Effects of Estradiol-17β Analogues on Activation of Estrogen Response Element Regulated Chloramphenicol Acetyltransferase Expression†

J. A. VanderKuur,‡ M. S. Hafner,‡ J. K. Christman,§ and S. C. Brooks*,‡

Department of Biochemistry, Wayne State University School of Medicine, and The Molecular Biology Program, Michigan Cancer Foundation, Detroit, Michigan 48201

Received September 21, 1992; Revised Manuscript Received April 16, 1993

ABSTRACT: These experiments were designed to examine the effect of structural modifications to the estradiol-17β (E_2) molecule on the estrogen response element (ERE) dependent activation of the thymidine kinase (tk) promoter. Estrogen receptor (ER) positive MCF-7 cells were transfected with plasmids containing one or two vitellogenin EREs inserted upstream of the tk promoter in p(–37)tk. Transient expression of the CAT gene in these constructs was measured after cells had been maintained for 36–42 h in the presence of E_2 or an E_2 analogue. E_2 induced CAT expression at levels as low as 10^{-13} M, with maximum induction at 10^{-11} M. CAT activity decreased at higher concentrations of E_2. Estratriene, which has low affinity for ER, was active only at micromolar concentrations. 3-Hydroxyestratriene displayed maximal activity at 10^{-9} M, with higher levels being less active. Still higher concentrations (10^{-7} M) of estratrien-17β-ol were required to induce maximum CAT activity. All positional and conformational alterations in the D-ring hydroxyl group of E_2 yielded active ligands. Movement of the phenolic hydroxyl group of E_2 to other positions on the A-ring produced dihydroxyestrogens with varied capacities to activate CAT (2-hydroxyestratrien-17β-ol produced maximum CAT activation at 10^{-11} M; 1-hydroxyestratrien-17β-ol required a 10^{-8} M concentration for maximum activity; 4-hydroxyestratrien-17β-ol gave maximum CAT activation at 10^{-6} M). Only those androstanediols or 5-androstenediols with a 3β-hydroxyl group were capable of activating CAT expression. CAT constructs with two consensus EREs placed 6 or 19 bp apart were equally active and displayed a response to E_2 or to the estrogen analogues similar to that of plasmids with a single consensus ERE. The concentrations of structurally modified estrogens which generated maximum CAT responses from these constructs were directly related to their affinity for ER. This contrasts with results obtained with the more complex regulatory regions of endogenous E_2-responsive genes in MCF-7 cells. It is suggested that transcriptional activation following the binding of ER–ligand complexes to the ERE can be modulated by interactions with factors bound to other cis regulatory elements. Such protein–protein interactions appear to be influenced by the structure of the ligand.

It has been demonstrated in this laboratory that discrete alterations in the structure of the E_2[1] ligand can either diminish the effect of the ER complex on gene activity or lead to differential stimulation of specific genes (VanderKuur et al., 1993; Pilat et al., 1993). This observation is consistent with the hypothesis that structural changes in estrogen modify the ligand–receptor complex in a manner which either influences its interaction with different EREs or affects the receptor interaction with other proteins required for activation of specific promoters. In this investigation, we have examined the effect of structural alterations in estrogen on transient CAT expression in MCF-7 cells transfected with a CAT plasmid with EREcs or one EREc and a mutated ERE upstream of a tk promoter.

The 13-bp palindrome ERE (GGTCACAGTGACC) of the *Xenopus laevis* vitellogenin gene was first described by Klein-Hitpass et al. (1986, 1988a,b). This nucleotide sequence, now referred to as the EREc, is sufficient to confer estrogen-dependent CAT induction on an ERE-tk-CAT expression vector transfected into MCF-7 cells (Klein-Hitpass et al., 1988a,b). EREc was shown to activate transcription in an orientation- and distance-independent manner (Klein-Hitpass et al., 1988a). Single EREs carrying point mutations were nonfunctional but could act synergistically to give an E_2 response when inserted as dimers separated by up to 18 bp (Klein-Hitpass et al., 1988b; Martinez & Wahli, 1989).

p(–37)tk was derived from pBLCAT2 (Luckow & Schutz, 1987) and contains 37 bp of the region immediately upstream of the start for transcription of the herpes simplex virus thymidine kinase gene (McKnight & Kingsbury, 1982). This minimal promoter contains the binding site for RNA polymerase II (TATA homology) with the distal promoter elements deleted (Strahle et al., 1988; Kaling et al., 1990; Wahli et al., 1989). A single ERE directly upstream of a TATA box does not require additional promoter elements to stimulate gene transcription in response to hormone. However, hormone response elements at a greater distance (several hundred nucleotides) from the promoter may require binding sites for additional transcription factors in order to activate transcription (Strahle et al., 1988, 1989; Schule et al., 1988).

† These investigations were supported in part by NIH Grant CA44771, the Lloyd and Marilyn Smith Fund, and institutional grants to the Michigan Cancer Foundation from the United Foundation of Greater Detroit.

* To whom correspondence should be addressed at the Department of Biochemistry, Wayne State University School of Medicine, 540 Canfield, Detroit, MI 48201.

‡ Wayne State University School of Medicine.

§ The Michigan Cancer Foundation.

[1] Abbreviations: E_2, estradiol-17β; ER, estrogen receptor; ERE, estrogen response element; EREc, concensus (vitellogenin) estrogen response element; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; PgR, progesterone receptor; GR, glucocorticoid receptor; GRE, glucocorticoid response element; TAF-2, transactivation function 2.

0006-2960/93/0432-7016$04.00/0

Table I: Nucleotide Sequences of JA12, JA1, and JV2 between the *Xba*I and *Bam*HI Restriction Sites[a]

JA12	5′-TCTAGAGGATCCAGGTCACAGTGACCTGGGCCCGGATCCGGGCCCAGGTCACAGTG/CCTGGCCC-3′
JA1	5′-TCTAGAAGGTCACAGTGACCTGTCTAGAGGATCCGGGCCCAGGTCACTGTGACCTGGATCC-3′
JV2	5′-TCTAGAGGTCACTGTGACCTAAGCTTAGGTCACAGTGACCTGATCC-3′

[a] The EREcs are underlined in each plasmid. EREcs were cloned into adjacent restriction sites, *Xba*I and/or *Bam*HI; both are part of the multicloning site. The remaining sequences are identical in the three plasmids. See Materials and Methods for construction of plasmids.

A number of investigators have examined the 5′-region of estrogen-regulated genes via CAT constructs. Cavailles et al. (1991) have studied the 5′-region of the cathepsin D gene in MCF-7 cells, utilizing constructs that included chimeric reporter genes with different fragments of the cathepsin D promoter region. They found a 240-bp sequence that activated transcription in response to the ER complex. Others (Anderson & Lingrel, 1989) employed the 5′-region of the rat T-kininogen gene in experiments with both dexamethasone and E_2. It was found that the hormonal stimulation of CAT response was determined by a 67-bp region that contained a GRE, an ERE, and a CACCC sequence. Finally, imperfectly palindromic EREs have been demonstrated to be inactive, or weakly active, with AP-1 transcription factors in fos–tk–CAT constructs (Weisz & Rosales, 1990) and in the β-globin promoter (Berry et al., 1989). Nevertheless, an imperfect ERE functioned well with the pS2 gene (Nunez et al., 1989).

MATERIALS AND METHODS

Steroids. Estratriene, estrone, E_2, estradiol-16α, and estradiol-17α were purchased from Research Plus, Inc. (Bayonne, NJ). Estratrien-17β-ol, 3-hydroxyestratriene, and 1-, 2-, and 4-hydroxyestratrien-17β-ol were synthesized according to published procedures (Horwitz et al., 1986; Dannenberg & Kohler, 1964; Palomino et al., 1990). All estrogens were purified by thin-layer chromatography and recrystallized to guarantee homogeneity. Each estrogen analogue was examined for contaminating estrogens, which were determined not to be present at a level greater than 1 part in 10^4 (VanderKuur et al., 1993).

5-Androstene-3β,17β-diol, 5α-androstane-3β,17β-diol, and 5α-androstane-3α,17β-diol were purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified as described by VanderKuur et al. (1993).

Construction of Plasmids. Cloning of the plasmids was performed following standard procedures as described in Maniatis et al. (1982). An oligonucleotide containing the EREc and a unique restriction site, *Apa*I, was obtained from Bio-Synthesis Inc. (Denton, TX) and cloned into the *Bam*HI site of the plasmids p(−37)tk and p(−37)tkERE (a gift from G. Schutz), yielding the two constructs JA12 and JA1, respectively (see Table I for sequences). JA12 contains a 25-bp insert with a mutated ERE between the TATA box and the EREc–*Apa*I oligonucleotide sequence in the *Bam*HI site of p(−37)tk. A third construct (JV2) consists of a 40-bp oligonucleotide containing two EREcs separated by 6 bases and flanked by the *Xba*I and *Bam*HI sites replacing the 12-bp *Xba*I-*Bam*HI fragment of p(−37)tk (see Table I for sequence). p(−37)tk was obtained by replacing the 169-bp *Bam*HI/*Bgl*II fragment of pBLCAT2 (Luckow & Schutz, 1987) with the *Bam*HI/*Bgl*II fragment of the tk linker mutant LS-47/−37 (McKnight & Kingsbury, 1982). The number and orientation of EREcs in each construct was verified by direct sequencing using the Sequenase version 2.0 kit from United States Biochemical Corp. (Cleveland, OH).

Cell Culture and Transfection. ER-positive MCF-7 cells were grown in closed T_{75} and T_{25} flasks in Eagle's minimal essential medium with Hanks' balanced salt solution, 2.5 mM L-glutamine, and 25 mM HEPES, supplemented with nonessential amino acids, 5 μg/mL genatmicin, and 5% donor calf serum. For maintenance, flasks were seeded with 1×10^6 (T_{25}) or 2×10^6 (T_{75}) cells, and the cells were grown to confluence before subsequent passage. For experiments, cells were plated at 500 000 cells per 60-mm dish 18 h prior to transfection, and incubated in a 5% CO_2 atmosphere. The cells were transfected using the strontium phosphate procedure described by Brash et al. (1987) modified by incubating the cells with 10 μg of plasmid DNA per dish for 2 h, followed by a 4-min incubation with 20% glycerol. The cells were treated with the various estrogens (dissolved in 5-50 μL of ethanol per 50 mL of media) for 36-42 h.

CAT Assay. Cells were lysed with four freezing and thawing cycles in 0.25 M Tris-HCl, pH 7.8. CAT assays were carried out as described by Gorman et al. (1982) with reaction mixtures containing 100–150 μg of supernatant protein and 0.1 μCi of ^{14}C-chloramphenicol (40–60 mCi/mmol; ICN Radiochemicals, Irvine, CA). The assays were incubated for 2 h at 37 °C. Acetylated chloramphenicol was separated by thin-layer chromatography, visualized by autoradiography, and then excised and quantitated by liquid scintillation counting. Protein concentrations were determined by utilizing the Pierce BSA assay kit (Rockford, IL). The pCH110 plasmid containing a functional *lacZ* gene (Pharmacia, Piscataway, NJ) was cotransfected in some experimentals to monitor transfection efficiency and assayed according to the manufacturer's protocol. The β-galactosidase standard and *o*-nitrophenyl β-galactoside were purchased from Boehringer Mannheim (Indianapolis, IN). Reactions were allowed to proceed overnight.

RESULTS

The response to E_2 of each of the dimer ERE CAT constructs used in this study was compared with the response of p(−37)tkERE and p(−37)tk. Maximum induction of CAT expression by each of the ERE-containing constructs was obtained at 10^{-11}M E_2. In a typical CAT assay, 1 mg of extract protein from MCF-7 cells transfected with p(−37)tkERE and cultured in the presence of 10^{-11}M E_2 acetylated 1.3% of input chloramphenicol in 2 h. Under the same conditions JA12, JA1, and JV2 converted ~24% of input chloramphenicol. Thus, all of the dimer constructs were ~19-fold more responsive to E_2 than p(−37)tkERE. This indicates that interaction of ligand–ER complexes at EREc and the mutated ERE in JA12 allowed a synergistic response to E_2 equivalent to that seen with two EREcs.

Levels of E_2 as low as 10^{-13}M stimulated CAT expression by JA12 (17%; data not shown), with maximum induction at 10^{-11}M (Figure 1A). Higher concentrations of E_2 were less efficient in CAT induction. Cotransfection with pCH110, which expresses β-galactosidase activity at a constant level regardless of E_2 concentration, confirmed that these results were not due to differences in transfection efficiency or cell viability (data not shown). In the presence of 10^{-11}M E_2, CAT expression from JA12 was 43-fold greater than that

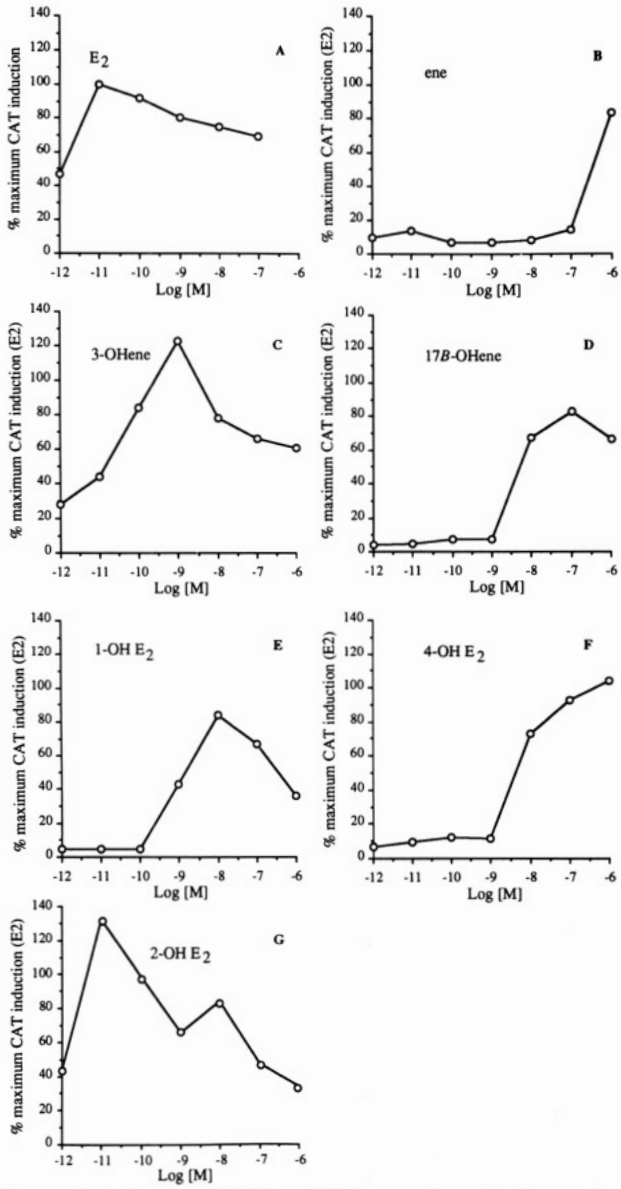

FIGURE 1: Induction of CAT activity in transfected MCF-7 cells by various estrogens. Cells were transfected with the JA12 plasmid and exposed to estrogens for 40 h as described in Materials and Methods. Shown are the effects of a concentration range (10^{-12}–10^{-6}M) of E_2 and each analogue. At each level of estrogen, CAT activity is plotted as the percent of maximum E_2 induction (10^{-11}M E_2, the positive control dish in each experiment). Estrogens examined: (A) E_2, (B) estratriene (ene), (C) 3-hydroxyestratriene (3-OHene), (D) estratrien-17β-ol (17β-OHene), (E) 1-hydroxyestratrien-17β-ol (1-OH E_2), (F) 4-hydroxyestratrien-17β-ol (4-OH E_2), and (G) 2-hydroxyestratrien-17β-ol (2-OH E_2).

from (–37)tk, which lacks an EREc. The two hydroxyl groups of the E_2 molecule makes a significant contribution to the affinity of this hormone for receptor (Brooks et al., 1987). Estratriene, which lacks hydroxyl groups, was ineffective in induction of the reporter gene except at a micromolar concentration (Figure 1B). Placement of one hydroxyl group on estratriene at either of the two hydroxyl positions in the E_2 molecule (3-phenolic or 17β) restored the ligand's capacity to stimulate CAT expression at the lower concentrations (Figures 1C,D and 2).

Position of the A-ring hydroxyl group was not important to the creation of dihydroxyestrogens with CAT induction competence. 1-, 2-, and 4-hydroxyestratrien-17β-ol each induced levels of CAT activity comparable to those obtained with E_2, although higher levels of the analogues with lower

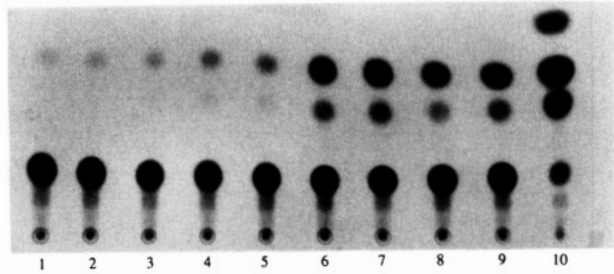

FIGURE 2: Autoradiogram showing CAT activity in MCF-7 cells transfected with the plasmid JA12 and treated for 40 h with estratrien-17β-ol over a concentration range of 10^{-12}–10^{-6}M: lane 1, minus E_2 (control); lane 2, 10^{-12}M; lane 3, 10^{-11}M; lane 4, 10^{-10}M; lane 5, 10^{-9}M; lane 6, 10^{-8}M; lane 7, 10^{-7}M; lane 8, 10^{-6}M; lane 9, 10^{-10}M E_2; lane 10, hormone-independent plasmid containing the Rous sarcoma virus (RSV) promoter with the CAT gene.

Table II: Induction of CAT Expression in MCF-7 Cells Transfected with JA12[a]

compd	concn[b] (M)	EC50[c] (10^{-9} M)
estradiol-17α	10^{-10}	0.0061
estradiol-16α	10^{-9}	0.20
ICI 164384	10^{-6}	1000
4-hydroxytamoxifen	10^{-6}	1000
5-androstene-3β,17β-diol	10^{-7}	10
5α-androstane-3β,17β-diol	10^{-8}	10
5α-androstane-3α,17β-diol	10^{-6}	1000

[a] Each 2-h incubation mixture contained 100–150 μg of protein from cell lysate. [b] Highest concentration examined or concentration which induced maximum activity. [c] EC50 is defined as the effective concentration which produced half-maximal (E_2) response. EC50 was determined according to the formula log [(% maximal response)/[100 – (% maximal response)]. Compounds that do not reach 50% maximum response have been given a very low, arbitrary property value (Cramer et al., 1988). EC50 for E_2 is 1.1×10^{-12} M.

ER affinity were required to maximally stimulate the transfected cells (Figure 1E,F,G). Changes in the orientation and position of the D-ring hydroxyl group had little effect upon CAT induction in relation to the activity of the parent estrogen, E_2 (Table II). 4-Hydroxytamoxifen and the pure antiestrogen ICI 164384 were inactive in this system (Table II).

The estrogenic 5-androstene-3β,17β-diol stimulated CAT expression in transfected MCF-7 cells (Table II). 5α-Androstane-3β,17β-diol was also active. However, the α-orientation of the A-ring hydroxyl (in 5α-androstane-3α,17β-diol) did not elicit CAT expression.

To determine whether ligand structure had the same effect on synergism between a mutated ERE and an EREc and synergism between two ERECs with different spacing relative to each other and the TATA box, the response of JA1 and JV2 to estrogen analogues was compared with that of JA12. The 3′ EREc in JA1 and JV2 is the same distance from the TATA box as the single EREc in p(–37)tkERE. The 3′-mutated ERE in JA12 is separated from the TATA box by one additional base pair. The 5′ ERECs in JA12 and JA1 are separated from the 3′ ERE by 18 and 19 bp, respectively. In JV2, the two ERECs are separated by 6 bp. No significant differences were observed between the response of the three plasmids (Table III). E_2 stimulated CAT activity in cells transfected with plasmids containing one EREc and a mutated ERE (JA12) to a level comparable to that obtained with plasmids containing two ERECs (JV2, JA1). The E_2 concentrations required for maximal activity were also identical. Similarly, all three plasmids responded in an equivalent manner when transfected cells were pulsed with estrogen analogues.

Table III: Induction of CAT Expression in MCF-7 Cells Transfected with JA12, JA1, or JV2[a]

		CAT activity[c]		
		plasmid[d]		
estrogen	concn[b] (M)	JA12	JA1	JV2
E_2	10^{-11}	25	23	24
estratriene	10^{-7}	0	0	0
3-hydroxyestratriene	10^{-9}	18	16	15
estratrien-17β-ol	10^{-7}	11	15	14
1-hydroxyestratrien-17β-ol	10^{-8}	24	20	13
2-hydroxyestratrien-17β-ol	10^{-8}	20	20	15
4-hydroxyestratrien-17β-ol	10^{-7}	16	13	12

[a] Each 2-h incubation mixture contained 100–150 μg of protein from cell lysate. [b] Highest concentration examined or concentration which induced maximum activity. [c] CAT activity expressed as percentage of chloramphenicol acetylated per mg of protein per 2-h incubation with standard conditions. Estrogen negative controls displayed a CAT activity of (3 ● 1)% (n = 7). [d] Plasmids are described in Materials and Methods.

DISCUSSION

Synergism between EREs and the Role of TAF-2 Interactions. These investigations confirm a previous report (Strahle et al., 1988) that E_2 can actively stimulate CAT expression in MCF-7 cells transfected with plasmids containing a single EREc in (–37)tk and demonstrate a synergistic response of equal magnitude in cells transfected with plasmids containing two EREs, regardless of whether both EREs are perfect or one ERE is perfect and the other is mutated.

In a comprehensive study of the effect of distance from the TATA box of a minimal adenovirus-2 major late promoter complex, Ponglikitmongkol et al. (1990) concluded that synergism between tandem EREs was dependent on both their affinity for the ER and their distance from the TATA box. They found an additive stereo-independent effect of tandem EREcs with the proximal ERE placed 65 bp upstream of this minimal promoter and a synergistic stereo-dependent effect of tandem EREcs with the proximal ERE placed 230 bp upstream. In contrast, our results, using a different minimal promoter, indicate a synergistic stereo-independent interaction between tandem EREcs placed with the proximal ERE only 19 or 20 bp from the TATA box. In JV2, the 15-bp EREcs were separated by 6 bp, creating a center-to-center distance of 21 bp, or two complete helix turns, thereby placing the EREcs on the same side of the helix. The two EREcs in JA1 and the EREc and mutant ERE in JA12 were separated by 34 and 33 bp, respectively, which does not give a perfect stereoalignment of EREs. This difference in spacing did not have a significant effect on ERE-dependent activation of the CAT gene in response to E_2 and its analogues. Homosynergism between TAF-2 domains of receptors bound at adjacent elements close to or at a distance from the TATA box does not require stereoalignment, while heterosynergism between TAF-1 and TAF-2 domains on different receptors does (Ponglikitmongkol et al., 1990). This suggests that the synergistic effect of tandem EREs in our constructs may be solely dependent on TAF-2 interactions.

The TAF-2 region of the ER steroid binding domain is believed to play a complex role in transcriptional regulation. Tora et al. (1989) demonstrated that receptors with TAF-2, but lacking TAF-1, acted synergistically in enhancing transcription from a minimal promoter with tandem receptor binding sites. Synergism between TAF-2 and transcription factors bound at upstream elements in more complex promoters has also been demonstrated (Tora et al., 1989; Kumar et al., 1987). Finally, interaction between ER and TFIIB appears

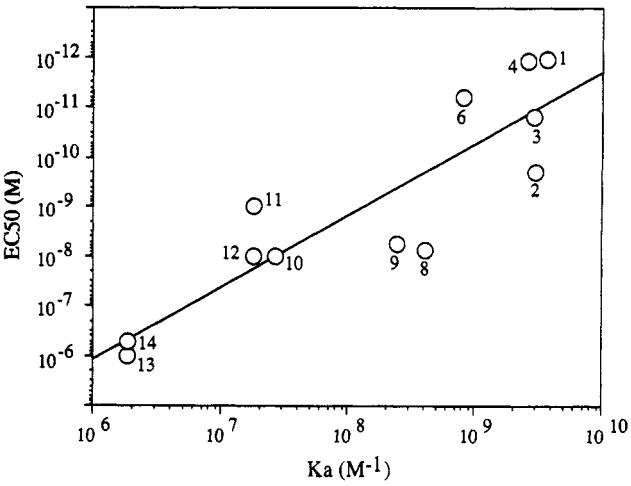

FIGURE 3: Relation of K_a of estrogen analogues to the induction of CAT expression in transfected MCF-7 cells. K_a's were determined by the competitive binding assay (VanderKuur et al., 1993). R^2 of the line determined by the activity of all estrogens is 0.78. Numbers refer to the following steroids: (1) E_2, (2) estradiol-16α, (3) 3-hydroxyestratriene, (4) 2-hydroxyestratrien-17β-ol, (6) estradiol-17α, (8) estratrien-17β-ol, (9) 4-hydroxyestratrien-17β-ol, (10) 5-androstene-3β,17β-diol, (11) 1-hydroxyestratrien-17β-ol, (12) 5α-androstane-3β,17β-diol, (13) 5α-androstane-3α,17β-diol, and (14) estratriene. EC50 is defined in Table II.

to be dependent on the TAF-2 domain (Kumar et al., 1987; Webster et al., 1988). It has recently been proposed that interactions between ER and TFIIB increase the stability of their binding to their cognate cis elements and that this leads to formation of a more stable preinitiation complex to which RNA polymerase II and other general factors can bind to activate high levels of gene transcription (Ing et al., 1992).

Absence of an Effect of Estrogen Structure on the Induction of CAT Activity. In our studies, variations in the presence and position of hydroxyl groups on the E_2 molecule created estrogens with a wide range of capacities to stimulate CAT activity. The observed activation was independent of estrogen structure, since each of the analogues examined in our studies induced maximal CAT expression at a concentration which was related to its affinity for receptor. A plot of the EC50 (the effective concentration which produced half-maximal response) of each estrogen versus the log K_a is shown in Figure 3. When administered at concentrations capable of affecting gene activity, all of the estrogen analogues were accessible to, and bound to, ER within the nuclear fraction of MCF-7 cells (VanderKuur et al., 1993).

On the basis of the mechanism proposed by Ing et al. (1992), these results suggest that, with each of the analogues tested, the limiting factor for transcriptional activation was the formation of a stable nuclear receptor complex, i.e., that once ligand-bound ER was bound to ERE, interaction between the TAF-2 domain of the ER and TFIIB occurred with the same affinity regardless of the ligand. The data also indicate that the alteration in isopotential over the A-ring of estrogen ligands did not interfere with either TAF-2 interactions involved in synergism between ERs bound at adjacent EREs or TAF-2-dependent interactions with TFIIB.

The fact that synergism between EREs occurred in our constructs is consistent with the proposal that a single EREc placed so close to the TATA box cannot mediate optimal stabilization and/or activation of the transcriptional initiation complex. However, it may also indicate a basic difference in interactions between ER and the transcriptional machinery at the minimal tk promoter and the minimal adenovirus-2

late promoter. In this regard, it is of interest that Klein-Hitpass et al. (1986, 1988a) and Martinez et al. (1987) did not find a stereo dependence for synergism of tandem EREcs placed ~90 bp upstream of the TATA box in the tk promoter. However, these constructs differed from both our constructs and those used by Ponglikitmongkol et al. (1990) in that they contained other upstream elements of the tk promoter.

In general, receptor binding is the primary factor in gene regulation by any ligand. However, other experiments carried out in this laboratory (VanderKuur et al., 1993; Pilat et al., 1993) have demonstrated that certain estrogen analogues (2- or 4-hydroxyestratrien-17β-ol) did not promote the induction of certain endogenous genes in MCF-7 cells (pS2, cathepsin D, and PgR) to the extent which would be predicted by the affinity of these analogues for ER. Decreased effectiveness brought about by the unnatural positioning of the A-ring hydroxyl group of these dihydroxyestrogens appears not to be related to alterations in the K_a of their ER complexes, nor were these estrogens prohibited from forming adequate levels of tightly bound nuclear ER (VanderKuur et al., 1993).

It remains to be determined why high-affinity estrogens with A-ring modifications have a diminished capacity for activating certain endogenous genes. Nevertheless, results from experiments reported herein with plasmids that carry only tandem EREs, a promoter devoid of known regulatory elements other than the TATA sequence, and a reporter gene have provided information relevant to understanding the phenomenon of structurally related ligand induction of estrogen-responsiveness genes. The estrogenic compounds examined in these studies possessed a number of steric divergences from the structure of E_2. Regardless of the structural alteration brought about by the oxidative state or placement of the D-ring hydroxyl group; the position (or absence) of the A-ring phenolic group; the twisting of the B-, C-, and D-rings (in 1-hydroxyestratrien-17β-ol; Palomino et al., 1990); or the saturation of the A-ring (in 5α-androstane-3β,17β-diol); all of the estrogenic compounds stimulated the CAT gene to a degree directly related to the K_a of the ligand (Figure 3).

Possible Role of Electronegative Isopotential above the A-Ring. Results of these and other studies which examined endogenous genes (VanderKuur et al., 1993; Pilat et al., 1993) suggest that the varied transcriptional effects of estrogen analogues do not reside in the hydrogen bonding and steric differences inherent in these structure, but rather to the electronegative isopotential generated by the unpaired electrons of the phenolic oxygen and the π-electron cloud of the aromatic ring. This electronegative isopotential occupies very different areas above the A-ring, depending upon the hydroxylated position or saturation of the aromatic A-ring (VanderKuur et al., 1993). Certain estrogens (e.g., estratrien-17β-ol), active in regulation of endogenous or reporter genes, possess only the π-electron cloud over and under the A-ring. Protrusion of this electronegative isopotential over position 4 (as in 4-hydroxyestratrien-17β-ol) inactivated the molecule for the induction of endogenous genes without affecting its ability to induce the CAT gene in JA12, JA1, and JV2. An electronegative isopotential extending over position 2 was a characteristic of a ligand (2-hydroxyestratrien-17β-ol) which stimulated the reporter gene in these plasmids, as well as the pS2 gene, while being less effective in inducing the PgR gene and marginally active in the regulation of cathepsin D (VanderKuur et al., 1993; Pilat et al., 1993).

The inability of 2- or 4-hydroxyestratrien-17β-ol to stimulate three endogenous genes as efficiently as they did the CAT gene in JA12, JA1, and JV2 may be related to the fact that additional transcription factors play a role in regulating the endogenous genes. A variety of additional cis-acting elements have been identified in the 5′-proximal region of the pS2 (Nunez et al., 1989), cathepsin D (Cavailles et al., 1989), and PgR (Katzenellenbogen & Norman, 1990) genes. Our results suggest that it is the interaction of the receptor complexes with 2- or 4-hydroxyestratrien-17β-ol with these other transcriptional regulatory proteins bound to complex natural promoters that is deficient, rather than the ability of ER–ligand to interact with the EREc or the transcriptional preinitiation complex.

There is ample evidence that the ability of mutated EREs to activate transcription can be enhanced by interaction with proteins bound at other regulatory sites (Martinez et al., 1989; Klein-Hitpass et al., 1988a; Wahli et al., 1989). Examination of endogenous genes has revealed the hormone-responsive regulatory regions to involve multiple DNA binding sites for steroid receptor and a variety of other protein factors, suggesting that steroid receptors interact with proteins binding at other DNA sequences. In fact, a single EREc functioned synergistically with the CAT reporter gene when flanked by natural sequences found in the vitA_2 promoter (Klein-Hitpass et al., 1988a). Similar interactions have been explored in detail for the glucocorticoid regulation of MMTV gene transcription (Ponta et al., 1985; Cordingley et al., 1987; Miksicek et al., 1989), tyrosine aminotransferase transcription (Jantzen et al., 1987; Reik et al., 1991), and tryptophan oxygenase transcription (Danesch et al., 1987; Schule et al., 1988). From these observations it is implied that the estrogen receptor leads to recruitment of transcription factors rather than activation of prebound transcription factors (Strahle et al., 1988). It is this function which may be facilitated by a properly activated TAF-2 or blocked by a TAF-2 bound by certain estrogen analogues (e.g., 2- or 4-hydroxyestratrien-17β-ol; VanderKuur et al., 1993; Pilat et al., 1993). Examination of the effects of estrogen structure on CAT expression in constructs which include palindromic and mutant EREs with certain other transcription regulatory elements is presently being pursued in this laboratory.

REFERENCES

Anderson, K. P., & Lingrel, J. B. (1989) *Nucleic Acids Res. 17*, 2835.

Berry, M., Nunez, A.-M., & Chambon, P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1218.

Brash, D. E., Reddel, R. R., Quanrud, M., Yang, K., Farrell, M. P., & Harris, C. C. (1987) *Mol. Cell. Biol. 7*, 2031.

Brooks, S. C., Wappler, N. L., Corombos, J. D., Doherty, L. M., & Horwitz, J. P. (1987) in *Recent Advances in Steroid Hormone Action* (Moudgil, V. K., Ed.) pp 443–466, Walter de Gruyter, Berlin, New York.

Cavailles, V., Garcia, M., & Rochefort, H. (1989) *Mol. Endocrinol. 3*, 552.

Cavailles, V., Augereau, P., & Rochefort, H. (1991) *Biochem. Biophys. Res. Commun. 174*, 816.

Cordingley, M. G., Riegel, A. T., & Hager, G. L. (1987) *Cell 48*, 26.

Cramer, R. D., Patterson, D. E., & Bunce, J. D. (1988) *J. Am. Chem. Soc. 110*, 5959.

Danesch, U., Gloss, B., Schmid, W., Schutz, G., Schule, R., & Renkawitz, R. (1987) *EMBO J. 6*, 625.

Dannenberg, H., & Kohler, T. (1964) *Chem. Ber. 97*, 140.

Gorman, C. M., Moffat, L. F., & Howard, B. H. (1982) *Mol. Cell. Biol. 2*, 1044.

Horwitz, J. P., Iyer, V. K., Vardhan, H. B., Corombos, J., & Brooks, S. C. (1986) *J. Med. Chem. 29*, 692.

Ing, N. H., Beekman, J. M., Tsai, S. Y., Tsai, M. J., & O'Malley, B. W. (1992) *J. Biol. Chem. 267*, 17617.
Jantzen, H. M., Strahle, U., Gloss, B., Stewart, F., & Schmid, W. (1987) *Cell 49*, 29.
Kaling, M., Weimar-Ehl, T., Kleinhans, M., & Ryffel, G. U. (1990) *Mol. Cell. Endocrinol. 69*, 167.
Katzenellenbogen, B. S., & Norman, M. J. (1990) *Endocrinology 126*, 891.
Klein-Hitpass, L., Schorpp, M., Wagner, U., & Ryffel, G. U. (1986) *Cell 46*, 1053.
Klein-Hitpass, L., Ryffel, G. U., Heitlinger, E., & Cato, A. C. B. (1988a) *Nucleic Acids Res. 16*, 647.
Klein-Hitpass, L., Kaling, M., & Ryffel, G. U. (1988b) *J. Mol. Biol. 201*, 537.
Klock, G., Strahle, U., & Schutz, G. (1987) *Nature 329*, 734.
Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R., & Chambon, P. (1987) *Cell 51*, 941.
Luckow, B., & Schutz, G. (1987) *Nucleic Acids Res. 15*, 5490.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Martinez, E., & Wahli, W. (1989) *EMBO J. 8*, 3781.
Martinez, E., Givel, F., & Wahli, W. (1987) *EMBO J. 6*, 3719.
McKnight, S. L., & Kingsbury, R. (1982) *Science 217*, 316.
Miksicek, R., Borgmeyer, U., & Nowock, J. (1987) *EMBO J. 6*, 1355.
Nunez, A. M., Berry, M., Imler, J. L., & Chambon, P. (1989) *EMBO J. 8*, 823.
O'Malley, B., (1990) *Mol. Endocrinol. 4*, 363.
Pilat, M. J., Hafner, M. S., Kral, L. G., & Brooks, S. C. (1993) *Biochemistry* (second paper of three in this issue).
Ponglikitmongkol, M., White, J. H., & Chambon, P. (1990) *EMBO J. 9*, 2221.
Ponta, H., Kennedy, N., Skroch, P., Hynes, N. E., & Groner, B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1020.
Reik, A., Schutz, G., & Stewart, A. F. (1991) *EMBO J. 10*, 2569.
Schule, R., Muller, M., Otsuka-Murakami, H., & Renkawitz, R. (1988) *Nature 332*, 87.
Strahle, U., Schmid, W., & Schutz, G. (1988) *EMBO J. 7*, 3389.
Strahle, U., Boshart, M., Klock, G., Stewart, F., & Schutz, G. (1989) *Nature 339*, 629.
Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., & Chambon, P. (1989) *Cell 59*, 477.
VanderKuur, J. A., Wiese, T., & Brooks, S. C. (1993) *Biochemistry* (first paper of three in this issue).
Wahli, W., Martinez, E., Carthesy, B., & Cardinaux, J.-R. (1989) *J. Steroid Biochem. 34*, 17.
Webster, N. H., Green, S., Jin, J.-R., & Chambon, P. (1988) *Cell 54*, 199.
Weisz, A., & Rosales, R. (1990) *Nucleic Acids Res. 18*, 5097.