Differential Induction of pS2 and Cathepsin D mRNAs by Structurally Altered Estrogens†

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ABSTRACT: The influence of structural alterations to the estradiol-17β (E_2) molecule on the induction of pS2 and Cathepsin D (Cath D) mRNAs has been examined by Northern analysis of RNA extracted from MCF-7 cells. Exposure of cultures to estratriene did not affect the level of expression of these estrogen-responsive genes. Addition of one hydroxyl group to estratriene at either of the hydroxylated positions of E_2 (3-phenolic or 17β) yielded monohydroxyestrogens which stimulated the synthesis of Cath D and pS2 mRNAs to a level comparable to that induced by 10^{-10} M E_2 and displayed a decrease in activity at the higher concentrations (10^{-8}–10^{-7} M) similar to that of the parent estrogen. Both of these genes were induced maximally by estrogens with D-ring alterations. Movement of the phenolic hydroxyl group of E_2 to other positions on the A-ring yielded ligands which were highly discriminatory in the induction of these messages. 1-Hydroxyestratrien-17β-ol was capable of stimulating maximal synthesis of both pS2 and Cath D mRNAs when added to cultures of MCF-7 cells at a concentration of 10^{-8} M. Placement of the phenolic hydroxyl at position 4 greatly diminished the induction of these two estrogen-responsive genes. On the other hand, positioning the A-ring hydroxyl group on carbon 2 yielded a ligand which brought about the induction of one gene (pS2) but was marginally effective in the induction of Cath D mRNA synthesis. 5α-Androstanediol and 5-androstenediol with a 17β-hydroxyl group were capable of inducing both genes, provided the 3-hydroxyl group was in the β-configuration. These results demonstrate that discrete changes in the structure of estradiol generate ligands (2- or 4-hydroxyestratrien-17β-ol) with affinities for the estrogen receptor which are not related to their capacity to regulate certain responsive genes (pS2 or Cath D). It is proposed that the observed discrimination between these two responsive genes is the result of variations in the receptor–analogue complex which are important in interactions with gene regulatory factors (e.g., estrogen response element and/or transactivation function 2). Furthermore, the spatial placement of the electronegative isopotential surrounding the aromatic A-ring of these estrogen analogues appears to be involved in the modulation of gene regulation.

Induction of the PgR[1] gene in MCF-7 cells has been shown by utilization of structurally altered estrogens to be independent of the affinity of the ligand for the ER (VanderKuur et al., 1993). Indeed, evidence suggests that structural variations in the ligand may interfere with ER dimerization or influence the manner in which the ER complex interacts with the ERE or TAF-2 region of the receptor. In order to further examine the effect of estrogen structure on the mechanism of receptor-mediated induction of genes, we have studied the regulation of mRNA transcription of two other estrogen responsive genes, pS2 and Cath D.

Cath D is a lysosomal aspartyl protease which has been found in a variety of normal tissues (Faust et al., 1985; Garcia et al., 1986; Augereau et al., 1988). However, levels of the Cath D mRNA are 8–50 times higher than normal in breast cancer cells (Capony et al., 1989) from which this gene has been cloned (Augereau et al., 1988). Unlike normal cells, these cells secrete the inactive pro-Cath D (52 kDa) and accumulate less of the intracellular 48-kDa form and the mature 34-kDa enzyme (Rochefort et al., 1987; Capony et al., 1989). The secreted peptide has been shown to act as a mitogen to steroid-deprived MCF-7 cells (Vignon et al., 1986). Endogenous Cath D, which is constitutively expressed, is further stimulated by E_2 (Westley & Rochefort, 1980; Morisset et al., 1986). Moreover, the E_2 effect on the Cath D gene may be brought about by growth factors known to be induced by estrogen (Cavailles et al., 1989). Studies of breast cancer patients have indicated that high concentrations of Cath D in tumors may be related to prognosis of the patient (Thorpe et al., 1989).

Transcriptional activation of the pS2 gene is a primary response of MCF-7 cells to estrogen (Masiakowski et al., 1982). The cDNA of the pS2 gene was first cloned from these cells in 1984 (Brown et al., 1984). Although the pS2 gene is expressed in other tissues (stomach mucosa; Rio et al., 1988), estrogen regulation of this gene has only been reported in ER-positive breast cancers, whereas the gene is not expressed in hormone-independent breast tumors and normal breast epithelial cells (Rio et al., 1987; Zajchowski et al., 1988). Survival analyses have shown that pS2 negativity was a significant predictor of early recurrence and death (Foekens et al., 1990). In addition to its response to estrogen, the pS2 gene is also regulated by a number of growth factors known to mediate the E_2 growth response (Nunez et al., 1989). Although the pS2 protein shares many similarities with several growth factors, there is no evidence that this E_2-dependent

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[1] Abbreviations: Cath D, cathepsin D; E_2, estradiol-17β; ER, estrogen receptor; ER_n, nuclear estrogen receptor; MEM, minimum essential medium; DCC, dextran-coated charcoal; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; bFGF, basic fibroblast growth factor; IGF-I, insulin-like growth factor I; ERE, estrogen response element; PgR, progesterone receptor; TAF-2, transactivation function 2.

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factor possesses mitogenic activity in breast cancer cells. The physiological role of this protein remains unknown.

EREs upstream of both the Cath D and the pS2 gene have been reported. The nucleotide sequence varies by one guanidine (substituted for adenine) in the 3′ end of the pS2 ERE in relation to the consensus ERE (Berry et al., 1989). A 240-bp fragment located in the 5′-proximal region of the Cath D gene has been shown to mediate transcriptional estrogen activation (Cavailles et al., 1991). The published sequence of this region (Redecker et al., 1991) contains two half-palindromes of the ERE separated by 80 nucleotides.

Cath D and pS2 represent two estrogen-responsive genes, cloned from human breast cancer cells, for which there exists information regarding their EREs and other regulators. In the experiments described herein, the capacity of a number of estrogen analogues to regulate the Cath D and pS2 genes are compared. Discrete changes in the estrogen molecule have been shown to discriminate between these two genes with regard to the regulatory potential of the ligand.

MATERIALS AND METHODS

Steroids. All steroids used in these investigations were synthesized in this laboratory except estratriene, estrone, E_2, estradiol-16α, and estradiol-17α, which were purchased from Research Plus, Inc. (Bayonne, NJ). 1-, 2-, and 4-hydroxyestratrien-17β-ol were synthesized according to published procedures (Palomino et al., 1990). The synthesis of estratrien-17β-ol and 3-hydroxyestratriene have also been reported (Horwitz et al., 1986; Dannenberg & Kohler, 1964). The purity of each estrogen analogue was guaranteed by thin-layer chromatography and crystallization. No analogue contained contaminating estrogens 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).

Cell Culture. A subclone of MCF-7 (designated E3; Butler et al., 1986) was maintained in Eagle's modified MEM supplemented with 5% donor calf serum which was not devoid of endogenous E_2, 0.5 μg/mL gentamicin sulfate, and 0.01 μg/mL phenol red. For experiments, cells were plated at approximately 10^6 cells/25-cm^2 tissue culture flask and allowed to grow for 2–3 days until approximately 80% confluent in MEM supplemented with 5% donor calf serum and 0.5 μg/mL gentamicin, without phenol red. Cells were then switched to media that contained calf serum which had been treated at 37 °C with DCC (Wiese et al., 1992) and screened for a minimum capacity to stimulate pS2 and Cath D mRNA transcription. Cultures were allowed to grow for 7 days. During this withdrawal period, the medium was changed and the cells were washed with phosphate-buffered saline (PBS, Sigma, St. Louis, MO) daily, to minimize residual E_2 contamination. The cells were then exposed to the estrogens (10^{-12}–10^{-6} M) for 24 h in the media containing DCC-treated serum. Experiments were carried out in duplicate.

RNA Isolation. Whole-cell RNA was extracted using a modified version of the one-step procedure published by Chomczynski and Sacchi (1984). Briefly, cells were homogenized in a guanidinium isothiocyanate solution (100 μL per 10^6 cells). Upon acidification with 1/10 vol of 2 M sodium acetate, pH 4.0, the homogenate was put through a phenol–chloroform extraction, followed by a chloroform wash of the aqueous layer. Two volumes of absolute ethanol was added to the aqueous layer, and the crude RNA was allowed to precipitate for several hours at –20 °C. Following centrifugation, the pellet was dissolved in a wash buffer (100 mM sodium acetate and 5.0 mM EDTA, pH 7.0) by heating in a 65 °C water bath for 10 min with agitation. Sodium acetate, 1/10 vol (3 M, pH 6), was added, and the samples were precipitated (–20 °C) with 2 vol of ethanol. After centrifugation, the pellet was next dissolved in a 0.5% SDS solution (300 μL), followed by the addition of 1/10 vol of RNA extraction buffer (100 mM sodium acetate and 100 mM EDTA, pH 5.1). Phenol was added (1:1, v/v), and the samples were allowed to incubate in a 65 °C water bath for two 5-min periods, with mixing of the tubes after the first period. After a 5-min cooling period at 25 °C, chloroform (1:1 v/v, with respect only to phenol) was added and the aqueous layer was extracted. This was again followed by a chloroform wash of the aqueous layer. Samples were precipitated as stated above. The pellet was dried and resuspended in diethyl pyrocarbonate (Sigma) treated water (200 μL per liter of water). Finally, to ensure purity and examine for degradation, a small aliquot from each sample was run on a 1.2% agarose gel stained with ethidium bromide.

Northern Analysis. The RNA was quantitated spectrophotometrically (260 nm), and 10 μg/lane was resolved by electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde and MOPS buffer [0.02 M 3-(*N*-morpholino)-propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0]. Denaturation of each RNA sample was carried out at 70 °C for 15 min in 15 μL of a solution containing 7.1 μL of formamide, 5.1 μL of formaldehyde, 2.8 μL of MOPS buffer (10× concentrated), and 10 μg of RNA in a total volume of 5 μL of DEPC-treated H_2O. The RNA was transferred onto a nylon membrane (Nytran, Schleicher and Schuell, Keene, NH) via capillary action overnight. The membrane was then immersed in a prehybridization solution consisting of 2 mL of 50% dextran sulfate, 4 mL of Northern prehybridization buffer (2× concentrated; 5 Prime-3 Prime Inc., Boulder, CO), 4 mL of deionized formamide (Gibco/BRL, Gaithersburg, MD), denatured yeast RNA, and sheared salmon sperm DNA (both at a final concentration of 40 μg/mL; 5 Prime-3 Prime) for 3–4 h at 42 °C. Next, a hybridization buffer was added, with all the components and volumes as described above except for the replacement of prehybridization buffer with 2× hybridization buffer (5 Prime-3 Prime) and the addition of dithiothreitol (10 mM) and three radioactive probes (see below). This mixture was allowed to incubate for 16–18 h at 42 °C, followed by several washes with SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0) in a 0.1% SDS solution at room temperature. The membrane was then more stringently washed at 50 °C with SSC diluted 1:10 with 0.1% SDS and was allowed to dry. After exposure of the membrane to film (Kodak Diagnostic Film X-OMAT, Rochester NY), the results were quantified with a Molecular Dynamics densitometer employing Imagequant software (Sunnyvale, CA). The recorded density units were directly related to the amount of RNA applied to each lane and to the radioactivity in each band from hybridized cDNA.

Preparation of DNA Probes for Northern Analysis. The cDNA for Cath D (a generous gift of B. Westley) had been cloned into the pUC19 plasmid, while both the pS2 and the 36B4 cDNA (graciously supplied by P. Chambon) were inserted into pBR322. These plasmids were transfected into competent cells of *Escherichia coli* strain DH5α. Plasmids were amplified, extracted, and purified using standard procedures. Restriction digests were performed (*Pst*I for pS2 and 36B4; *Kpn*I and *Hin*dIII for Cath D), and fragments

were purified by agarose gel electrophoresis. The inserts were cut out and recovered via electroelution for 10–12 h using an Elutrap (Schleicher and Schuell).

Radiolabeled probes of each cDNA were made using a commercially available random-primed labeling kit (Boehringer Mannheim, Indianapolis IN). A solution consisting of hexanucleotide primers, purified water, dATP, dGTP, dTTP, and [^{35}S]dCTP (NEN Du Pont, Boston, MA) was prepared, and the denatured cDNAs (roughly 100 ng) were added to these reactants along with Klenow fragment and allowed to incubate at 37 °C for 4 h. The reaction was stopped by the addition of 2 μL of 0.5 M EDTA, pH 8.0. Unincorporated nucleotides were separated from the probes by passing the reaction mixture over a G-25 Select D minicolumn (5 Prime-3 Prime). A 1.0-μL sample from each probe was counted in a Packard Tri-Carb 4530 scintillation counter to determine the radioactivity. Approximately 2.0×10^7 cpm of each probe was added to the hybridization buffer after denaturation at 95 °C for 10 min.

RESULTS

E_2 has been reported to stimulate the induction of pS2 mRNA (Masiakowski et al., 1982) and Cath D mRNA (Westley & Rochefort, 1980; Morisset et al., 1986) in MCF-7 cells. The reported magnitude of the effect of E_2 on the mRNA levels varied considerably. Presumably this variation is due to factors which contaminate serum-containing culture media in which the control cells are grown. Both genes have been shown to be regulated not only by E_2 but also by EGF, insulin, IGF-I, and bFGF (Cavailles et al., 1989; Nunez et al., 1989). Furthermore, the transcription of pS2 mRNA can be increased by factors which activate protein kinase C (Nunez et al., 1989). It was imperative that cultures of MCF-7 cells in which the effects of estrogen analogues were measured had been influenced minimally by the above factors. Therefore, batches of DCC-treated calf serum were screened for the ability to produce low control values of pS2 and Cath D mRNAs in MCF-7 cultures. The level of each mRNA in control (unpulsed) cells demonstrated the extent of activation of pS2 and Cath D genes in cells which had not been influenced by E_2. This quantity fluctuated more extensively for pS2 (±27% SD) and Cath D (±17% SD) in the control cells from a typical experiment than for housekeeping gene, 36B4 (±3% SD).

Increases in the mRNA values for each gene over control values in various cultures of MCF-7 cells pulsed with E_2 indicated the extent of estrogen regulation of transcription (Table I). Both genes in wild-type cultures of MCF-7 cells displayed an inconsistent response to E_2 (Table I), principally because the level of the mRNAs in control cells varied with passage number. A certain clone of MCF-7 cells (E3) exhibited a higher and more consistent E_2 stimulation of both the pS2 and the Cath D gene (Table I). Within an experiment, these cells showed little variation in the extent of E_2 induction of the pS2 (±2.9% SD) and Cath D (±4.5% SD) genes, with the unstimulated 36B4 displaying a ±5.2% (SD) range in values. The stimulation of pS2 mRNA by 10^{-10} M E_2 in these experiments was 3.3(0.8)-fold, while the Cath D mRNA increased 2.2(0.6)-fold in cultures of MCF-7 cells (E3 clone) exposed to this hormone. In these investigations the E3 clone was used since this clone responded to estrogens with a meaningful stimulation of both the pS2 and the Cath D gene, although a greater induction of each gene alone was experienced in other cultures of MCF-7 cells.

Stimulation of transcription of both the pS2 and the Cath D gene reached maximum values after a 12-h exposure of

Table I: Induction of pS2 and Cath D mRNAs by E_2 in Various Cultures of MCF-7 Cells[a]

MCF-7 culture[b]	passage no.	fold induction by E_2[c]	
		pS2 mRNA	Cath D mRNA
wt	161	4.0	3.9
wt	176	2.4	1.8
wt	289	4.7	2.8
wt	298	1.3	1.5
B	128	2.9	1.2
D	126	2.1	1.0
E3	182	3.3	2.3
E3	192	4.3	2.2
E3	196	4.5	2.5
E3	198	8.0	2.5
E3	200	5.7	2.3

[a] Cultures at confluence were pulsed with 10^{-10} M E_2 for 24 h prior to homogenization and RNA extraction. [b] wt, wild type; B, D, and E3 were clones of MCF-7 cells (Butler et al., 1986). [c] Fold increase of mRNAs from E_2-pulsed cultures over these messages in control cultures. Density units were measured on film exposed to hybridized ^{35}S-cDNA.

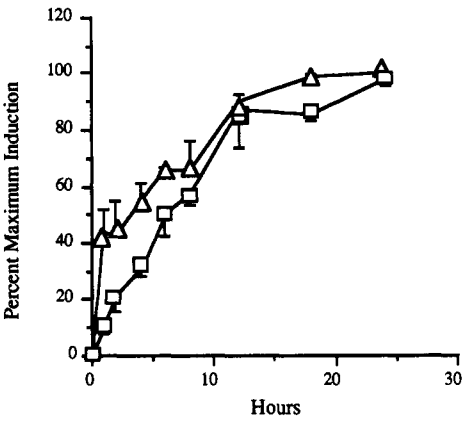

FIGURE 1: Time course of Cath D and pS2 mRNA induction in MCF-7 cells (E3 clone) pulsed with 10^{-10} M E_2. Confluent cultures which had been maintained in media devoid of E_2 for 7 days were pulsed with E_2 for the indicated times when the incubations were stopped. Cells were homogenized and RNA was extracted as described in Materials and Methods. Following Northern analysis of the Cath D and pS2 mRNAs, the data was plotted as percent maximum induction. Fold inductions of Cath D (△) and pS2 (□) messages were 4.0 and 19.6, respectively. Points indicate averages of two determinations. Ranges are shown by bars.

MCF-7 cultures to 10^{-10} M E_2 (Figure 1). The synthesis of these mRNAs remained at maximum levels from 12–24 h. In other experiments (data not shown) this stimulation was shown not to increase further for periods up to 72 h.

E_2 stimulation of pS2 and Cath D was maximal at a concentration of 10^{-10} M (Figure 2A). Higher levels of E_2 were less effective in elevating the synthesis of these two mRNAs. Removal of both hydroxyl groups from the E_2 molecule yielded a compound, estratriene, which had no effect on the pS2 and Cath D genes at concentrations as high as 10^{-7} M (Figure 2B). Without hydroxyls to interact with ER, estratriene has been shown to have an affinity for receptor too low to measure with the competitive binding assay (Brooks et al., 1987).

Placing a phenolic hydroxyl on position 3 of estratriene yielded an estrogen capable of inducing both the pS2 and the Cath D gene (Figure 2C). This monohydroxyestrogen was nearly as potent as E_2 in stimulating the synthesis of Cath D mRNA (maximum induction at 10^{-9} M) but less effective in the induction of pS2 mRNA (maximum effect between 10^{-11} and 10^{-9} M). The induction of both genes decreased at the higher concentrations of 3-hydroxyestratriene.

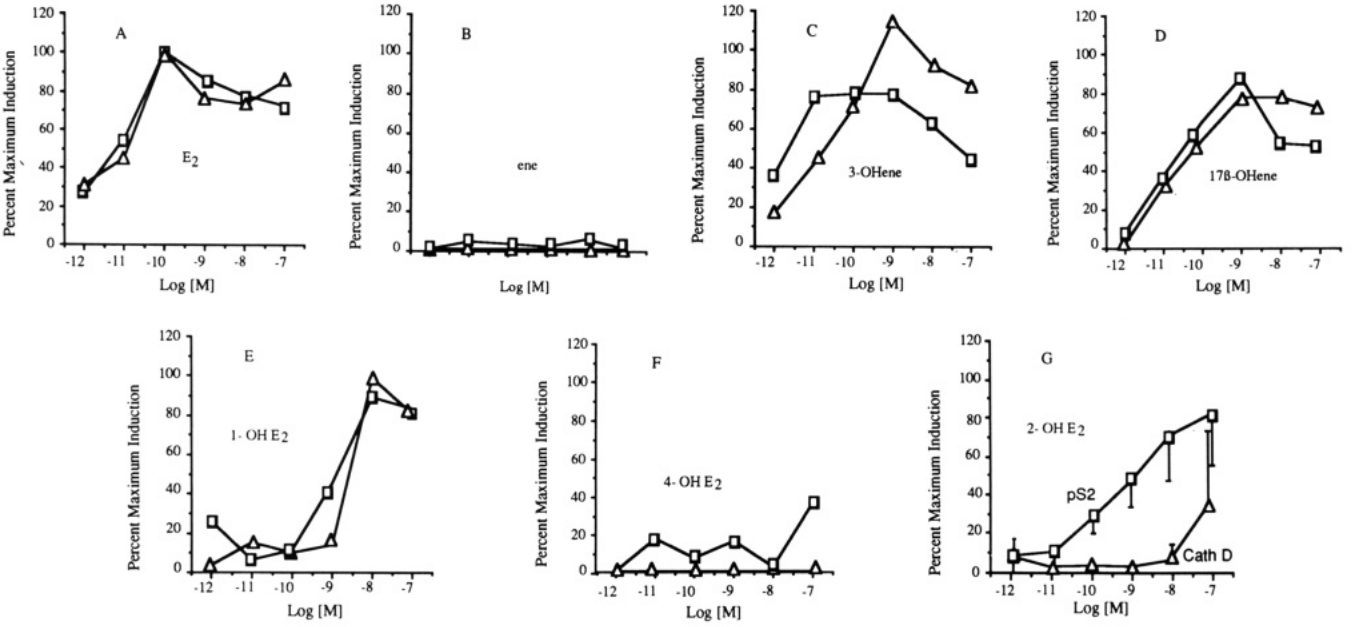

FIGURE 2: Induction of Cath D and pS2 mRNAs in MCF-7 cells (E3 clone) pulsed with different concentrations of estrogen analogues. Cultures were grown and treated with estrogens as described in Materials and Methods. RNA was extracted from cultures 24 h following pulse with estrogens, and the Cath D and pS2 mRNAs were determined by Northern analysis. Points represent averages of several determinations. Cultures were treated with (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), or (G) 2-hydroxyestratrien-17β-ol (2-OH E_2). In these experiments Cath D mRNA (△) was induced between 1.9- and 3.5-fold, and the stimulation of pS2 mRNA (□) synthesis was between 2.4- and 6.9-fold above control values. These experiments, which were carried out in duplicate, were repeated two to eight times. Points are averages of all experiments which examined each estrogen analogue. Bars in panel G represent the standard deviations (n = 4, Cath D; n = 15, pS2).

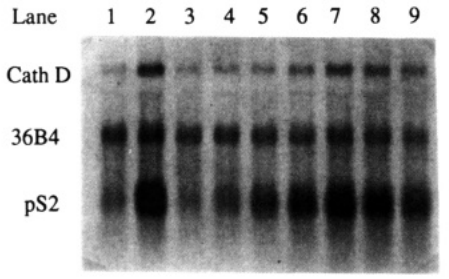

FIGURE 3: Representative Northern blot of the induction of Cath D and pS2 mRNAs by increasing concentrations of estratrien-17β-ol. Each Northern blot was hybridized with the ^{35}S-labeled cDNA of Cath D, pS2, and the housekeeping gene 36B4. RNA from control cells (lane 1) and cells pulsed with 10^{-10} M E_2 (lane 2) served as controls for the activity of estratrien-17β-ol. RNAs from MCF-7 cells pulsed with the following concentrations of estratrien-17β-ol are shown in the experimental lanes: 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. Films were exposed to membranes for 24 h.

The addition of D-ring hydroxyl group to estratriene yielded an active estrogen with slightly diminished capacity to stimulate both genes (maximum activity at 10^{-9} M; Figures 2D and 3). Metabolic hydroxylation of the aromatic ring of estratrien-17β-ol during the pulsing of cultures was unlikely since these MCF-7 cells have been shown to be devoid of the message for the required hydroxylation enzyme ($CytP450_{1A1}$; VanderKuur et al., 1993).

Repositioning the phenolic hydroxyl group on E_2 to other carbons of the A-ring produced estrogens with varying effects on the pS2 and Cath D genes. 1-Hydroxyestratrien-17β-ol was 100-fold less active than E_2. However, this dihydroxyestrogen stimulated both genes effectively when added to cultures of MCF-7 cells (E3 clone) at a concentration of 10^{-8} M (Figure 2E). On the other hand, 4-hydroxyestratrien-17β-ol was ineffective in stimulating the synthesis of Cath D mRNA and only slightly active in the induction of pS2 mRNA (Figure 2F). Interestingly, an estrogen with the phenolic hydroxyl on carbon 2 of estratrien-17β-ol was capable of forming a receptor complex which actively stimulated the pS2 gene while displaying minimal induction of Cath D mRNA (Figure 2G).

Table II: Induction of pS2 and Cath D mRNAs in MCF-7 Cells Pulsed with D-Ring Analogues of E_2

	concn[a] (M)		EC50[b] (10^{-9}M)	
estrogen	pS2	Cath D	pS2	Cath D
$E_2 17\alpha$[c]	10^{-9}	10^{-10}	0.086	0.071
$E_2 16\alpha$[d]	10^{-10}	10^{-10}	0.0093	0.040
estriol	10^{-10}	10^{-9}	0.012	0.040
estrone	10^{-10}	10^{-10}	0.15	0.085
ICI 164384	10^{-6} [e]	10^{-6} [e]	1000	1000
4-OH-tamoxifen	10^{-6} [e]	10^{-6} [e]	1000	1000

[a] Concentration of estrogen at peak gene stimulation. [b] Defined in the caption of Figure 4. EC50 for E_2 was 8.26×10^{-11} M for Cath D and 5.68×10^{-12} M for pS2. [c] Estradiol-17α. [d] Estradiol-16α. [e] Compounds which did not reach 50% maximal response were given an arbitrary property value (10^{-6} M; Cramer et al., 1988).

Changes in the position or oxidation state of the D-ring hydroxyl group of E_2 or the addition of a 16α-hydroxyl group produced estrogens with capacities for the induction of pS2 or Cath D mRNAs similar to that displayed by E_2 (Table II).

Elimination of the aromatic character of the A-ring of the estrogens, forming 5α-androstane-3β,17β-diol, yielded a steroid which was capable of stimulating both the pS2 and the Cath D gene (Table III). 5-Androstene-3β,17β-diol was also effective. However, changing the orientation of the 3β-hydroxyl group to the 3α-configuration eliminated the estrogenic function of the ligand with respect to the regulation of these two genes (Table III).

The antiestrogen ICI 164384 did not stimulate the synthesis of either Cath D or pS2 mRNA. 4-Hydroxytamoxifen displayed minimal estrogenic activity in the induction of both Cath D and pS2 mRNA (<25% over a concentration range of 10^{-11}–10^{-7} M).

Table III: Induction of pS2 and Cath D mRNAs in MCF-7 Cells Pulsed with Two 5α-Androstanediols and One 5-Androstenediol

steroid	concn (M)[a]		EC50[b] (10^{-9} M)	
	pS2	Cath D	pS2	Cath D
5-androstene-3β,17β-diol	10^{-8}	10^{-8}	6.68	13.8
5α-androstane-3β,17β-diol	10^{-8}	10^{-7}	16.3	1.00
5α-androstane-3α,17β-diol	10^{-7}	ND[c]	100	ND[c]

[a] Concentration of steroid at peak gene stimulation. [b] Defined in the caption of Figure 4. EC50 for E_2 was 8.26×10^{-11} M for Cath D and 5.68×10^{-12} M for pS2. [c] Not done.

DISCUSSION

Effect of Structure Modification on the Capacity of Estrogens to Regulate pS2 and Cath D mRNA Transcription. As previously reported (Westley & Rochefort, 1980; Masiakowski et al., 1982; Morisset et al., 1986), E_2 was a potent stimulator of both Cath D and pS2 mRNA synthesis in these experiments with MCF-7 cells. This natural estrogen brought about maximum induction of each gene at a concentration which is physiologically relevant (10^{-10} M; Figure 1A). Higher concentrations of E_2 were less efficient in inducing these messages. Either of the hydroxyl groups on the E_2 molecule was required for this activity. Whereas the configuration, oxidative state, or position of the D-ring hydroxyl group was not important in distinguishing regulation of the synthesis of Cath D and pS2 mRNAs, the position of the A-ring phenolic group did differentiate the stimulation of these genes. For example, 1-hydroxyestratrien-17β-ol actively elicited the synthesis of both mRNAs, and 4-hydroxyestratrien-17β-ol did not stimulate the synthesis of either the Cath D or the pS2 mRNA, while 2-hydroxyestratrien-17β-ol effectively induced the pS2 gene but was marginally active in Cath D regulation.

Role of Receptor Affinity. Most alterations to the E_2 molecule affect affinity of the ligand for ER (Hahnel et al., 1973; Hahnel & Twaddle, 1974). Binding affinities of the various estrogens and androstanediols employed in these experiments have been calibrated (Brooks et al., 1987; VanderKuur et al., 1993) and shown to decrease as much as 200-fold less than the K_a of E_2 (with the exception of estratriene and 5α-androstane-3α,17β-diol, which possessed affinities too low to measure). Nevertheless, the range of concentrations of these compounds which were added to cultures of MCF-7 cells was extended to 10^3-fold above the level (10^{-10} M) at which E_2 displayed its maximum stimulation of Cath D and pS2 mRNA synthesis. Therefore, each ligand examined would be expected to influence the responsive genes in this system, assuming in each case that the activity was related directly to the K_a for ER. All of these estrogen analogues brought about tight nuclear binding of receptor complex when added to cultures of MCF-7 (VanderKuur et al., 1993).

As presently understood, the receptor mechanism of estrogen activity in target cells would dictate that an estrogen should stimulate responsive genes to an extent which is related to the ligand's affinity for ER. Overall, plots which depict the capacity of each compound to stimulate the Cath D and pS2 genes show excellent correlations ($R^2 = 0.85$ for Cath D and 0.96 for pS2) with the binding affinity of the estrogens and androstanediols (Figure 4). Exceptions to this relationship were 2- and 4-hydroxyestratrien-17β-ol. 4-Hydroxyestratrien-17β-ol bound ER with an affinity comparable to, or greater than, a number of estrogens which activated these genes. Yet this dihydroxyestrogen, which was inactive in the regulation of either gene, formed a receptor complex that bound well to nuclear material in MCF-7 cells (VanderKuur et al., 1993). More interesting was the inability of 2-hydroxyestratrien-17β-ol to stimulate the synthesis of significant levels of Cath D mRNA while being effective in the regulation of the pS2 gene (Figure 2G). This dihydroxyestrogen bound receptor with an affinity which was 80% that of E_2 (VanderKuur et al., 1993). Furthermore, the 2-hydroxyestratrien-17β-ol receptor complex bound well to nuclei of MCF-7 cells when added to culture (VanderKuur, et al., 1993).

Results from these investigations have shown that the position of the A-ring phenolic group was important in gene regulation beyond the effect that this structural alteration may have had on receptor affinity. Whereas the localization of hydrogen bonding (of the phenolic hydroxyl group) may affect receptor affinity, there is no indication that this interaction between ligand and protein influenced gene regulation by the receptor complex (cf. estratrien-17β-ol, which stimulated both genes without phenolic hydroxyl groups).

X-ray crystallographic studies of the dihydroxyestrogens used in these studies have shown minor conformational aberrations in the four-ring structures (relative to E_2) brought about by electronic effects of the 2- and 4-phenolic groups (Palomino et al., 1990). On the other hand, 1-hydroxyestratrien-17β-ol displayed considerable twisting of the B-, C-, and D-ring as a result of the placement of the A-ring hydroxyl group. Although this steric modification affected this molecule's affinity for receptor, the 1-hydroxyestratrien-17β-ol–receptor complex fully regulated the Cath D and pS2 genes (Figure 2E). Steric alterations in the ligand brought about by the structural changes in the estrogen molecule, which affected receptor affinity, do not appear to contribute to the observed variations in gene regulation.

Possible Influence of Mutated EREs in Differential Ligand Effects. Sequence analyses indicate the following compositions of the EREs upstream from the Cath D and pS2 genes (Berry et al., 1989; Cavailles et al., 1991; Redecker et al., 1991):

5′-GGTCA-$(N)_3$-TGGCC-3′ (pS2)

5′-GGTCA-$(N)_{80}$-TGACC-3′ (Cath D)

5′-GGTCA-$(N)_3$-TGT/ACC-3′ (consensus)

Whereas the pS2 ERE differs from the consensus ERE by replacement of the T/A by a G in the 3′ half of the palindrome, the Cath D enhancer matches the consensus palindrome. However, unlike most responsive elements for estrogen-regulated genes [reviewed by Evans (1988)], the Cath D gene has 80 nucleotides separating the half-palindromes rather than the usual 3 nucleotides [detected by computer analyses of sequences reported by Cavailles et al. (1991), Redecker et al. (1991), and Evans (1988)]. Conceivably, the interaction of ER complexes with these different EREs could vary with ligand structure. Nevertheless, this does not appear to be an all-or-none phenomenon, since an appreciable quantity of the ER complex has been found to be tightly bound to nuclear material following a pulse with the inactive 4-hydroxyestratrien-17β-ol (VanderKuur et al., 1993).

Finally, it is believed that the ER complex interacts with the ERE as a dimer (Lannigan & Notides, 1989). Still, it is not likely that the A-ring regioisomers examined in these studies interacted with the dimerization region of the estrogen binding domain in a manner which prevented dimerization. Such a proposition could only ocur if ER dimerization was required for the induction of Cath D but not for stimulation of the pS2 gene (cf. 2-hydroxyestratrien-17β-ol, Figure 2G).

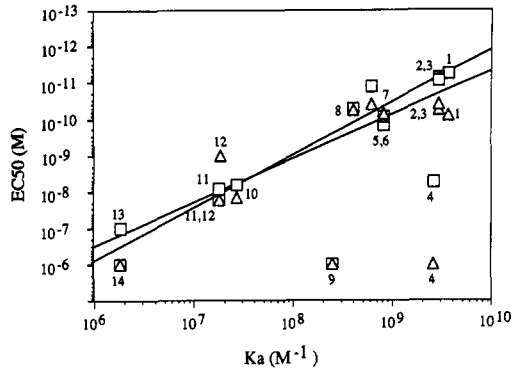

FIGURE 4: Relation of K_a of various estrogens and androstanediols to the induction of Cath D and pS2 mRNAs. K_a's were determined by competitive binding assay (VanderKuur et al., 1993). In order to relate the ability of each estrogen to induce Cath D and pS2 mRNA, the EC50 (defined as the effective concentration which produced a half-maximal response) was determined. The EC50 for each analogue was calculated as log [(% of maximal E_2 response)/[100 − (% of maximal E_2 response)]]. Compounds which did not reach 50% maximal response were given a low and arbitrary property value (Cramer et al., 1988). R^2 values of lines determined by Cath D data (△) and pS2 results (□) are 0.85 and 0.96, respectively, when points representing compounds 4 and 9 are omitted. Numbers refer to the following steroids: (**1**) E_2, (**2**) estradiol-16α, (**3**) 3-hydroxyestratriene, (**4**) 2-hydroxyestratrien-17β-ol, (**5**) estrone, (**6**) estradiol-17α, (**7**) estriol, (**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.

Possible Differential Influences of Structurally Altered Estrogens upon TAG-2. A potential explanation for the lack of regulatory activity exhibited by 4-hydroxyestratrien-17β-ol with the Cath D and pS2 genes may involve the TAF-2 region within the estrogen binding domain of ER (Webster et al., 1988; Lees et al., 1989). This region comprises a number of dispersed elements throughout the hormone binding domain brought together upon estrogen binding (Webster et al., 1989; Danielian et al., 1992). Point mutations or deletions of amino acids in TAF-2 (amino acids 282–595 and more specifically 538–552) have been shown to repress transcription (Tora et al., 1989; Berry et al., 1990; Danielian et al., 1992). It is believed that interaction of the ligand with TAF-2 may be required for the ER complex to influence adjacent transcription factors and ultimately responsive genes (Lees et al., 1989). To accomplish this, ligand binding may bring about an allosteric conformation that allows access of the TAF-2 region to other transcription factors (O'Malley, 1990; Murdoch & Gorski, 1991). Possibly, structural changes in the E_2 molecule could affect this essential function after binding to ER.

Molecular modeling of these estrogen analogues has demonstrated extensive variation in the location of electronegative isopotentials surrounding the aromatic ring of A-ring regioisomers (VanderKuur et al., 1993). As the π-electron cloud of the aromatic A-ring (all that was required for estratrien-17β-ol to stimulate the Cath D and pS2 genes) was extended over position 3 by the unpaired electrons on the 3-hydroxyl group of E_2, maximum gene induction occurred. Without the aromatic A-ring, it was even possible to stimulate these genes if the unpaired electrons of a 3-hydroxyl group were placed above the A-ring as in 5α-androstane-3β,17β-diol (Table II). When the electronegative isopotential protrudes over position 4, induction of these two genes (and PgR; VanderKuur et al., 1993) was hindered (Figure 2F). On the other hand, an electronegative isopotential directed over the 2-position of the ligand proved to be ineffective in the receptor-mediated induction of Cath D, marginally active in PgR stimulation (VanderKuur et al., 1993), and completely stimulatory to the pS2 gene (Figure 2G). It is conceivable that the binding of estrogens with vastly different geometric placement of the electronegative isopotential above the A-ring could differentially affect the organization of TAF-2 and thereby influence the interaction of this region of the ER with adjacent transcription factors.

Biological Significance. The results of these investigations suggest that structural variations in the estrogen ligand can influence the effect of the receptor complex on transcription of one responsive gene more than another. Furthermore, this differential induction of the pS2 and Cath D genes may be evoked via TAF-2. This could be rationalized if regulatory transcription factors which control separate genes are different. Such is the case for the Cath D and pS2 genes (Cavailles et al., 1989; Nunez et al., 1989).

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