys its function. Plasmid pTKCAT5A (11) which contains MMTV long terminal repeat

Table I: The estrogen receptor binds preferentially to the ERE

Construct	Relative binding affinity			Estrogen inducibility
	Exp. 1	Exp. 2	Exp. 3	
pBL-CAT8 ⁺	1.0	1.0	1.0	-
-821/-87	11.1			+
-482/-87	10.4	8.3		+
-356/-87	7.5			+
-331/-87	8.1			+
-331/-314	8.1			+
-324/-87	2.2	1.8		-
-317/-87	1.6			-
-260/-87		1.4		-
ERE wt	8.0		5.1 4.8	+
ERE mt 1	1.9		1.6 2.1	-
ERE mt 2			2.2 3.4	-
ERE mt 3			2.1 1.7	-
pTKCAT5A		1.5		-
Salmon DNA	1.1			

Estrogen receptor binding to linearized plasmid DNA was measured by the DNA cellulose competition assay as described in material and methods. From half reciprocal plots of percent bound receptor against amount of competitor DNA (see Figure 5) the amount of DNA needed for 50 percent competition was determined. All plasmids were compared to the vector pBL-CAT8⁺ whose relative binding affinity was defined as 1.0. In the three experiments shown different batches of nuclear extracts have been used. In experiment 3 two different preparations of each plasmid have been compared. The estrogen inducibility of the various constructs as analyzed in transfection experiments (13 and experiments described herein) is indicated. The various vitA2-tk-CAT constructs contain the indicated 5'flanking sequences cloned into the vector pBL-CAT8⁺. The ERE constructs are described in the legend of Figure 4. Plasmid pTKCAT5A contains MMTV long terminal repeat sequences (-428/-69) linked to the tk promoter (11).

Figure 6: The glucocorticoid receptor does not preferentially bind to the ERE. Glucocorticoid receptor binding to the vector pBL-CAT8⁺, the vit A2-tkCAT construct (-821/-87)⁸⁺ and the MMTV-GRE construct pTKCAT5A (11) was measured by the DNA cellulose competition assay as described in materials and methods.

sequences mediating both glucocorticoid (1,3,4) and progesterone (11) control has a low relative binding affinity for estrogen receptor (1.5). Taken together these results demonstrate, that estrogen receptor is the trans-acting factor, which interacts with the ERE at position -331 of the vit A2 gene.

ERE and GRE are two distinct regulatory elements

To find out whether the ERE is also bound by glucocorticoid receptor, we carried out a DNA cellulose competitive assay with labeled glucocorticoid receptor derived from rat liver cytosol. In this assay the vector pBL-CAT8⁺

Table II: The ERE does not mediate glucocorticoid control.

Construct	CAT activity			
	XC		MCF-7	
	- hormone	+ dex	- hormone	+ estrogen
pBL-CAT8+	0.9	0.8	0.1	0.1
-821/-87	0.6	0.7	0.3	5.9
-331/-87	0.7	0.6	0.3	12.2
-324/-87	0.9	0.8	0.6	0.5
pTKCAT5A	0.8	19.7	0.1	0.1

The indicated constructs were transfected transiently into rat XC cells or MCF-7 cells. XC cells were treated with 10⁻⁶M dexamethasone (dex) for 40 h. Estrogen treatment of MCF-7 cells was done as described (13). CAT activity is expressed as percent chloramphenicol acetylated per 100 ug protein under standard conditions.

or the estrogen-inducible vit A2-tk-CAT construct -821/-87 compete very inefficiently for binding the glucocorticoid receptor (Figure 6). In contrast, the plasmid pTKCAT5A containing the glucocorticoid response region of MMTV binds glucocorticoid receptor with high affinity, (Figure 6) but does not preferentially bind estrogen receptor (Table I).

To examine whether the specificity of steroid hormone receptor binding found in the in vitro binding assay is also reflected in functional assays, we transfected a number of vit A2-tk-CAT constructs into glucocorticoid-responsive rat XC cells (11). As shown in Table II, both the ERE containing constructs (-821/-87; -331/-87) and the construct lacking half of the ERE (-324/-87) do not respond to the synthetic glucocorticoid dexamethasone, whereas the MMTV-construct (pTKCAT5A), as expected, is clearly inducible (22 fold). On the contrary, in estrogen-responsive MCF-7 cells transfected with the MMTV-construct (pTKCAT5A) estrogen treatment does not increase CAT activity (Table II). Vit A2-tk-CAT clones containing a complete ERE show 20 to 40 fold estrogen induced CAT activity in the same experiment. In summary, these data clearly show that the estrogen receptor does not interact with the GREs of MMTV and similarly glucocorticoid receptor does not interact with the ERE of vitellogenin A2.

DISCUSSION

Our functional analysis demonstrates that 13 bp of the 5'flanking region of the vit A2 gene promoter separated from their natural flanking sequences are sufficient for estrogen control (Figure 4). The ERE of vit A2 is the palindrome GGTCACAGTGACC. This functionally defined ERE fits the consensus sequence derived by sequence comparison of regions preceding the transcription initiation site of estrogen regulated vitellogenin and apo-VLDLII genes (22). The EREs, which we have proven to be responsible for estrogen regulation of the *Xenopus* vit A1 and the chicken vit II genes, differ from the ERE of vit A2 only within the three central positions of the 13 bp palindrome (Figure 4). Therefore, by sequence comparison of the functional EREs of *Xenopus* vit A1, vit A2 and the chicken vit II, the consensus GGTCANNNTGACC can be derived. Most likely, any nucleotide can stand at the three central positions of the palindrome. We assume that the consensus represents the minimal sequence that constitutes a functional ERE. Our analysis of the three different ERE mutants (Figure 4) proves directly that both the first and eleventh position of the ERE are essential for its function.

Constructs in which the EREs are flanked by their natural sequences (A2 -331/-297; ch -625/-591; Figure 4) seem to be 2 to 3 fold higher inducible

than the plasmid containing only the ERE (ERE wt). This suggests that flanking sequences may modulate the regulatory potential of the ERE. However, a functional role for the conserved heptamer 'elegans box', which partially overlaps the ERE element of vit A2 (13), can be excluded since point mutations within this box do not significantly change the inducibility or basal expression of the relevant constructs (data not shown). We are aware that the extent of estrogen inducibility could be altered by minor changes due to insertion or deletion of sequences between the ERE and tk promoter. In fact, we observed that deletion of the 6 bp spacer between the ERE and the Bam HI restriction site at the border of the tk promoter in the construct ERE wt (Figure 4) results in a 2 to 3 fold reduction of estrogen induction of CAT activity (data not shown). The decreased inducibility could be the result of an unfavourable stereospecific alignment of the ERE and the tk promoter. Alternatively, SP1 factor binding to the distal SP1 element of the tk promoter (35), which in the case of the ERE wt construct is separated from the ERE by only 9 bp, could interfere with estrogen receptor binding.

Using essentially our approach and basic constructs, Wahli's group (14,15) showed that fragments of the *Xenopus* vit B1 and B2 5'flanking regions are able to confer estrogen-responsiveness to the tk-CAT hybrid gene. All five potential ERE copies contained in these genes differ in at least one position from the consensus ERE, e.g. the distal element of vit B1 at -555 corresponds to an inverted ERE mt1 copy, whereas the central (-334) and proximal (-314) copies correspond to the ERE mt2 type. By progressive 5' and 3' deletion analyses it could be demonstrated that fragments of the 5' flanking region of the start of transcription of vit B1 containing either the distal or the proximal element are not able to mediate estrogen regulation (14). The central copy by itself has not been tested. Our analysis proves, that not only the -555 and -314 copies but also the central copy (-334) of vit B1 as well as the two copies of the vit B2 gene are by themselves not sufficient for estrogen inducibility in our assay. Interestingly, estrogen control may be observed with constructs containing two of these imperfect ERE copies (14,15). We therefore speculate that estrogen regulation of the vit B1 and B2 genes is achieved by synergism of two imperfect ERE elements. In fact we have recently confirmed this assumption (42).

By a DNA cellulose competition assay we have demonstrated that the 13 bp ERE is sufficient for high affinity binding of the estrogen receptor (Table I). Generally, we observe a correlation between estrogen inducibility and a high estrogen receptor binding affinity (Table I). Since point mutations within either stem of the palindrome decrease both the binding affinity for estrogen receptor and function, we assume that interaction of the receptor(s)

with both stems of the ERE is required to form a functional ERE-estrogen receptor complex.

Jost et al. (36) have reported that partially purified estrogen receptor from chicken oviduct binds preferentially to a restriction fragment (C2) of the region preceding the chicken vit II gene. This report is in agreement with our binding data since fragment C2 contains a consensus ERE (position -625 to -613) which we have proven to show a high affinity for the estrogen receptor (Table I). Furthermore, we have demonstrated in transfection experiments, that a short region of fragment C2 that contains the ERE at position -625 confers estrogen inducibility to the tk-CAT hybrid gene (Figure 4, ch -625/-591). By DNaseI protection experiments Jost et al. (36) localized a putative estrogen receptor binding site at position -620 to -597 of the chicken vit II gene. This site does not include the left stem of the palindromic ERE but only the central positions and the right stem. Since one half of the palindrome as in deletion clone -324/-87 is neither sufficient for estrogen inducibility (Table II) nor shows high affinity binding of estrogen receptor (Table I) we would argue against the finding of Jost et al. (36) that it is the estrogen receptor that generates the footprint in their analysis. Our preliminary footprinting experiments with a fragment from the region upstream of the vit A2 gene show that after addition of MCF-7 nuclear extract all nucleotides of the ERE are protected against DNase I digestion.

Electron microscopy has revealed a protein-DNA complex at the ERE of the B2 vitellogenin gene (15). However, in this case it is not evident whether the estrogen receptor is part of the complex. With our approach we are confident, that the receptor labeled by the radioactive hormone interacts with the ERE. Since all our estrogen receptor preparations contain other proteins, we can not prove that the estrogen receptor binds directly to the palindrome or through an interactin with another protein that recognizes this palindrome. Whatever is the ultimate mechanism of binding to DNA by the estrogen receptor, our results demonstrate clearly that the intact ERE is required for this process.

By the DNA cellulose competition assay we cannot detect specific binding of glucocorticoid receptor to the ERE (Figure 6). Furthermore, various tk-CAT constructs containing an ERE when transfected into glucocorticoid-responsive rat XC fibroblasts do not respond to glucocorticoid hormone (Table II). Vice versa, a GRE containing construct (pTKCAT5A) does not bind estrogen receptor with significant affinity (Table I), and expression of this construct when transfected into estrogen-responsive MCF-7 cells cannot be induced by estrogen treatment. Taken together these data demonstrate that the ERE and the GRE are two distinct regulatory elements which interact selectively with

their corresponding steroid hormone receptors.

Sequence analyses of the GREs of a number of glucocorticoid regulated genes (2,8,37) have shown that the GRE like the ERE has a two fold rotational symmetry. However, in the case of the GRE it has not been tested whether the consensus sequence by itself is sufficient to mediate hormonal control. When the recently published 15 bp consensus for GREs (8) GGTACANNNTGTTCT and the functional 13 bp ERE consensus GGTCANNNTGACC are aligned at their center of symmetry, it is evident that the ERE differs only at four positions from the GRE. Obviously, the nonhomologous nucleotides contribute to the specificity of the regulatory elements for either glucocorticoid or estrogen receptor which we observed in our receptor binding (Table I and Figure 6) and functional studies (Table II). The finding that ERE and GRE despite their distinct functional specificity share some structural features correlates with the observation that the DNA binding domains of various hormone receptors are partially homologous (reviewed in 38).

After completion of our work Klock et al (43) published similar data.

ACKNOWLEDGMENTS

We acknowledge the gifts of ³H-triamcinolone acetonide labeled rat liver cytosol received from Dr.H.M.Westphal, Marburg, and the SP6-tk-CAT template from Dr.R.Miksicek, Heidelberg. We thank Jutta Weinmann and Gisela Schütz for their excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Ry 5/1-1).

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